AD_____

Award Number: DAMD17-99-1-9191

TITLE: Molecular Mechanisms of Estrogen and Antiestrogen Resistance

PRINCIPAL INVESTIGATOR: Robert R. Clarke, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057

REPORT DATE: July 2001

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Molecular Mechanism of Estrogen and Antiestrogen Resistance

5. FUNDING NUMBERS
DAMD17-99-1-9191

6. AUTHOR(S)
Robert R. Clarke, Ph.D.

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

E-Mail: clarker@georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

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

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

This is an Academic (Career Development) Award. Consistent with this award, the PI continues to "*..appraise critically the state of the science in a particular aspect of breast cancer research and to forge new avenues of investigation.*" In this application, the studies are focused on antiestrogen resistance. With respect to the career development aspects, several original studies and reviews relevant to this application have been published, others have been submitted for publication. We continue to apply state-of-the-art technologies to addressing resistance, including the development of novel approaches to data mining and analysis. The studies of interferon regulatory factor-1 (IRF-1) continue. We have now completed the initial studies of the IRF-1 transfected cells and have developed and begun to test a dominant negative IRF-1 construct. Immunohistologic methods for correlative studies on human breast tissues are being developed and we hope soon to study the expression of nucleophosmin, NFκB, IRF-1 and X-box binding protein -1 (hXBP-1) and any correlations with known prognostic markers. We also have explored the effects of *in utero* Tamoxifen exposure on mammary gland development and subsequent susceptibility to chemical carcinogenesis. The role of the steroid sulfatase in affecting estrogen dependence *in vitro* and *in vivo* also was explored. Data show that overexpression of this enzyme can release sufficient intracellular estrogens to support estrogen-dependent growth.

14. SUBJECT TERMS
Breast Cancer, estrogens, antiestrogens, drug resistance, hormone resistance, gene expression, microarrays, serial analysis of gene expression

15. NUMBER OF PAGES
106

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature Date

Table of Contents

Introduction

This is an Academic Award (Career Development Award). The purpose of this application is to free additional time for the Principal Investigator to *"..appraise critically the state of the science in a particular aspect of breast cancer research and to forge new avenues of investigation."* The PI will apply new, state-of-the-art technologies to identify key endocrine-regulated molecular pathways to apoptosis/proliferation. By identifying key components of these pathways, we may be able to predict response to first-line and crossover antiestrogenic therapies, and/or provide novel therapeutic strategies for antiestrogen resistant tumors.

Body

This is an Academic Award, for which a detailed research plan was not required. Since the award is to support academic development, the aims are not finite, *i.e.,* restricted only to the time frame or resources provided through this type of award. Furthermore, unlike a R01-style application, the amount of work proposed represents the efforts of a number of individuals and funded grants already active within the PI's laboratory, and both ongoing and future collaborations with other laboratories. Consistent with this, the proposed work requires substantially more than the time and financial resources provided by a single R01. Without describing the work in this manner, it was unclear how we could address the requirements of this new award category. The aims, amount of work proposed (which must, *e.g.,* go beyond the three year limit to satisfy the award requirements) and time frames were presented, in the original application, with these issues in mind. To prevent duplication and to limit the size of this report, published data are provided in the reprints, rather than being recapitulated in the text, and very preliminary data are described but not shown.

Aim 1: We will expand the MCF7/LCC1 and MCF7/LCC9 databases to a minimum of 30,000 tags/database. We also expect to establish a 30,000 tag database for MCF-7 cells growing with and without 17β-estradiol. Completion of all four databases will require longer than the three year period, since we also plan to perform functional studies on candidate genes identified from our comparisons of the MCF7/LCC9 and MCF7/LCC1 databases. For the purposes of this application's duration, we would consider this aim to have been successfully completed once the MCF7/LCC1 and MCF7/LCC9 databases have each reached a size of 30,000 tags. Time: years 1-3.

1. Initially, we chose to focus on completing the initial gene microarray studies. The MCF7/LCC1 and MCF7/LCC9 SAGE databases are of a sufficient size to warrant publication. We have approximately 11,000 tags in the MCF7/LCC1 database and approximately 13,000 in the MCF7/LCC9 database. **We have now submitted a manuscript describing these data and data from the initial gene microarray studies.** Once accepted, a copy will be provided either as a reprint or preprint in the next report.

Aim 2: We will continue to investigate the functional relevance of those genes/proteins that receive sufficient priority This will include transient transfection studies with promoter-reporter constructs (for transcription modulating factors) and stable transfections to assess functional relevance. We also will investigate clinical relevance by exploring expression in breast tumor biopsies, and correlating expression (or lack thereof) with established prognostic variables, *e.g.,* lymph node status, ER expression, S-phase/proliferation, tumor grade, disease free and overall survival and response to endocrine and cytotoxic chemotherapies. For the purposes of this application's duration, we would consider this aim to have been successfully completed if we can confirm the roles of *NPM*, NFκB, CRE/hXBP-1 and the IRF-1 polymorphism. Time: years 1-3.

We have continued to work on the candidate genes involved in estrogen and antiestrogen resistance from our 2D-gel, gene microarray and SAGE studies. These include nucleophosmin (*NPM*), interferon regulatory factor-1 (IRF-1), cAMP response element binding activities (CRE; induced by the human X-box binding protein-1 - hXBP-1) and nuclear factor kappa B (NFκB). We have completed studies showing that antiestrogen resistant MCF7/LCC9 cells, which overexpress NFκB transactivation (promoter-reporter activity), are more sensitive to the growth inhibitory effects of Parthenolide, a specific inhibitor of NFκB (Fig 1). Growth inhibition was assessed using a dye-based assay that effectively estimates cell number. These data are consistent with our hypothesis that increased NFκB activation in these cells contributes to their ability to survive prolonged antiestrogen exposure.

We have obtained commercial antibodies to hXBP-1, IRF-1, NPM and NFκB and begun to establish the experimental conditions necessary to obtain good immunohistochemical data on breast tumor tissues. We will use tissue microarrays to do correlative science studies and explore the association among protein expression and clinical data. We have obtained tissue microarrays commercially, from in-house arrays, and from NIH. Preliminary studies suggest that the hXBP-1, NPM and NFκB antibodies should work well. Data with the first commercial IRF-1 antibody was equivocal. We have requested other IRF-1 antibodies (both commercial and from academic institutions).

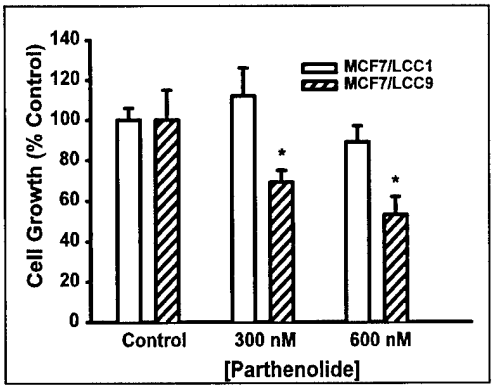


Fig 1: *Parthenolide inhibits proliferation of MCF7/LCC9 but not MCF7/LCC1 cells. *p<0.01 vs. control, n=4.*

In the reporting period, we chose to focus primarily on studies of IRF-1. We have completed our studies of the IRF-1 transfectants. The full length IRF-1 cDNA was cloned into an expression vector placing its expression under the direction of the constitutive CMV promoter. IRF-1 was overexpressed using standard transfection methods (1). Overexpression of IRF-1 inhibits the rate of cell

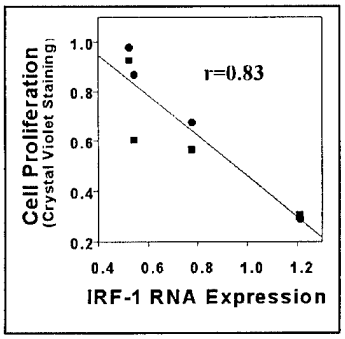


Fig 2: *IRF-1 expression is inversely correlated with the rate of cell proliferation. Squares = data from expt 1; circles=data from expt 2.*

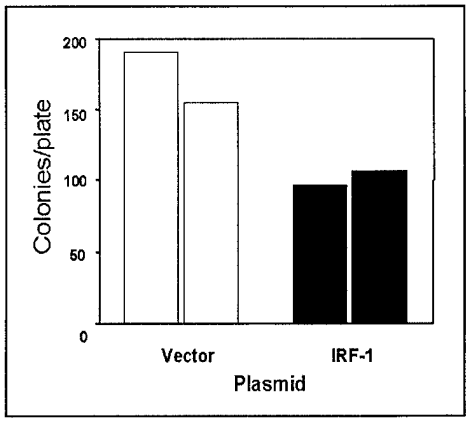


Fig 3: *Reduced anchorage-dependent colony formation in pooled IRF-1 transfectants compared with G418-resistant controls. data from two independent experiments.*

proliferation (Fig 2) and anchorage-dependent colony formation in antiestrogen and estrogen responsive MCF-7 cells (Fig 3). In Fig 2, we estimated the cell population doubling time and plotted these as a function of IRF-1 mRNA expression. A correlation coefficient was estimated, showing that the higher the level of IRF1 expression, the slower the cells grow (r=0.83). In Fig 3, we measured anchorage-dependent growth and counted the number of discrete colonies. IRF-1 transfectants produced fewer colonies than controls. These data are consistent with IRF-1 acting as a tumor suppressor gene in breast cancer, and are consistent with our initial hypotheses.

In other preliminary studies we found a new IRF-1 polymorphism MCF-7 cells. Subsequently, we found the same polymorphism in several biopsies of primary human breast tumors and cell lines (Table 1). The role of this polymorphism, which changes an A at base 4396 to a G, is unclear because this does not affect amino acid sequence. However, it creates a putative splice donor site in the IRF-1 transactivation domain. Additional studies are in progress.

Tissue	**n**	**+/+**	**+/-**	**-/-**	**mutant**	**allele freq***
Cell Lines	17	8	2	7	53% (9/17)	47% (16/34)
Tumors (breast)	18	13	5	0	28% (5/18)	14% (5/36)
Normal (PBL)	30	27	3	0	10% (3/30)	5% (3/60)

*allele frequency (χ^2=26.19, p<0.001)

Table 1: Prevalence of the A4396G IRF-1 polymorphism

We have a first draft of a manuscript describing the IRF-1 studies and reporting the IRF-1 polymorphism.

We have designed, built and tested a dominant negative IRF-1 construct (dnIRF-1) comprising the IRF-1 cDNA without the transactivation domain. Fig 4 shows preliminary data that dnIRF-1 is a potent inhibitor of endogenous and interferon-induced IRF-1 transcriptional activities (promoter reporter assay). These were done using standard transient transfection methods. We also stably overexpressed dnIRF-1 using the same vector and methods for overexpressing IRF-1. These cells have been injected into NCr athymic nude mice, and we are currently assessing the ability of dnIRF-1 overexpression to increase the tumorigenicity of MCF-7 cells. Initial studies suggest that these transfected cells grow more quickly *in vitro* and may be more tumorigenic *in vivo*. We hope to complete these studies and submit a manuscript on dnIRF-1 within the next 12 months. The mature data will be included in our next annual report.

Studies with the IRF-1 null mice from Jackson Laboratories and our own *NPM* transgenic mice were curtailed due to a general *Helicobacter* infection in our *Vivarium*. We have been able to keep some mice treated with DMBA prior to the outbreak, but had to stop other studies until the infection was eliminated. This delay will likely cost 12-18 months in this aspect of our research program.

Aim 3: We will continue to integrate the emerging experimental data into our molecular transduction schemes, and amend these as appropriate. Clearly, this will require substantial ongoing effort to integrate the studies from the more broad-based projects, *e.g.*, SAGE and gene array, with the more focused and functional studies, *e.g.*, those specifically addressing the function of *NPM* and IRF-1 polymorphism. For the purposes of this application's duration, we would consider this aim to have been successfully completed once we have established the likely validity of the *NPM*/IRF-1 signaling components (p53 independent). Time: years 1-3.

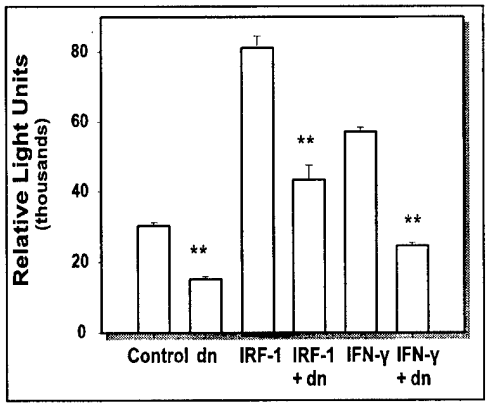


Fig 4: *Activity of the IRF-dominant negative (dn). **p<0.001 for dn vs. ctr and effects of dn on IRF-1 & IFN-γ (n=4).*

We have increased significantly our collaborations with the informatics group at Catholic University of America and developed a new algorithm for normalizing gene expression microarray data. This is essential given the microarray studies described in the original application. The data normalization approach is based on our implementation of regression through the origin, which also allows for direct normalization to a reference array (2). The regression is described by:

$$y_i = ax_i + b \qquad \textbf{(Eq. 1)}$$

where, the data points in the floating data set are $\{x_1,x_2,x_3...,x_i\}$, and those in the reference set are $\{y_1,y_2,y_3...,y_i\}$. This applies an approach similar to the "boosting" principle but differs from that of Chen *et al.* (3) in two ways. First, rather than forcing b=0, both *a* and *b* are iteratively estimated. Secondly, genes for normalization are iteratively selected, rather then predefined as non-differentially expressed genes (Chen *et al.* predefined control genes prior to analyis). There is some risk in predefining "control" genes, particularly if this is based on expected function. For example, GAPDH is often included as a "control/housekeeping" gene but has been reported to have prognostic value in some breast cancers (4). Thus, its expression must vary among breast and possibly other tumors.

We apply a bootstrap approach, starting with all genes, which alternates between estimating normalization coefficients and identifying an interim control gene subset for normalization. A factoring-shifting approach estimates regression coefficients at each iteration, based on an interim control gene subset defined by a window function. The window function, which decays with alternate iterations, rejects outliers and measures the consistency of the matched neighborhoods, *i.e.,* the corresponding data for each gene in the reference and floating gene sets. By setting the window over the center of the scatter plot, genes with very high or very low levels of expression are excluded from guiding normalization. Convergence is achieved when $b=0$ and $a=1$. For those cases where $b \approx 0$, our algorithm should arrive at a solution very similar to that of Chen *et al.* Where $b \neq 0$, our algorithm should provide a more robust normalization. **A manuscript describing this new algorithm has been submitted for publication.**

We have now completed a pilot study to optimize tissue acquisition and processing for breast core needle biopsies, such that we can begin to array prospective samples from clinical trials. This will allow us to begin testing any predictive models we build based on the *in vitro* gene expression microarray studies. **We have a draft manuscript prepared and will include a full manuscript in the next annual report.** The completed manuscript will contain all the technical, and methodologic details.

Briefly, human breast cancer xenografts were used to evaluate several processing methods for prospectively collecting adequate amounts of high quality RNA for gene expression microarray studies. Samples were assessed for the preservation of tissue architecture and the quality and quantity of RNA recovered. An optimized protocol was applied to a small study of core needle breast biopsies from patients, in which we compared the molecular profiles from cancer with those from noncancer biopsies. Gene expression data were obtained using Research Genetics, Inc. Named Genes Gene arrays. Data were visualized at the top level using a novel multidimensional scaling method (5) and simple hierarchical clustering (6). Data dimensionality was reduced by simple statistical approaches. Predictive neural networks were built using a multilayer perceptron and evaluated in an independent data set from mastectomy specimens.

Processing tissue through RNA*Later*™ (Ambion) preserves tissue architecture when biopsies are washed for 5 min on ice with ice-cold PBS. Cell margins are clear, tissue folding and fragmentation are not observed, and integrity of the cores is maintained, allowing optimal pathological interpretation and preservation of diagnostically important information. Adequate concentrations of high quality RNA are recovered, biopsies producing a median of 1.34 μg total

RNA. Snap freezing or the use of RNA*Later*™ does not affect RNA recovery or the molecular profiles obtained from biopsies. Neural network predictors were built using the gene expression microarray data and accurately discriminate between predominantly cancer and noncancer breast biopsies in both the training and independent data sets.

The approaches generated in these studies provide a simple, safe and effective method for prospectively acquiring and processing breast core needle biopsies for gene expression studies. Gene expression data from these studies can be used to build accurate predictive models that separate very different molecular profiles. The data establish the utility and effectiveness of these approaches for future prospective studies.

We continue to array the cell lines to identify differential gene expression associated with acquired antiestrogen resistance. We recently acquired access to the Affymetrix technology and are in the process of re-arraying samples with this platform rather than the nylon filter-based Research Genetics arrays.

Since intratumor generation of estrogens likely contributes to acquired antiestrogen resistance, we completed a study where we overexpressed the steroid sulfatase cDNA in MCF-7 cells and determined the effects of overexpression on endocrine responsiveness. The experimental details and results are provided in the published paper **(reprint included in appendix)**. Briefly, 17β-estradiol sulfate induces expression of the progesterone receptor mRNA only in STS Clone 20 cells (MCF-7 cells transfected with the sulfatase cDNA), while estrone sulfate produces the greatest stimulation of anchorage-independent growth in these cells (compared with vector controls). STS Clone 20 cells retain responsiveness to antiestrogens, which block the ability of estrogen sulfate to increase the proportion of cells in both the S and G2/M phases of the cell cycle.

Consistent with these *in vitro* observations, only STS Clone 20 cells exhibit a significant increase in the proportion of proliferating tumors in nude ovariectomized mice supplemented with 17β-estradiol sulfate. We used a study design where STS Clone 20 were injected on one side and empty vector control cells on the other side. The primary activity *in vivo* appears to be from intratumor STS, rather than hepatic STS, since the vector cells do not produce tumors in mice receiving estradiol sulfate but the transfectants are tumorigenic. Surprisingly, 17β-estradiol sulfate appears more effective than 17β-estradiol when both are administered at comparable concentrations. This effect, which is seen only in STS Clone 20 cells, may reflect differences in the cellular pharmacology of exogenous estrogens compared with those released by the activity of intracellular STS. These studies directly demonstrate that intratumor STS activity can support estrogen-dependent tumorigenicity in an experimental model, and may contribute to the promotion of human breast tumors (7).

We also continue limited studies of P-glycoprotein and its role in resistance. While we have shown previously that P-glycoprotein does not confer resistance to Tamoxifen (8), preliminary data suggest that it can confer resistance to Faslodex (ICI 182,780). We recently described a novel method for looking at the interactions between P-glycoprotein and its substrates. Studies with the

antiestrogens are planned for the future. **A manuscript describing this technology is included in the appendix.**

Each of these aims represent ongoing studies within the PI's laboratory and each will continue beyond the limitations of this award. We will continue to evaluate new methodologies and adapt our approaches and integrative studies in the light of published work from other laboratories. ***In this latter regard, the award will specifically allow the PI to spend more time critically appraising the state of science in the area of resistance to estrogens and antiestrogens in breast cancer.***

Key Research Accomplishments (bulleted)

- Submitted manuscript describing data from gene microarray and SAGE studies based on the data presented in the previous report. These data show the altered regulation of X-box binding protein-1, NFκB, NPM and IRF-1 in acquired antiestrogen resistance (manuscript submitted).

- Completed development of a new algorithm based on regression through the origin for normalizing gene expression microarray data (manuscript submitted).

- Published a major review on cellular and molecular mechanisms of antiestrogen resistance.

- Completed and published a study of the role of the estrone sulfatase and its potential to affect the endocrine responsiveness of human breast cancer cells. Data show that tumors can use the steroid sulfatase to convert sufficient sulfated estrogens to free estrogen to support cell growth *in vitro* and *in vivo.*

- Completed a pilot study applying gene expression microarrays to human core breast needle biopsies and prepared a manuscript. The data show that samples collected in RNALater can be used for gene expression microarray analyses, that high quality gene expression data can be obtained and that these expression data can be used to build neural network predictors that can accurately identify the phenotype of unknown samples as being cancer or noncancer.

Reportable Outcomes
Reportable outcomes are presented as A. Manuscripts, Abstracts and Presentations; B. Other Professional Activities; C. Degrees; and D. Funding Applied for Based on Work Supported by this Award.

A. Manuscripts, Abstracts and Presentations

Consistent with the goals of allowing the PI to spend time reevaluating his field, the PI has recently published a major review entitled "Cellular and Molecular Pharmacology of Antiestrogen Action and Resistance" in the peer review journal *Pharmacological Reviews*. A copy of this review and other published articles are included in the appendix. "In press" articles are not included in the appendix.

Manuscripts (published since last annual report)

1. **Clarke, R.** "Sex steroids in the mammary gland." *J Mammary Gland Biol Neoplasia,* 5: 245-250, 2000 (Appendix).

2. Hilakivi-Clarke, L.A., Cho, E., Onojafe, I. **& Clarke, R.** "Maternal exposure to tamoxifen during pregnancy increases mammary tumorigenesis among female offspring." *Clin Cancer Res* 6: 305-308, 2000 (Appendix).

3. Poola, I., Chatra, S., Koduri, S. & **Clarke, R.** "Identification of twenty alternatively splioced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targetd primer approach." *J Steroid Biochem Mol Biol* 72: 249-258, 2000 (Appendix).

4. **Clarke, R.,** Hilakivi-Clarke, L.A. & Trock, B. "Dietary and environmental sources of estrogenicity and breast cancer risk." *Biologist,* 48: 21-26, 2001 (Appendix).

5. Hilakivi-Clarke, L.A., Cho, E., deAssis, S., Olivo, S., Ealley, E., Bouker, K.B., Welch, J.N., Khan, G., **Clarke, R.** & Cabanes, A. "Maternal and prepubertal diet, mammary development and breast cancer risk." *J Nutr,* 131:154-157, 2001.

6. Lu, L., Leonessa, F., **Clarke, R.** & Wainer, I.W. "Competitive and allosteric interactions in ligand binding to P-glycoprotein as observed on an immobilized P-glycoprotein liquid chromatographic stationary phase." *Mol Pharmacol,* 59:62-68, 2001 (Appendix).

7. **Clarke, R.,** Leonessa, F., Welch, J.N., & Skaar, T.C. "Cellular and molecular pharmacology of antiestrogen action and resistance." *Pharmacol Rev*, 53: 25-72, 2001 (Appendix).

8. James, M.R., Skaar, T.C., Lee, R.Y., MacPherson, A., Zwiebel, J.A., Ahluwalia, B.S., Ampy, F. **& Clarke, R.** "Constitutive expression of the steroid sulfatase gene in estrogen-dependent MCF-7 human breast cancer cells." *Endocrinology,* 142:1497-1505, 2001 (Appendix).

9. **Clarke, R.** "Human tumors in animal hosts". In: *"Cancer Handbook",* Nature Publishing Group Reference Ltd., London, U.K., in press.

10. **Clarke,** R. & Dickson, R.B. "Animal models of endocrine responsive and unresponsive breast cancers". In: *"Endocrine Management of Breast Cancer"*, Eds: Robertson, J. & Hayes, D.F., Isis Medical Media Ltd., Faringdon, U.K., in press.

Abstracts

11. Guthrie, N., Hasegawa, S., Manners, G.D., Lippman, M.E., **Clarke, R.** & Vandenberg T. "Effect of citrus limonoids on human breast cancer cell growth in culture and in nude mice". *DOD Breast Cancer Research Program* pp504, 2000.

12. Pu, L.-P., Skaar, T.C., Leonessa, F. **& Clarke, R.** "Tumor suppressor genes in breast cancer". *DOD Breast Cancer Research Program* pp108, 2000.

13. Lee, R.Y., Skaar, T.C., Gu, Z., Leonessa, F. **& Clarke, R.** "Acquiring resistance to 9-*cis*-RA and 4HPR in breast cancer is not associated with the loss of RARα and RXRα mRNA expression". *DOD Breast Cancer Research Program* pp448, 2000.

14. Leonessa, F., Kim, J.-H.**& Clarke, R.** "C7 progesterone analogs for MDR1 reversal in breast cancer". *DOD Breast Cancer Research Program* pp710, 2000.

15. Nava, V., Murthy, S., Olivera, A., Poulton, S., Stoica, A., Martin, M.B., **Clarke, R.** & Spiegel, S. "Sphingosine kinase-1 promotes estrogen-dependent tumorigenesis of MCF-7 breast cancer cells through inhibition of apoptosis and induction of proliferation" *Keystone Symposium on Molecular Basis of Cancer: Signaling to Cell Growth and Cell Death.* Jan 9-14, 2001.

16. Chen, J., Chen, Y., Leonessa, F., **Clarke, R.,** Xu, X.-M., Liu, N., Underhill, C.B., Creswell, C. & Zhang, L. "Effect of tachyplesin on MDR overexpressing tumor cells." *Proc Am Assoc Cancer Res* 42: 812, 2001.

17. Welch, J.N., Chrysogelos, S. **& Clarke, R.** "Expression and function of the epidermal growth factor receptor in breast cancer cells exposed to chemotherapy." *Proc Am Assoc Cancer Res* 42: 938, 2001.

Presentations

1. 14th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Québec City, Quebec, ***Canada*** (2000)

2. Department of Oncology, Queen's University of Belfast, Belfast, ***U.K.*** (2000)

3. Federal Drug Administration, (Biometrics), Rockville, Maryland, ***U.S.A.*** (2000)

4. Indiana University Cancer Center, Indianapolis, Indiana, ***U.S.A.*** (2000)

5. University of Michigan Cancer Center, Ann Arbor, Michigan, ***U.S.A.*** (2000)

6. Fox Chase Cancer Center, Philadelphia, Pennsylvania, ***U.S.A.*** (2000)

7. National Cancer Institute, N.I.H., Specialized Programs of Research Excellence: 9th SPORE Investigators Workshop, Omni Shoreham Hotel, Washington, DC, ***U.S.A.*** (2001)

B. Other Professional Activities

Study Section Memberships and Other Grant Reviews

1. Member, American Institute for Cancer Research grant review study section "Panel I".

2. Member, NIH Site Visit Team that reviewed P01-CA94934-01, "Breast cancer drug discovery" at the University of Pittsburgh, Pittsburgh, PA (2001).

3. Member, NIH Site Visit Team that reviewed P01-CA64255-06, "Mechanisms of the hormonal prevention of breast cancer" at Baylor College of Medicine, Houston, TX (2000).

4. Member, U.S. Army Medical Research and Materiel Command Clinical and Experimental Therapeutics-1 study section.

5. Member, N.I.H. Grant Review Study Section "Chemical Pathology: Oncological Sciences Initial Review Group Special Emphasis Panel" ZRG2 SSS-1.

6. Member, Cancer Research Foundation of America's grant review panel.

7. Mail Review (Merit applications), Department of Veterans Affairs, Veterans Administration.

8. Mail Reviewer, Fighting Blindness, Dublin, Republic of Ireland.

9. Member, California Breast Cancer Research Program Study Section "Etiology & Prevention" (1999, 2001).

Election to Professional Societies
The PI was elected as a Fellow of the Royal Society of Medicine (U.K.) in 2001 based on overall contribution ot medical research (this is not a medical qualification), including the publications resulting from the work covered in this award..

The PI also was recently appointed as an Associate editor of *Cancer Research.*

C. Degrees
In 1999, the Principal Investigator was awarded the degree of Doctor of Science by his *alma mater*, The Queen's University of Belfast (United Kingdom). The DSc degree is awarded for a thesis containing significant research in breast cancer published since award of a PhD degree. In the U.K. and many other countries, a DSc is considered a "higher" degree than a PhD. This was reported in the previous annual report.

D. Funding Applied for Based on Work Supported by this Award
In direct support of this application, the PI successfully submitted an IDEA award to the DOD to fund studies into identifying the molecular mechanisms of antiestrogen resistance. In his role as mentor, the PI assisted and encouraged one of his predoctoral fellows to apply to the DOD for funding to study the role of EGF-receptor signaling in resistance to systemic therapies in breast cancer. This also was successfully funded. These applications were described in the previous annual report. The following grants have been awarded since the previous annual report.

1. **Co-Principal Investigator:** N.I.H. R21/R33-CA893231: "Intelligent mapping of gene expression profiles" for the further development of Cluster Analysis by standard Finite Normal Mixture Modeling using Akaike Information Criterion and Minimal Description Length analyses. Total funds awarded for this study: $600,000. Principal Investigator - Joseph Wang, PhD. This was originally funded as theR21 phase of a R21/R33. We recently successfully recompeted for the R33 phase.

2. **Principal Investigator:** NIH SBIR R41-GM61401-01A1 (Subcontract): "Development of multi-receptor LC stationary phases" to study the development of novel methods for identifying P-glycoprotein substrates. Total funds awarded for this subcontract: $59,800. Principal Investigator: William P. Purcell, PhD; President and CEO, Molecular Design International, Inc.

Conclusions

We have made considerable progress in addressing our proposed aims. The time made available to the PI through this Academic Award has resulted in several relevant publications and reviews, the ability to attract significant additional funding related to the research, and the generation of preliminary data that should lead to further publications in the coming year. The PI also continues to participate in other related professional activities. The time necessary to complete and successfully submit his DSc thesis, while not envisioned in the original application, also would not have been available had this award not been forthcoming. The PI will attempt to continue this level of productivity/activity in the remaining two years of support.

References

1. Sambrook, J., Fritsch, E. F., and Maniatis, M. Molecular Cloning: A laboratory Manual, 2 ed. NY: Cold Spring Harbor, 1989.
2. Wang, Y., Lu, J., Lee, R. Y., and Clarke, R. Iterative normalization of cDNA microarray data. IEEE Trans Info Tech Biomed, submitted.
3. Chen, Y., Dougherty, E. R., and Bittner M.L. Ratio-based decisions and the quantitative analysis of cDNA microarray images. J Biomed Optics, *2*: 364-374, 1997.
4. Revillion, F., Pawlowski, V., Hornez, L., and Peyrat, J. P. Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. Eur.J.Cancer, *36*: 1038-1042, 2000.
5. Wang, Y., Lin, S. H., Li, H., and Kung, S. Y. Data mapping by probabilistic modular networks and information theoretic criteria. IEEE Trans Signal Processing, *46*: 3378-3397, 1998.
6. Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad.Sci U.S.A, *95*: 14863-14868, 1998.
7. James, M. R., Skaar, T. C., Lee, R. Y., MacPherson, A., Zwiebel, J. A., Ahluwalia, B. S., Ampy, F., and Clarke, R. Constitutive expression of the steroid sulfatase gene supports the growth of MCF-7 human breast cancer cells in vitro and in vivo. Endocrinology, *142*: 1497-1505, 2001.
8. Clarke R., Currier, S., Kaplan, O., Boulay, V., Gottesman, M. & Dickson, R.B. "Effect of P-glycoprotein expression on sensitivity to hormones in MCF-7 human breast cancer cells" *J Natl Cancer Inst* 84: 1506-1512, 1992.

Appendices

Representative Reprints (reprints are not yet available for all publications)

Journal of Mammary Gland Biology and Neoplasia, Vol. 5, No. 3, 2000

Introduction and Overview: Sex Steroids in the Mammary Gland

Robert Clarke[1,2]

This issue of the *Journal of Mammary Gland Biology and Neoplasia* is entitled, "Sex Steroid Receptors," a subject of central importance in the biology of both normal and neoplastic mammary tissues. Since several issues of this journal would be required to deal with the topic in depth, the current issue primarily focuses on the essential roles of the naturally occurring estrogens and progesterone and their receptors. The nonovarian biosynthesis of estrogens is reviewed, as is the relative importance of estrogen receptors, coregulators of ER action, and the utility of ER in directing therapy in the breast cancers of both women and men. The issue concludes with a discussion of the role of progesterone and its receptors in the mammary gland.

In the mammary gland, estrogens can influence growth, development and function, responses reflecting their ability to influence the choice of mammary epithelial cells to proliferate, differentiate or die. These effects are induced by estrogenic exposures that occur either naturally, and/or because of environmental exposures, throughout life (1–5). Several of the factors affecting breast cancer risk in humans, and that implicate altered sex steroid exposures, are shown in Table I. While the relative risk conferred by each factor is small, i.e., generally less than two-fold, several factors are relatively common, and many women have more than one risk factor. While the exact risk factors may vary, each endocrine factor that increases risk has an approximate relative risk (RR) = 1.1–2.0. Ovariectomy before thirty-five years of age reduces breast cancer risk (RR = 2.1–4.0). Early menarche and late menopause would be expected to increase both the number of menstrual cycles and total lifetime estrogen exposure. Multiparity and prolonged lactation may reduce the number of targets for transformation, perhaps as a result of the associated differentiation events that occur within the breast. However, a significant short term increase in risk is associated with each pregnancy, perhaps reflecting the mitogenic effects of the high estrogenic environment of pregnancy on pre-existing breast lesions.

An association between increased serum estrogen concentrations and postmenopausal breast cancer is well established (6–8). This finding is consistent with the increased risk associated with hormone replacement studies seen in most large studies and meta analyses (1,9–12). Obesity, which is associated with an increased production of estrone by adipocytes, increases the risk of postmenopausal breast cancer (13). In contrast, the risk of premenopausal breast cancer is reduced in young obese women (14), but these women do not exhibit increased serum estrogen levels. Elevated serum estrogen concentrations are not clearly associated with the risk of developing premenopausal breast cancer (15).

Given the importance of estrogens in affecting breast cancer risk, this issue begins with two articles that describe the role of estrogen biosynthesis within both normal and neoplastic breast tissues. The biosynthetic pathways are described in detail in the articles by Miettenen *et al.* and Simpson. Essentially, adrenal androgens, primarily androstenedione in postmenopausal women, are aromatized to estrone. Estrone may be converted to its inactive sulfate by sulfotransferases and released again by the steroid sulfatase. Finally, the metabolism of estrone to 17β estradiol completes the biosynthetic pathway (16). Estrogen biosynthesis, both peripheral and within the breast, likely contributes to the high 17β estradiol

[1] W405A Research Building, Departments of Oncology and Physiology and Biophysics, Georgetown University School of Medicine, 3970 Reservoir Rd. NW, Washington, D.C. 20007.

[2] To whom correspondence should be addressed at E-mail: clarker@gunet.georgetown.edu

1083-3021/00/0700-0245$18.00/0 © 2000 Plenum Publishing Corporation

Table I. Some Estrogen-based Risk Factors for Breast Cancer

Exposure[a]	Measurement
Endogenous estrogens	High concentrations of serum or urinary estrogens (increase postmenopausal breast cancer risk—association unproven for premenopausal breast cancer)
	Early age (≤11 years) at menarche (increased risk)
	Late age (≥55 years) at natural menopause (increase risk of postmenopausal breast cancer)
	Postmenopausal obesity (increases estrogen production—high body mass index may be primarily associated with an increased risk of ER+/PR+ tumors—see Ref. 12)
	Premenopausal obesity (associated with lower risk of premenopausal breast cancer but not an increase in serum estrogen levels)
Exogenous estrogens	Oral contraceptives (increased risk of premenopausal breast cancer)
	Estrogen + progestogen replacement therapy. Recent evidence suggest that this is a more potent risk factor than estrogen alone-based therapies (increased risk of postmenopausal breast cancer)
Parity	Nulliparity (increased risk)
	Multiparity (reduces lifetime risk, as does prolonged lactation)
Diet/Lifestyle	Alcohol (may increase serum estrogens and breast cancer risk)
	Fat (controversial but can increase serum estrogens in some studies; may be restricted to an increase in the risk of developing breast cancer postmenopause)
	Physical exercise reduces risk (can delay onset of menarche, the number of ovulatory menstrual cycles, and serum estrogen levels—effect may be most apparent in premenopausal women)

[a]Data adapted from Hulka and Stark (13) unless otherwise noted in the text. Whether a risk factor primarily affects risk of disease in the premenopause or postmenopause is indicated.

concentrations in the tumors of both premenopausal and postmenopausal women. These concentrations range from below the limit of detection to approximately 5 μM (17). However, it is likely that most tumors contain ≤1 nM estradiol. The clinical utility of inhibitors of estrogen biosynthesis, particularly the use of aromatase inhibitors as second line endocrine therapies for postmenopausal patients, is well established (18). These drugs apparently inhibit both peripheral and intratumor aromatase activities.

In the first of these two reviews, Simpson describes the production of aromatase in mammary adipose tissues. This enzyme converts the ketone at position C3, in the A-ring of specific adrenal androgens, to a hydroxylated (at position C3) aromatic ring. Aromatization of testosterone produces estradiol, whereas estrone is produced when androstenedione is the substrate. Convincing evidence is provided for both the regulation of the aromatase gene by cytokines, and the use of different promoters in normal versus neoplastic tissues. The clinical relevance of aromatase activity is discussed, the article concluding with the suggestion that selective aromatase modulators (SAMs) could be developed. These compounds would take advantage of several aspects of aromatase biology, including tissue selective aromatase promoter usage and their differential activation by tissue specific signaling pathways.

In the second article, Miettenen *et al.* describe the importance of several enzymes involved in estrogen metabolism, including brief discussions of the aromatase and steroid sulfatase enzymes. Specific emphasis is placed upon the role of the 17β-hydroxysteroid dehydrogenases, which convert their biologically weaker substrates to more potent 17β-hydroxylated metabolites. The type 1 enzyme can use the estrone product of aromatase as a substrate. Thus, the reductive activity of 17β-hydroxysteroid dehydrogenase type 1 converts estrone's ketone at position C17 to a -hydroxyl, producing 17β-estradiol. The structures of both the gene's regulatory elements, and the functional domains of its encoded protein, are carefully described for 17β-hydroxysteroid dehydrogenase type 1.

When considered together, the two reviews of estrogen biosynthesis provide a compelling indication of the likely origins and importance of intratumoral estrogens. While there is clear evidence that tumors also can accumulate serum-derived estrogens (19,20), little doubt remains regarding important roles for both the intratumor activities of aromatase and 17β-hydroxysteroid dehydrogenase type 1. While only briefly discussed, there also is evidence implicating other enzymes. Many breast cancer cells express both steroid sulfotransferases and the steroid sulfatase (21). The former enzymes can inactivate steroids by replacing the C3 hydroxyl with a sulfate. Since the functional group on the steroidal A-ring is

generally believed important for receptor recognition and initial binding (3), sulfated estrogens are biologically inactive. However, the sulfate group can be removed by the steroid sulfatase, releasing the biologically active estrogen. The clinical relevance of the steroid sulfatase and sulfotransferases has not been studied in the same depth as aromatase. Nonetheless, these enzymes also may play an important role in affecting the availability and activity of intratumor estrogens.

Signaling induced by estrogenic ligands is dependent upon the expression and activation of the nuclear estrogen receptors (ER). A comparison of mice lacking ERα with those lacking only ERβ, implicate ERα as the predominant mediator of estrogenic effects on mouse mammary gland morphogenesis. ERα is required for ductal growth, since knock-out of ERα in mice leads to the generation of mammary glands reminiscent of those seen in newborn females [see (22) for recent review]. The mammary glands in these mice do not undergo the changes normally seen after puberty. In marked contrast, the mammary epithelia in adult ERβ knock-out mice appear normal, fill the mammary fat pads, and undergo apparently normal developmental changes associated with pregnancy and lactation. These data implicate ERα as the primary mediator of the effects of estrogens in the mouse mammary gland (22). The extent to which these ER knock-out models predict the relative importance/function of each ER in other species is unclear. Both the normal human breast and rat mammary gland express ERβ, whereas this expression is already very low/undetectable in some normal mouse mammary glands (22,25). Others detect wild type ERβ and ERβ splice variant mRNAs in the lactating mouse mammary gland, but it is not known if these are translated into functional protein (37).

While nongenomic effects of steroids have been described in several tissues (26,27), it is widely believed that the major function of sex steroids in the mammary gland is to alter the transcriptional regulatory activities of their nuclear receptors. Thus, three consecutive articles review the expression of ER in breast tumors, in both women and men, and the possible need for a significant reassessment of our understanding of ER function in the light of the recently described ERβ.

Ali and Coombes consider the role of ERα in breast cancer, with ER structure and function briefly reviewed. ERα expression is shown to correlate more strongly with response to antiestrogens and a good prognosis than ERβ. The lack of a clear role for splice variants of ERα in either response or resistance to endocrine therapy, or in predicting clinical outcomes, also is discussed. Phosphorylation of ERα is described, the discussion concluding with a description of the possible role of protein kinases in producing a ligand-independent activation of ERα that could contribute to endocrine resistance. The authors finally conclude that some breast cancer cells may become resistant to endocrine therapies through an adaptive response to the selective pressure of the treatment.

The article by Olsson focuses specifically on a comparison of breast cancers in men and women. An analysis of the literature on breast cancers in men is presented and extended with data from the author's institution. Both similarities and differences are noted between breast tumors arising in women and men. For example, lobular and mucinous carcinomas are rarely reported in male breast cancer cases. Age at diagnosis tends to be approximately ten years later in men compared with women. However, there have been few large studies of male breast cancer, particularly where the cases have been directly compared with breast cancers in women.

The third paper reviewing the role of estrogen receptors provides a provocative assessment of the field from the perspective of ER. Warner *et al.* suggest that ERβ has opposite effects to ERα, and may be antiproliferative when activated in breast cancers. Evidence for both the tissue specificity of ERβ expression and action is presented. These observations suggest that, in the mammary gland, estrogen action is dependent upon the ration of ERα:ERβ splice variant (ERβ ins). Four estrogen responsive phenotypes are predicted, based on the relative expression of the ERs, each eliciting a different response to estrogenic stimulation. The possible roles of ERβ in development and function of normal and neoplastic tissues are discussed, providing a unique perspective on estrogen action.

The next two articles relate to factors that may modulate ER function. The review by Safe *et al.* describes the aryl hydrocarbon receptor (AhR), which binds the environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with high affinity. In the immature and ovariectomized rodent uterus, TCDD inhibits some estrogenic events. The authors also describe that, in breast cancer cells, TCDD blocks the estrogenic regulation of several estrogen-regulated genes including c-fos and pS2.

Safe *et al.* discuss how the putative "antiestrogenic" effects mediated by AhR may involve cross

talk between the AhR and ER. One possibility is the activation of a proteasome-dependent degradation of ER. 5′-regulatory regions called inhibitory dioxin responsive elements, which bind AhR, also may disrupt ER action. The precise mechanism appears complex. For example, the authors suggest that dioxin responsive elements may bind AhR in orientations that could either disrupt formation of a productive ER-regulated transcription complex and/or inhibit its function. Finally, the possibility that selective AhR modulators (SAhRMs) could be useful is presented. The authors indicate that several such compounds are currently under development/investigation.

The second of these two articles looks at specific cellular factors that affect ER function. Receptor coregulators can be considered as either coactivators or corepressors of receptors' transcriptional activities. The number of these coregulators continues to increase, and Edwards provides an extensive review and update of this rapidly changing field. The structure and function of receptor coregulators are discussed, as is their possible contribution to estrogen responsiveness and endocrine resistance. With the possible effects of interactions with other receptor systems, such as the AhR (see Safe *et al.* in this issue), retinoic acid receptors (28,29) and peroxisome proliferator-activated receptors (30), changes in coregulator expression/function likely significantly contribute to the biological consequences of steroid hormone receptor activation and function.

It is apparent that the patterns of proteins expressed within a cell, often called the cellular context (31), substantially affect steroid hormone receptor function. This context may be relatively fluid in both normal and neoplastic cells, continually changing in response to external signals. The importance of tumor-stromal interactions has been well documented. For example, many breast tumors are characterized by a marked stromal response (desmoplasia), frequently comprising reticuloendothelial cells (32). Some of these infiltrating cells secrete growth factors, cytokines, and even estrogens. Receptors for many of these secreted molecules are present both within and on the plasma membranes of tumor cells. Similar interactions between normal mammary epithelia and stroma are well documented, some being described in detail in this issue (see reviews by Lydon *et al.* and Warner *et al.*).

Paracrine interactions between stromal and epithelial cells likely initiate cell signaling within normal and malignant epithelial cells. Thus, stromal cells may alter the cellular context of their adjacent epithelial partners (31). Some of these perturbations may affect the transcription, translation and/or modification of molecules required, and/or necessary but not sufficient, for optimal signaling through ER-regulated events. Others may interact with ER signaling pathways, downstream of active ER-regulated transcription complexes, to affect outcomes. For example, the receptors for several estrogen regulated growth factors, including members of the epidermal growth factor (EGF) family, have tyrosine kinase activities that activate mitogen activated protein kinase (MAPK) signaling (see Ali and Coombes in this issue). MAPK activity, which is induced downstream of the receptor in an EGF-R signaling pathway (33,34), can phosphorylate ER and produce a ligand-independent ER activation (35). This observation may partly explain the apparent estrogenic effects of EGF, and why these are lost in ERα null mice (36). A possible cycle between ER, activated independent of ligand, and growth factor signaling, perhaps driven by paracrine/autocrine interactions, could arise in some cells.

The role of estrogens and their receptors has been widely studied with respect to mammary gland development and breast cancer risk. The role of progestins and their receptors also has received attention. This issue concludes with an appraisal of the role of progesterone and progesterone receptors in mammary gland biology. Lydon *et al.* provide a valuable synthesis of the activities of progesterone, particularly in the pregnancy-associated ductal proliferation and lobuloalveolar differentiation of mammary epithelium. Evidence is presented demonstrating that progesterone's effects are specific, progesterone having effects on both proliferation and differentiation within the gland. Much of the supporting evidence is obtained from extensive studies in progesterone receptor (PR) null mice, which are described in some detail.

These authors also investigated the relative importance of epithelial-stromal and epithelial-epithelial interactions in mediating the effects of progesterone. Data obtained from PR null mice using innovative mammary gland transplantation studies, clearly implicate inter-epithelial interactions as being critical in these activities. Thus, a model of progesterone's function is described where a paracrine interaction occurs in which PR expressing cells direct the proliferation and/or differentiation of adjacent epithelial cells that do not express PR.

The articles in this issue of the Journal provide insight into several critical issues concerning the role of sex steroids in mammary gland biology. While

much of the discussion relates to estrogens, the critical role of progesterone in mammary gland biology is now becoming more apparent (see Lydon *et al.* in this issue). Interactions between estrogens and progesterone (and 17-hydroxyprogesterone) have been known for many years. For example, mitogenesis in the normal adult mammary gland occurs during the luteal phase of the menstrual cycle. Thus, estrogens alone may be insufficient for this proliferative event, which likely requires the increases in both estrogens and progesterone that concurrently occur only in the luteal phase. Further evidence of the importance of progesterone is evident in recent studies of hormone replacement therapy. There is now compelling data that the risk of developing breast cancer is significantly higher in women taking estrogen + progestin-based hormone replacement therapies, when compared with women taking "unopposed" estrogenic therapies (9,10).

Several fields reviewed in this issue continue to advance rapidly, particularly in the areas of steroid hormone receptor function and the cellular/molecular biology of cell-cell interactions. As aspects of these areas of investigation come together, we will begin to better understand how both intracellular and intercellular signaling affect steroid hormone receptor function, and how the consequences of these interactions determine the biology and physiology of the normal and neoplastic mammary gland.

REFERENCES

1. Collaborative Group on Hormonal Factors in Breast Cancer (1997). Breast cancer and hormone replacement therapy: Collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. *Lancet* **350:**1047–1059.
2. Collaborative Group on Hormonal Factors in Breast Cancer (1996). Breast cancer and hormonal contraceptives: Collaborative reanalysis of individual data on 53,297 women with breast cancer and 100,239 women without breast cancer from 54 epidemiological studies. *Lancet* **347:**1713–1727.
3. R. Clarke, L. A. Hilakivi-Clarke, E. Cho, M. R. James, and F. Leonessa (1996). Estrogens, phytoestrogens and breast cancer. *Adv. Exp. Biol. Med.* **401:**63–86.
4. L. A. Hilakivi-Clarke, R. Clarke, I. Onojafe, M. Raygada, E. Cho, and M. E. Lippman (1997). A high fat diet during pregnancy increases mammary epithelial density and breast cancer risk among femal rat offspring. *Proc. Natl. Acad. Sci. U.S.A.* **94:**9372–9377.
5. L. A. Hilakivi-Clarke, I. Onojafe, M. Raygada, E. Cho, R. Clarke, and M. E. Lippman (1996). Breast cancer risk in rats fed a diet high in n-6 polyunsaturated fatty acids during pregnancy. *J. Natl. Cancer Inst.* **88:**1821–1827.
6. F. Berrino, P. Muti, A. Micheli, V. Krogh, R. Sciajno, P. Pisani, S. Panico, and G. Secreto (1996). Serum sex hormone levels after menopause and subsequent breast cancer. *J. Natl. Cancer Inst.* **88:**291–296.
7. A. Zeleniuch-Jacquotte, P. Toniolo, M. Levitz, R. E. Shore, K. L. Koenig, S. Banerjee, P. Strax, and B. S. Pasternack (1995). Endogenous estrogens and risk of breast cancer by estrogen receptor status: A prospective study in postmenopausal women. *Cancer Epidemiol. Biomarkers Prev.* **4:**857–860.
8. P. G. Toniolo, M. Levitz, A. Zeleniuch-Jacquotte, S. Banerjee, K. L. Koenig, R. E. Shore, P. Strax, and B. S. Pasternack (1995). A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J. Natl. Cancer Inst.* **87:**190–197.
9. C. Schairer, J. Lubin, R. Troisi, S. Sturgeon, L. Brinton, and R. Hoover (2000). Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk. *J. Am. Med. Assoc.* **283:**485–491.
10. C. Magnusson, J. A. Baron, N. Correia, R. Bergstrom, H. O. Adami, and I. Persson (1999). Breast-cancer risk following long-term oestrogen- and oestrogen-progestin-replacement therapy. *Int. J. Cancer* **81:**339–344.
11. K. K. Steinberg, S. J. Smith, S. B. Thacker, and D. F. Stroup (1994). Breast cancer risk and duration of estrogen use: The role of study design in meta-analysis. *Epidemiology* **5:**415–421.
12. M. Sillero-Arenas, M. Delgado-Rodriguez, R. Rodigues-Canteras, A. Bueno-Cavanillas, and R. Galvez-Vargas (1992). Menopausal hormone replacement therapy and breast cancer: A meta-analysis. *Obstet. Gynecol.* **79:**286–294.
13. S. Hulka and A. T. Stark (1995). Breast cancer: Cause and prevention. *Lancet* **346:**883–887.
14. S. M. Enger, R. K. Ross, A. Paganini-Hill, C. L. Carpenter, and L. Bernstein (2000). Body size, physical activity, and breast cancer hormone receptor status: Results from two case-control studies. *Cancer Epidemiol. Biomarkers Prev.* **9:**681–687.
15. T. J. Key, D. Y. Wang, J. B. Brown, C. Hermon, D. S. Allen, J. W. Moore, R. D. Bulbrook, I. S. Fentiman, and M. C. Pike (1996). A prospective study of urinary oestrogen excretion and breast cancer risk. *Brit. J. Cancer* **73:**1615–1619.
16. J. L. Falany and C. N. Falany (1996). Expression of cytosolic sulfotransferases in normal mammary epithelial cells and breast cancer cell lines. *Cancer Res.* **56:**1551–1555.
17. D. S. Millington (1975). Determination of hormonal steroid concentrations in biological extracts by high resolution mass fragmentography. *J. Steroid. Biochem.* **6:**239–245.
18. M. Dowsett (1997). Future use of aromatase inhibitors in breast cancer. *J. Steroid Biochem. Mol. Biol.* **61:**261–266.
19. S. Masamura, S. J. Santner, P. Gimotty, J. Georger, and R. J. Santen (1997). Mechanism for maintenance of high breast tumor estradiol concentrations in the absence of ovarian function: Role of very high affinity tissue uptake. *Breast Cancer Res. Treat.* **42:**215–226.
20. W. R. Miller (1997). Uptake and synthesis of steroid hormones by the breast. *Endocrine-Related Cancer* **4:**307–311.
21. J. R. Pasqualini, B. Schatz, C. Varin, and B. L. Nguyen (1992). Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer. *J. Steroid Biochem. Mol. Biol.* **41:**323–329.
22. J. F. Couse and K. S. Korach (1999). Estrogen receptor null mice: What have we learned and where will they lead us? [published erratum appears in *Endocrine Rev.* **20**(4):459]. *Endocrine Rev.* **20:**358–417.

23. E. Enmark, M. Pelto-Huikko, K. Grandien, S. Lagercrantz, J. Lagercrantz, G. Fried, M. Nordenskjold, and J.-A.Gustafsson (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J. Clin. Endocrinol. Metab.* **82**:4258–4265.
24. S. Saji, E. V. Jensen, S. Nilsson, T. Rylander, M. Warner, and J. A. Gustafsson (2000). Estrogen receptors alpha and beta in the rodent mammary gland. *Proc. Natl. Acad. Sci. U.S.A* **97**:337–342.
25. J. F. Couse, J. Lindzey, K. Grandien, J. A. Gustafsson, and K. S. Korach (1997). Tissue distribution and quantitative analysis of estrogen receptor- alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* **138**:4613–4621.
26. D. Duval, S. Durant, and F. Homo-Delarche (1983). Nongenomic effects of steroids. Interactions of steroid molecules with membrane structures and functions. *Biochim. Biophys. Acta* **737**:409–442.
27. M. Farhat, S. Abi-Younes, R. Vargas, R. M. Wolfe, R. Clarke, and P. W. Ramwell (1992). Vascular nongenomic effects of estrogen. In P. W. Ramwell, G. Rubanyi, and E. Schillinger, (eds.), *Sex Steroids and the Cardiovascular System*, Springer-Verlag, Berlin, pp. 145–159.
28. C. M. Klinge, D. L. Bodenner, D. Desai, R. M. Niles, and A. M. Traish (1997). Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements *in vitro*. *Nucleic Acids Res.* **25**:1903–1912.
29. A. K. Rishi, Z. M. Shao, R. G. Baumann, X.-S. Li, M. S. Sheikh, S. Kimura, N. Bashirelahi, and J. A. Fontana (1995). Estradiol regulation of the human retinoic acid receptor a gene in human breast carcinoma cells is mediated via an imperfect half-palindromic estrogen response element and Sp1 motifs. *Cancer Res.* **55**:4999–5006.
30. H. Keller, F. Givel, M. Perroud, and W. Wahli (1995). Signaling cross-talk between peroxisome proliferator-activated receptor/retinoid X receptor and estrogen receptor through estrogen response elements. *Mol. Endocrinol.* **9**:794–804.
31. R. Clarke and N. Brünner (1996). Acquired estrogen independence and antiestrogen resistance in breast cancer: Estrogen receptor-driven phenotypes? *Trends Endocrinol. Metab.* **7**:25–35.
32. R. Clarke, R. B. Dickson, and M. E. Lippman (1992). Hormonal aspects of breast cancer: Growth factors, drugs and stromal interactions. *Crit. Rev. Oncol. Hematol.* **12**:1–23.
33. C. Xing and W. Imagawa (1999). Altered MAP kinase (ERK1,2) regualtion in primary cultures of mammary tumor cells: Elevated basal activity and sustained response to EGF. *Carcinogenesis* **20**:1201–1208.
34. A. M. Tari, M. C. Hung, K. Li, and G. Lopez-Berenstein (1999). Growth inhibition of breast cancer cells by Grb2 down-regulation is correlated with inactivation of mitogen-activated protein kinase in EGFR, but not in ErbB2, cells. *Oncogene* **18**:1325–1332.
35. S. Kato, H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, D. Metzger, and P. Chambon (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**:1491–1494.
36. S. W. Curtis, T. Washburn, C. Sewall, R. P. DiAugustine, J. Lindzey, J. F. Couse, and K. S. Korach (1996). Physiological coupling of growth factor and steroid receptor signaling pathways: Estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **93**:12626–12630.
37. B. Lu, E. Leygue, H. Dotzlaw, L. J. Murphy, L. C. Murphy, and P. H. Watson (1998). Estrogen receptor-β mRNA variants in human and murine tissues. *Mol. Cell. Endocrinol.* **138**:199–203.

PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 72 (2000) 249–258

The Journal of
Steroid Biochemistry
&
Molecular Biology

www.elsevier.com/locate/jsbmb

Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach

Indra Poola[a,b,*], Sailaja Koduri[a], Shubha Chatra[a], Robert Clarke[c]

[a]*Department of Pharmacology, Howard University School of Medicine, 520 W. Street, NW, Washington, DC 20059, USA*
[b]*Department of Biochemistry and Molecular Biology, Howard University School of Medicine, Washington, DC 20059, USA*
[c]*Lombardi Cancer Center and Department of Physiology and Biophysics, Georgetown University Medical School, Washington, DC 20007, USA*

Received 12 July 1999; accepted 20 December 1999

Abstract

Estrogen receptor (ER) alpha splice variant transcript profiles were analyzed by RT PCR in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6, and three ER positive malignant breast tumors using targeted primers which specifically anneal to the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that largest possible transcripts were amplified between exons 1 and 8. The results described here show that each splice specific primer amplified not only the single exon deleted transcript but also a number of related transcripts that have deletions in various combinations of exons. The exon 2Δ specific primer amplified five transcripts that have deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4–5, and exons 2 and 4–6. The exon 3Δ specific primer amplified two transcripts that have deletions in exon 3, and exons 3 and 7. The exon 2–3Δ specific primer amplified three products that have deletions in exons 2–3, exons 2–3 and 7 and exons 2–3, 5 and 7. The exon 4Δ specific primer amplified two products that have deletions in exon 4, and exons 4 and 7. The exon 5Δ specific primer amplified three transcripts, that have deletions in exon 5, exons 5 and 2, and exons 5, and 2–3. The 6Δ specific primer amplified only one transcript that has a deletion in exon 6. The 7Δ specific primer amplified four transcripts, that have deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5. None of the above splice specific primers amplified the wild type ER sequences. The six ER positive cell lines differed in the patterns of the variant transcripts and among the three ER negative cell lines analyzed, only MDA-MB-435 showed the presence of exon 2Δ and exon 4Δ transcripts. Analyses in the tumor samples indicated that the above transcripts are extensively modified.

Keywords: ER alpha splice variants; Splice targeted primers; Sequential exon deletions; Breast cancer cell lines and tumors; Distant exon deletions

1. Introduction

The ER alpha mRNA undergoes alternate splicing, generating transcripts containing single, double or multiple exon deletions. The presence of ERα transcripts with deletions in exons 2-, 3-, 2–3, 2–5, 4-, 5-, 6- and 7 has been described in breast cancer cell lines and normal- and malignant breast tissue samples [1–4]. Although the exact function(s) of these splice variants is not established, it has been hypothesized that the splice variant mRNAs may result in proteins that differ in activity. These may differentially modulate the ER signalling pathway in normal tissues. Consequently, changes in the balance of these transcripts could perturb the ER signaling pathway and contrib-

* Corresponding author. Tel.: +1-202-806-5554; fax: +1-202-806-5553/4453.
E-mail address: poola@garvey.pharm.med.howard.edu (I. Poola).

0960-0760/00/$ - see front matter
PII: S0960-0760(00)00033-9

Nomenclature

ER	estrogen receptor	Exon Δ	exon deletion
PgR	progesterone receptor	AX	anti-sense
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	SX	sense

ute to tumor progression. Several studies suggested that the expression of certain exon deletion transcripts is deregulated during breast tumorigenesis. It was shown that the exon 5 deletion transcript was significantly elevated in ER^- PgR^+ breast tumor tissues [5]. Elevated levels of exon 7 splice transcripts have also been reported in $ER^+/PR^-/pS2$ compared to ER^+/PgR^+ tumors [6]. It has been reported that expression of the exon 3-deleted mRNA is reduced in breast tumor tissue compared with normal tissue [7]. Differential expression of exon 5 and exon 7 deletion transcripts also seem to influence the estrogen responsiveness in breast cancer cell lines [8]. All these reports suggest that expression of some ER variants is altered in human breast tumors and may contribute to tumorigenesis, tumor progression and response to hormones. Therefore, it is important to qualitatively and quantitatively investigate the levels and pattern of ER splice variant expression between normal and neoplastic tissues, and amongst groups of tumors with different characteristics. Yet, there are no specific methods available which can precisely detect and quantify the alternatively spliced ER molecules.

Conventionally, the ER exon deletion variant transcripts are characterized by co-amplification with the wild type sequences using reverse transcription polymerase chain reaction (RT-PCR) approaches which by virtue of specific primer design are focussed on small regions of the known wild type mRNA. However, there are several practical limitations to this approach. Firstly, the threshold of detection — since the wild type transcripts are present in large excess to alternatively spliced molecules, a competitive amplification occurs amongst the wild type and all the alternatively spliced transcripts. Detection of products corresponding to alternatively spliced molecules depends upon the relative expression levels of their mRNA species within the sample. Thus, spliced transcripts expressed at low levels may fall below the threshold of detection. Secondly, this approach cannot distinguish those mRNAs with multiple deletions in distant exons. For example, an ER transcript which has deletions in exons 2 and 7 cannot be distinguished from transcripts having single deletions in exon 2 or exon 7 by this method, and finally transcripts with similar sized deletions cannot be distinguished by gel exclusion chromatography.

To circumvent all the above described limitations, we have developed a new approach to characterize the alternatively spliced molecules. This involves the targeted amplification of the alternatively spliced molecules as separate gene populations without co-amplification of wild type molecules using specific primers designed for the alternative splice junctions [9]. In the current study, we analyzed the ER single, double, and multiple exon deletion variant transcripts in breast cancer cell lines and tumors by RT PCR using the splice targeted primers. We show here that each splice specific primer amplifies not only the single exon deleted transcript but also a number of related transcripts with deletions in various combinations of exons. Our results also show that several alternatively spliced molecules are either missing or extensively modified in tumor samples.

2. Materials and methods

AmpliTaq PCR core kits and QIAquick gel extraction kits were obtained from QIAGEN, Santa Clara, CA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. Reverse transcriptase kits were purchased from Applied Biosystems. The pCR™2.1-TOPO cloning vector was obtained from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, MD. The total RNA samples from breast cancer cell lines and tumors were prepared using Trizol reagent (Gibco-BRL). The integrity of all the RNA preparations was confirmed by electrophoresis and ethidium bromide staining and amplification of the constitutively expressed gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The ER status of all the tumors used in the current study was determined immunohistochemically by Oncotech laboratories using monoclonal antibodies against the NH_2 terminal (A/B region) of the receptor. The six tumors used were ER positive by the above immunohistochemical method.

2.1. Targeted primers for the amplification of single, double and multiple exon deletion variant cDNAs of ER

We have previously shown that the primers targeted at the alternate splice junctions that have a minimum of three out of four unique bases at the extreme 3′ end

will specifically amplify the spliced junction without amplifying the flanking wild type exons and in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction [9]. The splice specific primers used in the current study were designed based on these principles. The splice specific primers used for amplifying 2Δ, 3Δ, 2–3Δ, 4Δ, 5Δ, 6Δ, and 7Δ were ER SX1/3, 5′ CGCCGGCATTC-TACAG 1/3 GACAT 3′ (positions, exon 1, bp 669–684, and exon 3, bp 876–880), ER SX2/4, 5′ AAGA-GAAGTATTCAAG 2/4 GGATA 3′ (positions, exon 2, bp 860–875 and exon 4, bp 993–997), ER SX1/4, 5′ GCCGGCATTCTACAG 1/4 GGATAC 3′ (positions, exon 1, bp 670–684 and exon 4, bp 993–998), ER SX3/5, 5′ GTGGGAATGATGAAAGGTG 3/5 GCTTT 3′ (positions, exon 3, bp 974–992 and exon 5, bp 1329–1333), ER AX4/6, 5′ ATTTTCCCTGGTTC 6/4 CTGGCAC 3′ (positions, exon 6, bp 1481–1468 and exon 4, bp 1328–1322), ER AX 5/7, 5′ CAGAAATGTGTACACTC 7/5 CTGT 3′ (positions, exon 7, bp 1618–1603 and exon 5, bp 1468–1465) and ER AX6/8, 5′ CTCCATGCCTTTGTTA 8/6 CAGAA 3′ (positions, exon 8, bp 1801–1786 and exon 6, bp 1601–1597), respectively. The partner primer for 2Δ, 3Δ, 2–3Δ, and 4Δ splice specific primers was ERA, 5′ GCACTTCATGCTGTACAGATGC 3′ (position, exon 8, bp 1822–1801) and for 5Δ, 6Δ, and 7Δ primers was ERS, 5′ TGCCCTACTACCTGGAGAACG 3′ (position, exon 1, bp 615–635). The sequence and locations of all the primers described here are based on the full length ER cDNA sequence published by Green et al. [10].

2.2. Reverse transcription and PCR

The total RNA was reverse transcribed to cDNA using Maloney Murine Leukemia Virus reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 μg of total RNA, 2.5 units of MuLV reverse transcriptase, 1 mM each of dNTPs, 2.5 μM random hexamers, 20 U of RNAse inhibitor, 5 mM $MgCl_2$ and 1 × PCR buffer in a total volume of 20 μl. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 min, followed by incubations at 42°C for 15 min, 99°C for 5 min and finally 5°C for 5 min. The polymerase chain reactions were performed in an automatic thermal cycler (MJ Research) as described previously [11] in a 25 μl reaction volume containing the cDNA reverse transcribed from 250 ng of total RNA, 1 × PCR buffer, 1 × Q solution, 200 μM each of dNTPs, 2 μM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The GAPDH was amplified using a sense primer, 5′ AAGGCTGA-GAACGGGAAGCTTGTCATCAAT 3′ (position, exon 3, bp 241–270), an anti- sense primer, 5′ TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3′ (position, exon 7, bp 740–711) [12] and cDNAs prepared from reverse transcription of 25 ng of total RNA. To amplify the exon deletion variant cDNAs in the tumor samples, PCRs were performed using cDNAs prepared from reverse transcription of 500–750 ng of total RNA. The PCR conditions were initial denaturation for 1 min at 95°C followed by 94°C for 1 min, annealing for 1 min at the specified temperature depending on the primer pair used, extension for 2 min at 72°C for 40 cycles and final extension for 10 min at 72°C. The annealing temperature for 2Δ, 2–3Δ, 4Δ and 6Δ specific primers was at 61°C, for 3Δ and 7Δ primers at 55°C and for 5Δ specific primer at 65°C.

2.3. Detection and sequence analysis of PCR products

To detect the PCR amplified ER splice variant products from cell lines, an aliquot (4–7 μl) was electrophoresed in 1% agarose gels in Tris-acetate EDTA buffer and detected by ethidium bromide staining. To detect the PCR products of GAPDH, 1 μl was electrophoresed and the ER splice variant products amplified from tumor samples, 12–25 μl of the products were analyzed on the gel. To determine the identity of the PCR amplified ER splice variant products, they were electrophoresed in 1.2% agarose gels and purified individually using the QIAquick gel extraction kit. The purified products were cloned into pCR™2.1-TOPO vector and sequenced by cycle sequencing method on an automated DNA sequencer (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

3. Results

We analyzed the ER single, double, and multiple exon deletion transcripts by RT PCR using primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that the largest possible transcripts were amplified between the exons 1 and 8. This permitted the amplification of not only the single exon deletion transcripts but also those with multiple deletions in distant exons. The PCR analyses were carried out in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, and LCC9 and three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6. Three ER positive breast tumor samples were also included to test the applicability of splice targeted primer approach in analyzing the above transcripts in clinical samples. The results described here on the analysis of various alternatively spliced ER transcripts were repeated in 20 experimen-

tal trials with cell lines and three trials with tumor samples.

3.1. Analysis of exon 2Δ transcripts

The exon 2Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 1. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. The ER positive cell lines, MCF-7, ZR-75, LCC1, LCC2, and LCC9, amplified three major bands of sizes about 960, 780, and 640 bp. The cell line T47D did not amplify the 960 band, instead it amplified two products which are higher than 960 bp. All six ER positive cell lines amplified several minor bands ranging from 480–330 bp. Unexpectedly, one of the three ER negative cell lines tested, MDA-MB-435, also amplified 960, 640 and 480 bp bands and three additional bands that showed lower mobility than the 960 bp band. Tumor 3 did not amplify any product. Tumor 2 amplified minor bands at 640 and 480 bp and tumor 1 amplified only the 480 bp one as a minor band. To determine the identify of the above products, the PCR products from LCC1 cells were cloned and sequenced. The 960, 780, 640, 480 and 330 bp products were identified as ER transcripts with deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4–5, and exons 2 and 4–6, respectively (Fig. 1B). It was also found that the exons 2Δ and 4–6Δ product had 20 bps missing in exon 7. Fig. 1(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.2. Analysis of exon 3Δ transcripts

The exon 3Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 2. Lanes M1 contain Gibco-BRL 100 bp ladders. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes about 845 and 661 bp. The ER negative cell lines and two of the tumors in the study did not amplify these two bands. Only one of the three tumors (Tumor 5) amplified the 845 bp but not 661 bp product. To determine the identity of the above products, the PCR products from LCC1 cell line were cloned and sequenced. The 845 and 661 bp products were identified as ER transcripts that have deletions in exon 3, and exons 3 and 7, respectively (Fig. 2B). Fig. 2(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.3. Analysis of exons 2–3Δ transcripts

The PCR product profiles of exon 2–3Δ transcripts

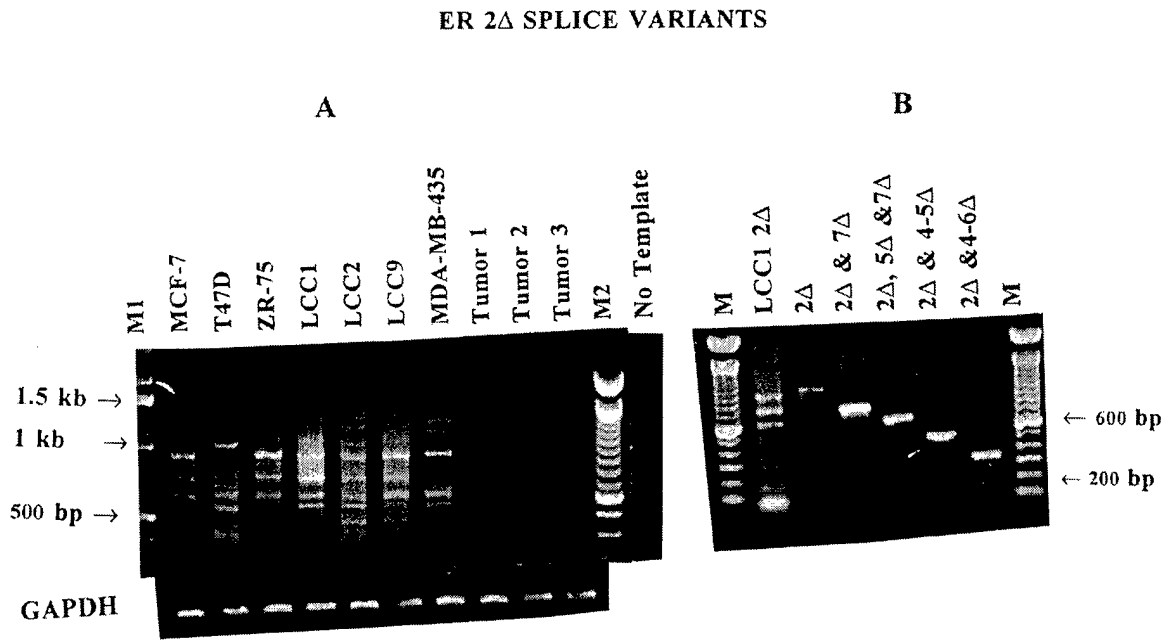


Fig. 1. Analysis of ER exon 2Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2Δ specific primer. The ER exon 2Δ transcripts were analyzed using the specific sense primer, ER SX1/3, and an anti-sense primer ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435, and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lanes M have 100 bp ladders.

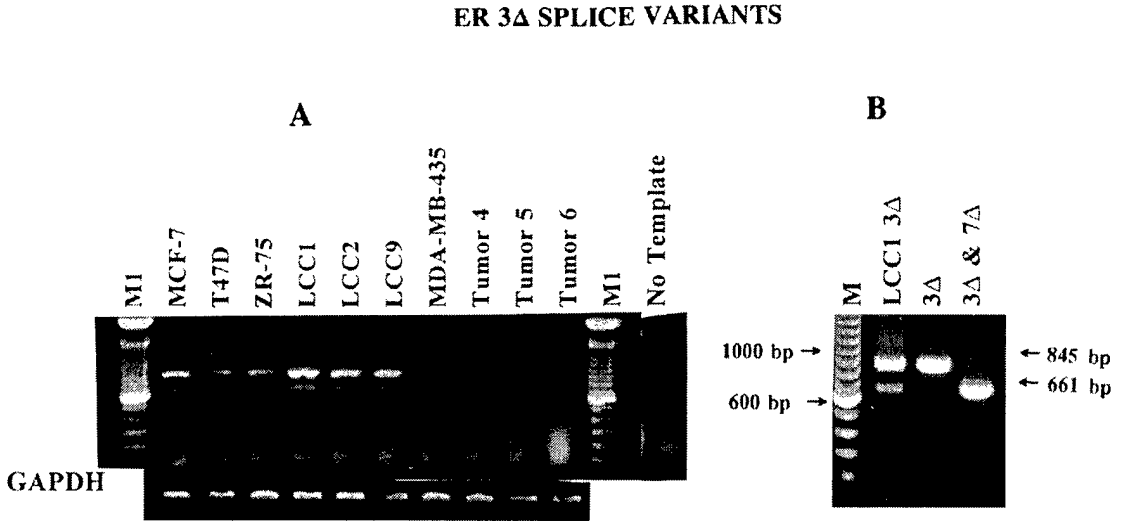


Fig. 2. Analysis of ER exon 3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 3Δ specific primer. The ER exon 3Δ transcripts were analyzed using ER SX2/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

in seven cell lines and three tumors are shown in Fig. 3(A). The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. All the six ER positive cell lines amplified three products with approximate sizes of 840, 660 and 520 bp. Two minor bands between 840 and 660 bp are also seen. One of the three ER negative cell line, MDA-MB-435, generated a minor product slightly bigger than the 840 bp product. To determine the identities of 840, 660 and 520 bp products, the PCR products from LCC1 were cloned and sequenced. The 840, 660, and 520 bp products were identified as ER transcripts with deletions

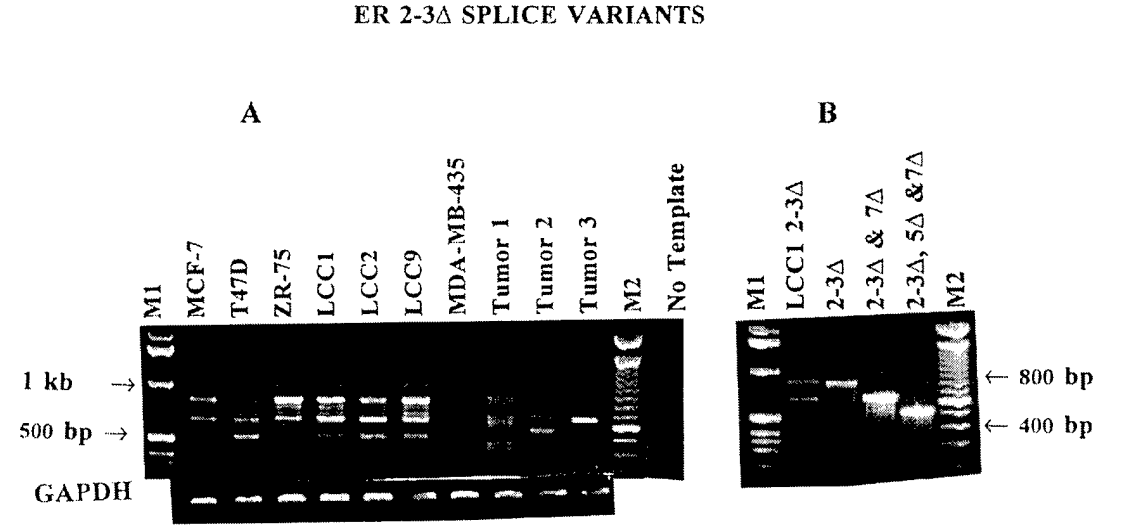


Fig. 3. Analysis of exons 2–3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2–3Δ specific primer. The exon 2–3Δ transcripts were analyzed using ER SX1/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. In both A and B panels, lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis.

in exons 2–3, exons 2–3 and 7, and exons 2–3, 5 and 7, respectively (Fig. 3B). Tumor 1 generated three bands of which two corresponded to exons 2–3Δ, and exons 2–3Δ and 7Δ. The third band showed slightly higher mobility than the exons 2–3Δ, 5Δ and 7Δ product. Tumor 2 amplified two bands of approximate sizes 700 and 550 bp, which are slightly higher than the exons 2–3Δ and 7Δ, and exons 2–3Δ, 5Δ and 7Δ products. The third tumor generated only the exons 2–3Δ and 7Δ product. Fig. 3(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.4. Analysis of exon 4Δ transcripts

The exon 4Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 4. The lane M1 contains Gibco-BRL 100 bp ladder. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes about 512, and 328 bp. One of the three ER negative cell lines, MDA-MB-435, also amplified faint bands of 512 and 328 bp. All three of the tumors tested amplified these two products. To identify the above products, the PCR products from LCC9 cell line were cloned and sequenced. The 512, and 328 bp products were identified as ER transcripts with deletions in exon 4, and exons 4 and 7, respectively (Fig. 4B). Fig. 4(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.5. Analysis of exon 5Δ transcripts

The profiles of exon 5Δ transcripts in seven cell lines and three tumors are shown in Fig. 5. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. All the ER positive breast cancer cell lines except MCF-7 amplified one major product and two minor products of approximate sizes, 730, 540 and 420 bp, respectively. The MCF-7 and all the three ER negative cell lines did not generate any products. To determine the identity of 730, 540 and 420 bp products, the PCR products from ZR-75 were cloned and sequenced. The 730-, 540- and 420 bp products were identified as ER transcripts having deletions in exon 5, exons 5 and 2, and exons 5 and 2–3, respectively (Fig. 5B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above three products and an additional product between exon 5Δ and the exons 5Δ and 2Δ products. Tumor 2 amplified one product between exon 5Δ and exons 5Δ and 2Δ products similar to tumor 1 and two products of approximate sizes 500 and 350 bp. Tumor 3 amplified only the 500 and 350 bp products. Neither tumor 2 nor 3 amplified the major single deletion product. Fig. 5(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.6. Analysis of exon 6Δ transcripts

The profiles of exon 6Δ transcripts in seven cell lines and three tumors are shown in Fig. 6. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders,

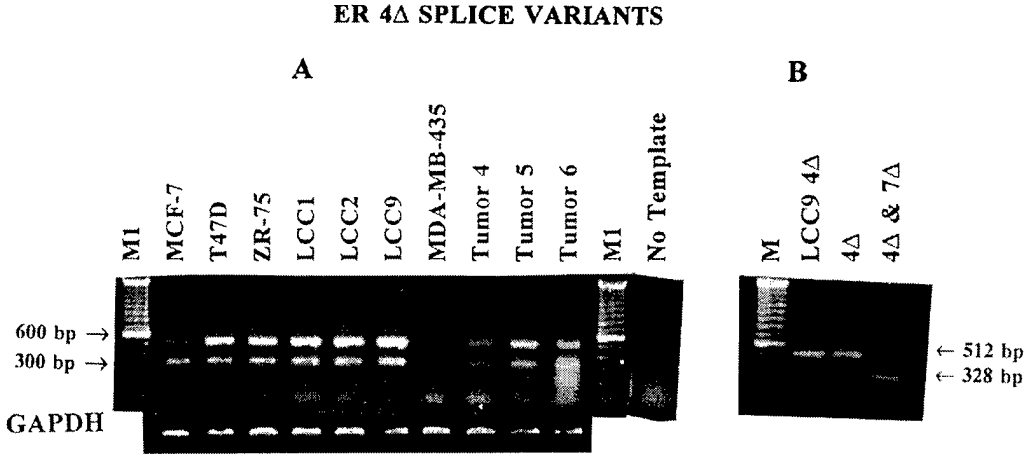


Fig. 4. Analysis of ER exon 4Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 4Δ specific primer. The ER exon 4Δ transcripts were analyzed using ER SX3/5 and ERA. To determine the identity of various PCR products, the products from LCC9 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

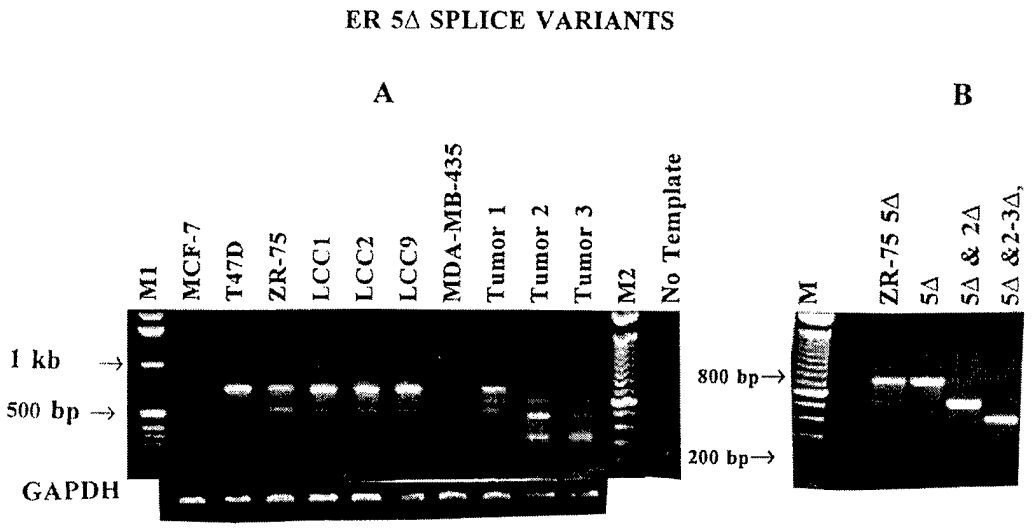


Fig. 5. Analysis of exon 5Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 5Δ specific primer. The exon 5Δ transcripts were analyzed using ER AX4/6 and a sense primer ERS. To determine the identity of various PCR products, the products from ZR-75 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the GiBco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

respectively. All ER positive breast cancer cell lines amplified one major product of approximate size 866 bp. It was identified as the transcript that has a deletion in exon 6 (Fig. 6B). None of the ER negative cell lines amplified any product. We could not detect any double or multiple deletion transcripts with 6Δ primer. The three tumors analyzed did not amplify any products (Fig. 6A). Fig. 6(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.7. Analysis of exon 7Δ transcripts

The profiles of exon 7Δ cDNAs in seven cell lines and three tumors are shown in Fig. 7. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders,

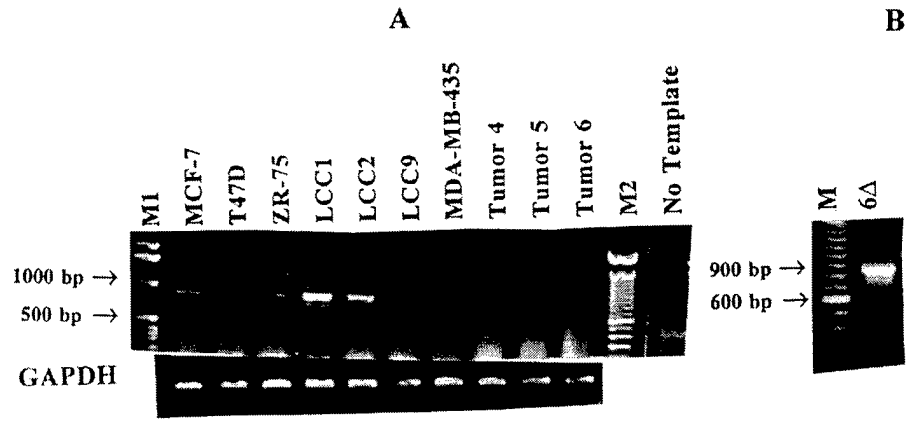


Fig. 6. Analysis of exon 6Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 6Δ specific primer. The exon 6Δ transcripts were analyzed using ER AX5/7 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435, and the tumors 4, 5, and 6. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR product as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

respectively. All the six ER positive breast cancer cell lines generated a major 1 kb band and a minor band of approximately 665 bp. The cell line LCC2 generated an additional two minor bands of sizes 560 and 410 bp. The cell line LCC1 also generated 560 bp minor band and LCC9 generated the 410 bp minor band. In all these cell lines, several closely spaced minor bands were visualized between 1 kb and 665 bp products. To determine the identities of 1 kb, 665, 560 and 410 bp products, the PCR products from LCC1 were cloned and sequenced. They were identified as ER transcripts with deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5, respectively (Fig. 7B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above four products, similar to LCC1 cell line. However, the exons 7Δ and 4Δ product is seen as a major band and the single deletion 1000 bp product as a minor band. Tumor 2 gave a similar profile to tumor 1, and tumor 3 did not amplify any product. Tumor 3 was previously shown not to have any exon 7Δ transcript when analyzed by co-amplification with wild type sequences between exon 4–8 [13]. Fig. 7(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

4. Discussion

In the current study we applied a novel approach to specifically amplify a particular category of alternatively spliced ER molecules, from a pool of other alternatively spliced and wild type ER genes, using primers which anneal to the spliced junctions. We used primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ transcripts. The results described above on the identities of various transcripts, amplified by the seven splice specific primers, are summarized in Table 1. Each splice specific primer amplified not only the single exon deleted transcript but also a number of related cDNAs that have deletions in various combinations of exons. None of the splice specific primers amplified the wild type ER sequences. The seven specific primers amplified a total of 20 transcripts, of which 14 had double or multiple exon deletions. Although single, a few double, and multiple deletion variants have been described, most of the double and multiple deletion transcripts described here were not previously reported.

Our results show that 10 of the 20 transcripts identified have exon 7 deletion, suggesting that this is the most frequently deleted exon. Examination of the products amplified by exon 2Δ, exon 3Δ, and exon 4Δ specific primers indicated a trend in the deletion of exons. In all these cases, the double deletion transcript identified had the deletion of exon 7 (Figs. 1B, 2B and 4B). A similar trend was seen for the exons 2–3Δ primer amplified products (Fig. 3B). These results suggest that initial deletion of a particular exon is mostly followed by the deletion of exon 7. Interestingly, the exon 7Δ specific primer recognized only one of the double deletion products, the exons 7Δ and 4Δ (Fig. 7B). This preferential amplification may be due

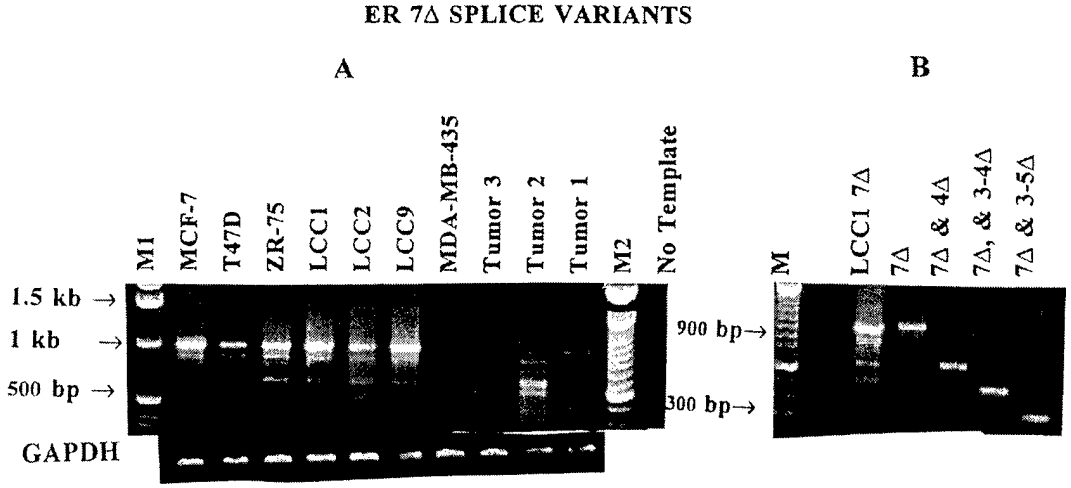


Fig. 7. Analysis of exon 7Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 7Δ specific primer. The exon 7Δ transcripts were analyzed using ER AX6/8 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

to competition among various transcripts. The detection of double deletion transcripts, the exons 5Δ and 7Δ, and exons 6Δ and 7Δ, was not possible in our studies because of the 5Δ and 6Δ specific primers design. The data presented here also show that the third largest cDNA amplified by 2Δ and exons 2–3Δ specific primers had the deletion of exon 5, suggesting that the third most common exon to be deleted in a transcript after the deletion of exon 7 is the exon 5. These observations also indicate that alternative splicing of the ER transcript takes place in a sequential manner, rather than at random. The 3Δ targeted primer did not amplify the triple deletion transcript, which lacked exons 3, 5, and 7 in our studies, probably due to its low abundance. The 4Δ primer did not amplify because of its unique design.

Among the seven targeted primers tested, only 2Δ and 7Δ primers amplified the transcripts with deletions in consecutive exons (Figs. 1B and 7B, respectively and the Table). The profile of these transcripts suggests that after the deletion of exon 2 in a transcript, if the second deletion is initiated at exon 4, the deletions seem to proceed up to exon 5 or 6. Similarly, after exon 7 deletion, if the second deletion is initiated at exon 3, the deletions seem to proceed up to exon 4 or 5. Examination of the other multiple deletion transcripts indicated that none of those had single exon 3Δ, instead, the deletion of exon 3 appears to be associated with either exon 2 or exon 4 deletion (Fig. 3B and 7B, respectively).

The results presented in Fig. (1)–(7) show some differences between estrogen dependent and independent ER positive cell lines in the patterns of variant transcripts. The LCC1, LCC2, and LCC9 are estrogen independent cell lines derived from the estrogen-sensitive parent cell line, MCF-7, after exposure to steroidal (ICI 182, 780)- or non-steroidal (Tamoxifen) anti-estrogens [14], [15]. These three cell lines did not show any differences in variant expression, suggesting that no ER remodeling is associated with either acquired Tamoxifen [14] or Tamoxifen and ICI 182,780 crossresistance [15]. In contrast, there seems to be some differences in ER variant expression associated with acquired estrogen-independence in these cells. For example, all three of the estrogen-independent cells contain the exons 7Δ, and 3–4Δ and exons 7Δ, and 3–5Δ transcripts. These are absent in the parental MCF-7 cells, and in the T47D and ZR-75 cells. Loss of exon 7 might be expected to affect ligand binding as might deletion of exon 5 and possibly exon 4. The entire hinge region would be lost in the 3–4Δ and 3–5Δ containing transcripts. Elimination of the ligand binding domain and part of the hinge region can produce transcriptionally active protein [16], overexpression of which could contribute to estrogen independence. While expression of the exons 7Δ, and 3–4Δ and exons 7Δ, and 3–5Δ transcripts is associated with acquired estrogen-independence, their function and whether significant amounts of these proteins are made, remain unclear. Another major difference observed is the absence of exon 5Δ, exons 5Δ and 2Δ and exons 5Δ, and 2–3Δ transcripts in the parental MCF-7 cells (Fig. 5A). It is possible that, these cells are estrogen dependent, in part, because of the absence of 5Δ transcript, which was reported to possess ligand independent transcriptional property. However, absence of 5Δ transcript alone may not determine the estrogen dependency because this transcript is detected in both T47D and ZR-75. It is possible that several splice variants, and their relative amounts to the wild type alpha receptor and the amounts of beta receptor in a given cell may influence estrogen dependency rather than a single transcript.

The exon deletion transcript analysis in tumor samples showed very interesting findings. In the cell lines, the most abundant product each specific primer amplified was the single deletion product and the second most abundant product was the double exon deleted transcript in the case of exon 2Δ, exon 3Δ, exon 4Δ, exon 5Δ and exon 7Δ. In the case of exons 2–3Δ specific primer, they are double and triple exon deleted transcripts. However, different primers gave different results in tumor samples. When three tumors were analyzed with exon 7Δ specific primer, two tumors showed the presence of four transcripts similar to the cell lines. However, the ratio of each transcript appears to be different compared to the cell lines. In the case of exon 2Δ transcripts, only two tumors

Table 1
Identities of twenty ER alpha spliced variants amplified by seven targeted primers

No.	Splice specific primer	cDNAs amplified
1.	ER SX1/3	2Δ, 2Δ & 7Δ, 2Δ, 5Δ & 7Δ, 2Δ & 4–5Δ and 2Δ & 4–6Δ
2.	ER SX2/4	3Δ and 3Δ & 7Δ
3.	ER SX1/4	2–3Δ, 2–3Δ & 7Δ and 2–3Δ, 5Δ & 7Δ
4.	ER SX3/5	4Δ and 4Δ & 7Δ
5.	ER AX4/6	5Δ, 5Δ & 2Δ, and 5Δ & 2–3Δ
6.	ER AX5/7	6Δ
7.	ER AX6/8	7Δ, 7Δ & 4Δ, 7Δ & 3–4Δ, 7Δ & 3–5Δ

showed the presence of minor bands and none of them amplified the single or double deletion products. When analyzed for the exons 2–3Δ containing transcripts, only one of the tumors generated 2–3Δ product, and the other two amplified the multiple deletion products, that appear to have other modifications, such as base pair insertions/deletions (Fig. 3A). Similar observations were made when analyzed for exon 5Δ transcripts (Fig. 5A). In summary, 5Δ and 2–3Δ transcripts are altered for base pair deletions and alterations, 2Δ, 3Δ and 6Δ transcripts are mostly absent, 7Δ transcript ratios are altered and 4Δ transcripts are unchanged in the tumor samples. These results suggest that the patterns and levels of ER variants undergo extensive alterations in tumor tissues.

The results presented in the current study clearly demonstrate the efficacy of the novel approach for analyzing the ER splice variant transcripts in the cell lines and tissue samples using targeted primers designed at alternate splice junctions. We believe that the new approach described here will be useful in: (1) delineating the functional roles of ER exon deletion variants in estrogen induced signal transduction processes, (2) analyzing the changes in the profiles of splice variants in the tumor tissues compared to normal tissues, (3) evaluating their role in tumorigenesis, tumor progression and loss of hormone dependency, (4) predicting prognosis and response to anti-hormone therapy, and finally (5) developing tissue specific synthetic estrogens and anti-estrogens.

Acknowledgements

This work was supported by grants from the Susan G. Komen breast cancer foundation and the Department of Defence (DAMD 17-94-J-4495) to I.P.

References

[1] W.L. McGuire, G.C. Chamness, S.A.W. Fuqua, Estrogen receptor variants in clinical breast cancer, Mol. Endocrinol. 5 (1991) 1571–1577.

[2] U. Pfeffer, E. Fecarotta, G. Vidali, Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and MCF-7 cells, Cancer Res. 55 (1995) 2158–2165.

[3] M. Gotteland, G. Desuty, J.C. Delarue, L. Liu, E. May, Human estrogen receptor messenger RNA variants in both normal and tumor tissues, Mol. Cell Endocrinol. 112 (1995) 1–13.

[4] E. Leygue, A. Huang, L.C. Murphy, P.H. Watson, Prevalence of estrogen receptor variant messenger RNAs in human breast cancer, Cancer Res. 56 (1996) 4324–4327.

[5] S.A.W. Fuqua, S.D. Fitzgerald, G.C. Chamness, et al., Variant human breast tumor estrogen receptor with constitutive transcriptional activity, Cancer Res. 51 (1991) 105–109.

[6] S.A.W. Fuqua, S.D. Fitzgerald, G.C. Chamness, et al., Inhibition of the estrogen receptor action by a naturally occurring variant in human breast tumors, Cancer Res. 52 (1992) 483–486.

[7] I. Ercnburg, B. Schachter, Y. Mira, R. Lopez, L. Ossiwski, Loss of an estrogen receptor isoform (ER alpha delta 3) in breast cancer and the consequence of its reexpression: interference with estrogen-stimulated properties of malignant transformation, Mol. Endocrinol. 11 (1997) 2004–2015.

[8] D.M. Klotz, C.G. Castles, S.A.W. Fuqua, L.L. Springs, S.M. Hill, Differential expression of wild type and variant ER mRNAs by stocks of MCF-7 breast cancer cells may account for differences in estrogen responsiveness, Biochem. Biophys. Res. Commun. 210 (1995) 609–615.

[9] D.M. Williams, S. Koduri, Z. Li, W.D. Hankins, I. Poola, Primer design strategies for the amplification of alternatively spliced molecules, Anal. Biochem. 271, (1999) 194–197.

[10] S. Green, P. Walter, V. Kumar, A. Krust, J.-M. Bornert, P. Argos, P. Chambon, Human estrogen receptor cDNA: sequence, expression, and homology to v-erb-A, Nature 320 (1986) 134–139.

[11] I. Poola, D.M. Williams, S. Koduri, J. Ramprakash, R.E. Taylor, W.D. Hankins, Quantitation of estrogen receptor mRNA copy numbers in breast cancer cell lines and tumors, Anal. Biochem. 258 (1998) 209–215.

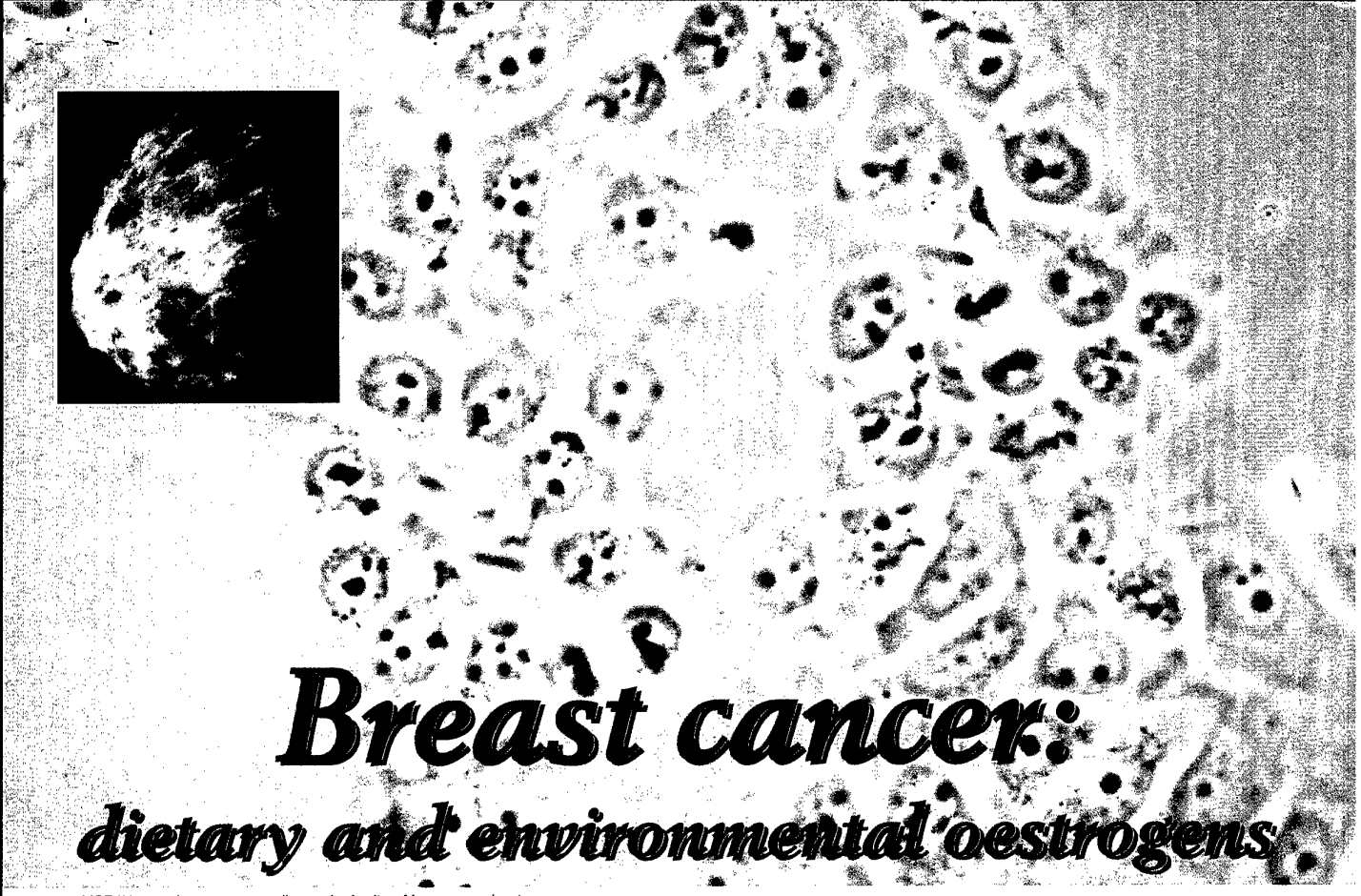
[12] L. Ercolani, B. Florence, M. Denaro, M. Alexander, Isolation and complete sequence of functional human glyceraldehyde-3 phosphate dehydrogenase cDNA, J. Biol. Chem. 263 (1988) 15,335–15,338.

[13] S. Koduri, S.A.W. Fuqua, I. Poola, Alterations in the estrogen receptor alpha mRNA in the breast tumors of African American women, J. Cancer Res. Clinical Oncol. (2000) in press.

[14] N. Brunner, T.L. Frandsen, C. Holst-Hansen, M. Bei, E.W. Thompson, M.E. Lippman, R. Clarke, MCF-7/LCC2: A 4-hydroxytamoxifen resistant human breast cancer variant which remains sensitive to the steroidal antiestrogen ICI 182,780, Cancer Res. 53 (1993) 3229–3232.

[15] N. Brtinner, B. Boysen, S. Jirus, T.C. Skaar, C. Holst-Hansen, J. Lippman, T. Frandsen, M. Sprang-Thomsen, S.A.W Fuqua, R. Clarke, MCF7/LCC9: An antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen, Cancer Res. 57 (1997) 3486–3493.

[16] V. Kumar, S. Green, G. Stack, M. Berry, J.R. Lin, P. Chambon, Functional domains of human estrogen receptor, Cell 51 (1987) 941–951.



Breast cancer: dietary and environmental oestrogens

MCF-7 human breast cancer cells growing in vitro. Mammogram inset.

Robert Clarke, Leena Hilakivi-Clarke and Bruce Trock
The Vincent T Lombardi Cancer Center, Washington DC

Breast cancer is among the most common of the cancers that occur in women living in western societies. It is a much-feared disease and the risks are confusing and often badly reported. Oestrogen levels are a known risk factor. But is it possible to lower the risk? And where are all the oestrogens coming from?

The incidence of breast cancer is high among women in western societies. The disease has a high mortality rate when local treatment (surgery and radiotherapy) does not produce a cure. A major problem for oncologists is the management of the cancer once it has spread beyond the breast (metastatic disease). Many treatments, with the possible exception of hormone-based therapies, are toxic and often produce severe side effects. The most effective approaches to eradicating this disease may come from the area of prevention. To identify effective preventive treatments, it is important to determine the cause(s) of the disease, or at least identify controllable factors that can reduce disease risk. While several risk factors are known, most cannot easily be influenced. To complicate things further, many patients present with few, if any, of the known risk factors.

More than 200 years ago, the Italian physician Ramazzini observed an increased incidence of breast cancer among nuns. It is now well established that never having had children is associated with an increased breast cancer risk. Conversely, risk is reduced if a woman has her first full term pregnancy at a young age (<20 years) and further reduced by extended breastfeeding and multiple pregnancies. One hundred years ago the Scottish physician Beatson described the beneficial effects of removing a woman's ovaries on the progress of breast cancer in premenopausal women. This remains an effective treatment but has been largely replaced by either the use of drugs that block oestrogen biosynthesis or action, or by destruction of the ovaries by irradiation. Breast cancer risk is increased both in women who begin menstruating at a young age (<12 years) and in women who enter the menopause at an older age (Hulka and Stark, 1995). Together, these observations show a clear case for ovarian oestrogens in breast cancer risk.

If the case for internal oestrogens is well established, what then of external oestrogens? This area is controversial, often simply because the data are sparse and contradictory. Nonetheless, oestrogenicity is pervasive in our environment and has been associated with several adverse effects. Plant chemicals with oestrogenic activities (phyto-oestrogens) are responsible for diseases such as clover disease, which causes severe infertility in sheep. Contamination of alligator habitats with man-made oestrogenic chemicals (xeno-oestrogens) affects the sexual development of alligators.

The two primary classes of oestrogenic compounds in our environment are phyto- and xeno-oestrogens. The first class is natural: phytochemicals in plants and plant products, which probably provide some benefit to the plants in which they occur. (It has been suggested that they help protect plants by interfering with the reproductive cycle or

development of parasitic insects.) The second class of compounds (called either environmental oestrogens, or xeno-oestrogens) includes pesticides, industrial waste products and other man-made chemicals and pollutants.

Oestrogenicity and oestrogen receptors

Oestrogens are ligands for (or compounds that bind to) two nuclear hormone receptors, the alpha (ERα) and beta (ERβ) oestrogen receptors. These are the products of two different genes. In the cancerous breast, ERα expression may predominate. It is likely that most of the oestrogenic effects in the mature gland are mediated by ERα. The role of ERβ is less well understood, the gene being cloned relatively recently. ERs are nuclear transcription factors, that is they regulate the expression of other genes. They do so by binding to specific DNA sequences called oestrogen responsive elements (EREs). Coregulator proteins are also recruited and affect the transcription of the adjacent gene (Figure 1). There are many oestrogen-regulated genes, including the progesterone receptor, the protease cathepsin D, the epidermal growth factor receptor and several growth factors that stimulate cell proliferation (mitogens). The precise oestrogen-regulated genes that drive the proliferation of normal and neoplastic breast tissues are not known.

Phyto-oestrogens

If we define an oestrogen in terms of its ability to bind and activate ER and, consequently, regulate gene expression, then we also can loosely apply this simplistic definition to phytochemicals. Thus, a phyto-oestrogen could be any plant-derived compound that is capable of activating ER (Clarke *et al.*, 1996). From the perspective of breast cancer research, there are probably 'good' phyto-oestrogens and 'bad' phyto-oestrogens. The timing and dose of exposure may be crucial, meaning that the same compound is 'good' in some circumstances and 'bad' in others. Phyto-oestrogens can also have other properties. For example, genistein (found in soy and some soy products) is a phyto-oestrogen and an inhibitor of both topoisomerase II and some tyrosine kinases. In many cases it may be difficult to distinguish between these mechanisms, particularly when an oestrogenic pathway includes the regulation of tyrosine kinase activities.

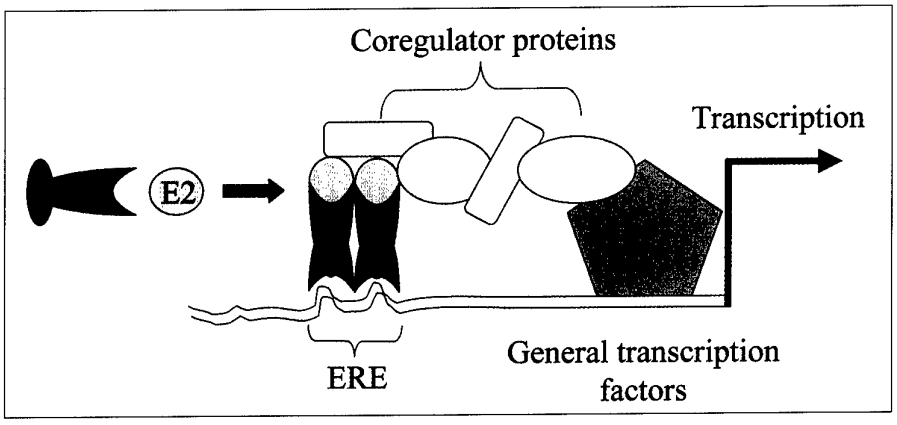


Figure 1: How the oestrogen receptor works. The receptor binds ligand, then binds DNA as a dimer. Other coregulator proteins are recruited, such that the oestrogen receptor and its associated proteins ultimately regulate the activities of the cell's general transcription apparatus assembled at the gene's promoter.
Ligand = 17β-estradiol, any phyto-oestrogen, or xeno-oestrogen; ER=oestrogen receptor; ERE=oestrogen responsive element. GTA = general transcription apparatus.

Where do you find phyto-oestrogens and what is the level of exposure?

There are several groups of plant oestrogens. These include the lignans such as enterolactone, isoflavones such as genistein, and fungal mycotoxins such as zearalenone (see Table 1 and Figure 2). Lignans and isoflavones are found in whole grain and soy products, fruits and berries (Table 1). Levels of exposure to these phyto-oestrogens can be high and they are a major source of oestrogenicity in prepubertal and postmenopausal women. Other sources come from the conversion of adrenal androgens (male sex hormones) to oestrogens in peripheral adipose (fat) tissues and, for some postmenopausal women, hormone replacement therapy.

In soy products the major oestrogenic phyto-oestrogens are the isoflavones genistein and daidzein. They are actually present as the glycosides (sugar containing) genistin and daidzin but the sugar group is removed by gut microflora, leaving genistein and daidzein. They are then absorped into the circulation. Genistein exposure is approximately1.5–4.1 mg/person (~0.05–0.1 mg/kg) in Asia, and approximately 20 times less (at most ~0.05 μg/kg) in the US and EU. These levels reflect the marked dietary differences among these populations. It has been suggested that the difference in soy consumption may contribute to the lower incidence of breast cancer in Oriental countries. However, this is probably a simplistic interpretation.

Zearalenone has also begun to attract attention. Zearalenone is mainly produced by the mould *Fusarium graminearum*. It is found in a variety of host plants and soil debris. It is present as a contaminant in stored cereals, *e.g.*, barley, wheat, corn, corn flakes, and rice, at concentrations from 35–115 g/kg. Individuals living in the US are exposed to 1–5 mg/day (0.02–0.1 mg/kg/day) of zearalenone, a level comparable to the exposure to genistein in the East.

Some alcoholic beverages contain phyto-oestrogens. Bourbon contains the phyto-oestrogens biochanin A and β-sitosterol, whereas beer contains genistein. In grapes and wines the potentially active compound is resveratrol, which is believed to act as a natural antifungal. Consumption of wines is associated with reduced risk of cardiovascular disease. Since oestrogens have protective effects in this context, these could be partly produced by the oestrogenicity in some alcoholic beverages. Generally, alcohol consumption is associated with an increased risk of developing breast cancer. The precise mechanism is unclear but alcohol is known to increase serum oestrogen levels, in addition to the oestrogenic activity of any phyto-oestrogens present.

Indole-3-carbinol is found in *Brassica* species, such as broccoli, and has been identified as a weak phyto-oestrogen. Its most important activity may be to alter the metabolism of the natural oestrogens to less chemically reactive oestrogen metabolites (Telang *et al.*,1997).

The lignans enterolactone and enterodiol are formed by the action of gut microflora on precursors present in grains, seeds, berries and nuts. Women

Isoflavones	Lignans	Lactones	Stilbenes	Coumestans
soy beans, beer, bourbon, peas, fruit	grains, seeds, berries, nuts	Barley, wheat, corn, rice, peas, seeds	grapes wines	soy beans peas, beans

Table 1. Some sources of oestrogenic phytochemicals (phyto-oestrogens).

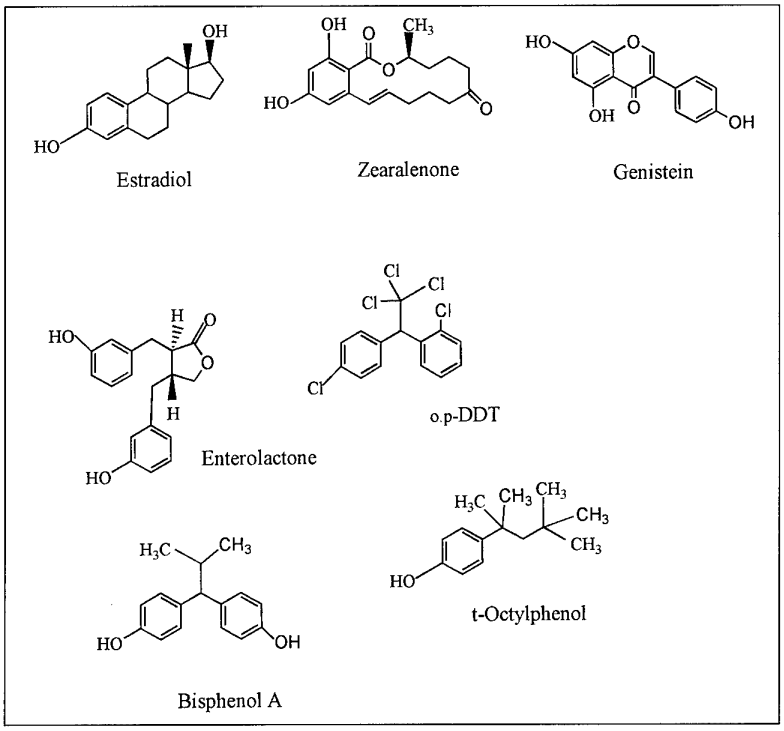


Figure 2: Structure of 17B-estradiol and selected phyto-oestrogens and xeno-oestrogens. The compounds in this figure and/or in the text are:
bisphenol A = 4,4'isopropylidenediphenol
daidzein = 4',7-dihydroxyisoflavone
DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DDT = 1,1,1-trichloro-2-2-bis(4-chlorophenyl)ethane
17B-estradiol = 1,3,5-estratriene-3,17B-diol
genistein = 4',5,7-trihydroxyisoflavone
octylphenol = 4-tert-octylphenol
resveratrol = trans-3,4',5-trihydroxystilbene
zearalenone = 2,4-dihydroxy-6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid-μ lactone

who excrete high levels of lignans have a lower breast cancer risk. Excretion levels tend to be high in some vegetarians and populations that consume high amounts of whole grain products (Adlercreutz, 1990).

Studies of soy and breast cancer risk

Several case-control studies have explored the soy/ breast cancer hypothesis but the data are often contradictory or unclear. Four out of eight studies found no statistically significant association. One small study found an association for Japanese soup but not for tofu, despite both foods having comparable genistein/ isoflavone levels. The lack of an association with tofu consumption was subsequently contradicted, with a significant effect in both premenopausal and postmenopausal women being reported. However, another study failed to find any association with soy protein intake and breast cancer risk in postmenopausal patients, reporting a protective association only in premenopausal women (see Table 2).

We have recently combined these studies using a statistical technique called meta analysis. This allowed us to explore the data to determine if there were any clear associations not apparent from a simple reading of the individual studies. The results from this analysis found no effect on postmenopausal breast cancer risk, and only a small (20%) reduction in the risk of developing breast cancer in the premenopause (Table 2). In contrast, the beneficial effects of soy consumption on reducing the risk of cardiovascular disease are much clearer (Potter, 1995).

Lab trials indicate a mixed influence. Some animal studies find a protective effect, some no effect, and some a significant increase in breast cancer risk associated with soy/ genistein consumption. Genistein also stimulates human breast cancer cell proliferation *in vitro* and can support the growth of human breast cancer xenografts in immunodeficient mice (Hsieh *et al.*, 1998). Human volunteers fed soy milk experienced changes in their menstrual cycling consistent with a potent oestrogenic exposure. Soy consumption also induces clearly oestrogenic changes in women's breast nipple aspirate fluid. These symptoms might suggest an increase in breast cancer risk.

Genistein and the timing of exposure

Why should genistein produce such diverse and potentially conflicting results, and what might the potential risks or advantages be of exposure? We and others have begun to look at the timing of exposure, to determine whether this can affect breast cancer risk.

Oestrogens are required for the successful maintenance of pregnancy, the levels increasing throughout pregnancy to peak at birth. The concentrations of oestrogens vary considerably among pregnant women. The cause of this variability is not known but may be dietary. There is some evidence that higher oestrogen levels during pregnancy are associated with daughters' increased breast cancer risk. Daughters of mothers who suffered from pre-eclampsia/ eclampsia during pregnancy, which is associated with low levels of oestrogens, have a lower breast cancer risk. Conditions associated with high oestrogen levels (such as high birth weight and infant jaundice) lead to higher risks.

Administering oestradiol (an ovarian oestrogen) or genistein to pregnant rats increases the susceptibility of their female offspring to chemically-induced mammary cancers. In addition, the age of onset of sexual maturation is accelerated (Hilakivi-Clarke *et al.*, 1997). These exposures increase the number of structures within the mammary glands known to be targets for chemical carcinogens. The more targets, the greater the probability that one will be transformed and develop into a breast tumour.

The situation is quite different if the exposure to genistein occurs after birth but before sexual maturation. When genistein is administered during this period, female rats have a reduced susceptibility (Murrill *et al.*, 1996; Hilakivi-Clarke *et al.*, 1999). Data indicate that this exposure is sufficient to override the increased risk associated with exposure during pregnancy. When considered together it is clear that timing is everything.

How do these studies relate to human exposures and

Population	Exposure	Association[1]	Citation
212 patients (cases) 212 hospital (controls)	total soybean products	none	Hirohato *et al.* (*J Natl Cancer Inst Monogr 69*:187–190, 1985)
534 patients (cases) 534 community (controls)	total soy protein	none	Yuan *et al.* (*Br J Cancer 71*:1353–1358, 1995)*
300 patients (cases) 300 community (controls)	total soy protein	none	Yuan *et al.* (*Br J Cancer 71*:1353–1358, 1995)
86 spouses of patients	weekly intakes	tofu[2]: none miso soup: p=0.046	Nomura *et al.* (*Am J Clin Nutr 31*: 2020–2025, 1978)
1,186 patients (cases) 23,163 (controls)	miso soup	pre[3]: OR1.16 (0.98, 1.37) (borderline increased risk)	Hirose *et al.* (*Jpn J Cancer Res 86*: 146–154, 1995)
222 patients (cases) 222 sisters (controls)	tofu intake	OR=0.5 (0.2-1.1) (borderline protective effect)	Witte *et al.* (*Breast Cancer Res Treat 42*:243–251, 1997)
200 patients (cases) 420 hospital (controls)	soy protein	post: none pre: -ve	Lee *et al.* (*Lancet 337*: 1197–1200, 1991)
597 patients (cases) 996 community (controls)	tofu intake	post: none pre: OR=0.84 (0.70, 0.99) all cases: OR=0.83 (0.72, 0.95)	Wu *et al.* (*Cancer Epidemiol Biomarker Prev 5*: 901-906, 1996)
Meta analysis	N/A	pre: OR=0.80 (0.71–0.90)	White, Hilakivi-Clarke, Clarke, Trock: submitted

Table 2. Soy epidemiological studies. While neither a complete list, nor a full description of the data in each study (which would be beyond the scope of this article) these data demonstrate the variability in the epidemiological literature regarding the association between soy consumption and breast cancer risk. The meta analysis, which derives an overall estimate by combining all studies, includes some reports not in this table.

[1] Most studies performed logistic regression and obtained an odds ratio (OR) to describe the association between exposure and breast cancer risk. The OR is an approximation of the relative risk and is presented with its 95% confidence interval. In these studies, the association is protective when the OR <1 and indicates an increase in breast cancer risk when the OR >1. Generally, the OR is significant when the confidence interval does not encompass 1.

[2] Generally, tofu has a higher isoflavone content than soy drinks, but levels comparable to, or slightly lower than, miso and other Japanese soups.

[3] Pre=premenopause; post=postmenopause.

breast cancer risk? In eastern countries, soy exposure is likely to be throughout life. This should have either no effect, or even be modestly protective. Feeding soy to infant girls might reduce their breast cancer risk in later life. We may not have to wait a lifetime to find out. Soy-based infant formulas (baby milk) have been widely available and extensively used for several decades. These contain very high levels of phyto-oestrogens (Setchell *et al.*, 1997). The children exposed to these formulas are already becoming old enough to determine whether there is a reduction in premenopausal breast cancer, if the data can be obtained.

Zearalenone

The mycotoxin zearalenone also fulfils our definition of an oestrogen. Zearalenone has been used as a contraceptive, as an oestrogen replacement therapy for postmenopausal women, and as an anabolic agent to enhance growth in cattle and lambs. The growth of MCF-7 human breast cancer cells *in vitro* is stimulated by zearalenone. This mycotoxin has also been reported to enlarge the mammary gland and induce spontaneous mammary tumours in mice. In rat studies, we found that *in utero* exposure to zearalenone did not affect susceptibility of the female pups to mammary carcinogenesis. However, like genistein, prepubertal exposure was protective.

What is so different between zearalenone and genistein?

Why these differences between genistein and zearalenone occur, and why timing is so important, is not entirely clear. One possibility is that coregulators recruited into the ER protein complex (Figure 1) could differ depending on the ligand. We also should not exclude the possibility that the oestrogenic effects of these compounds reflect differences in how they activate ERα and ERβ. For example, genistein has a much higher affinity for ERα than zearalenone (Table 3), while oestradiol has a similar affinity for both ERα and ERβ. These data suggest that not all oestrogens are created equal.

Environmental oestrogens (xeno-oestrogens)

Perhaps the most widely studied xeno-oestrogens are the organochlorines. These compounds are ubiquitous in the environment. The organochlorines are lipophilic (fat loving), slow to metabolise, and persist in adipose tissues. They can accumulate up the food chain and into the human diet. Their widespread occurrence and tendency to bioaccumulate, has raised concern that they may produce chronic, low-level oestrogenic stimulation, resulting in an increased risk of breast cancer.

The most widely implicated organochlorines are the chlorinated pesticides, *e.g.*, DDT (Figure 2) and its metabolites, the polychlorinated biphenyls (PCB), and the polychlorinated dibenzo compounds (PCD). DDT was first produced as an insecticide more than 50 years ago. While banned in the US in 1972, DDT is still commonly used in many developing countries. It is readily metabolised to the more stable and lipophilic DDE. Other implicated chlorinated pesticides include kepone, methoxychlor, hexachlorobenzene, hexachlorocyclohexane, chlordane, toxaphene, aldrin and dieldrin.

The PCBs have been produced commercially for more than 60 years for uses such as flame retardants, insecticides and lubricants. Their production was discontinued in the US in 1977. Contamination of the environment continued to occur through waste disposal and leakage. While not commercially manufactured, PCDs occur as contaminants and by-products in a number of production and combustion processes.

DDT, DDE and many PCBs have been found in breast milk, serum and adipose (fat) tissue. The ability to detect these compounds in human milk suggests that they may accumulate in breast adipose tissue and could be passed to breast-fed infants. In contrast, PCDs are found at very low levels in human tissue or blood. Discontinuation of the use of the chlorinated pesticides and PCBs is being reflected in decreasing levels in most of the US population.

Other possible xeno-oestrogens have recently begun to attract attention. Among the most potent are bisphenol A (which is used in the manufacture of polycarbonate) and octylphenol (Figure 2). Bisphenol A can leach into foods from packaging.

Do xeno-oestrogens affect breast cancer risk?

The long-term, gradual increase in breast cancer incidence in the US and EU has been put forth as evidence for risk from xeno-oestrogen exposure. However, this increase has occurred during a time of changes in reproductive patterns, diet, and occupational roles for women. Each of these could also influence breast cancer risk. The low level of breast cancer among Japanese women, despite a high body burden of organochlorines, has been cited as evidence against the xeno-oestrogen hypothesis. However, the positive effects of Japanese diet and reproductive patterns may overcome a small risk from organochlorines, if such a risk existed. Overall, there is no convincing evidence that the oestrogenic effects of organochlorines increase the risk of human breast cancer.

Case-control studies have drawn the most attention and controversy. These are studies in which biological specimens from women with breast cancer (cases) are compared with those from healthy women (controls). There have been twelve case-control studies to date but only five had reasonably adequate sample sizes.

Of the four methodologically strongest studies, two observed a statistically significant result, while two did not. At this time, there is no clear indication that exposure to organochlorine pesticides or PCBs is a significant risk factor for breast cancer. Citations for these trials can be found in (Clarke *et al.*, 1998).

Phyto-oestrogens		
Compound	ERα RBA[1]	ERβ RBA
Zearalenone	1.2×10^{-2}	ND
Zearalenone (zearalenone metabolite)	0.2	0.2
Genistein	0.05	0.36
Coumestrol	0.94	1.84
Resveratrol	10^{-4}	ND
Xeno-oestrogens		
p,p'-DDE	10^{-5}	ND
Bisphenol A	5×10^{-4}	3.3×10^{-3}
Methoxychlor	10^{-4}	1.3×10^{-4}
Octylphenol	10^{-3}	ND
β-hexachlorohexane	4×10^{-4}	ND
Butylated hydroxyanisole	10^{-6}	ND
Estimated Relative Oestrogenic Exposures[2]		
Contraceptive pill	100%	
Hormone replacement therapy	20%	
Phyto-oestrogens	6%[3]	
Xeno-oestrogens	1.5×10^{-8}%	

Table 3. Relative oestrogenic potencies and exposures. The data are derived from several sources. ND = no data.

[1] RBA = relative binding affinity based on the Kds as estimated by Martin *et al.* (*Endocrinology* **103:** 1860-1867, 1978; Kuiper *et al.* (*Endocrinology* **138:** 863-870, 1997) and Gehn *et al.* (*Proc Natl Acad Sci USA* **94:** 14138-14143, 1997) where 17β-estradiol = 1.

[2] These are primarily derived from the mass balance estimates of Safe (*Environ Health Perspect* **103:** 346-3512, 1995) taking the oestrogenicity of the contraceptive pill = 100%.

[3] The estimate for phyto-oestrogens is based on an average RBA = 10^{-3}. This could be higher if the population is primarily exposed through soy or zearalenone/zearalenol and possibly lower in other populations.

Oestrogens versus phyto- versus xeno-oestrogens

In premenopausal women, the ovarian oestrogens are likely to provide the major source of oestrogenicity. Phyto-oestrogens, particularly where the exposure is substantial, could provide sufficient oestrogenicity to affect ovarian function. Such an effect might reduce both natural oestrogen levels and breast cancer risk.

In postmenopausal women, in western populations, oestrogen biosynthesis in adipose tissues, and any hormone replacement therapy, probably provide the main oestrogenic exposure. However, exposure to some phyto-oestrogens may be sufficient to provide an almost equivalent contribution to oestrogenicity. In postmenopausal women in Asia, when hormone replacement therapy is not administered, the oestrogenicity of phyto-oestrogens may predominate.

In some pre-existing breast tumours, the levels of the natural oestrogens are relatively high because the tumours can synthesise oestrogens from adrenal androgen precursors. The potentially protective effects associated with soy consumption suggest that genistein, or another component of soy, might function as an antiproliferative or even an anti-oestrogen. However, there is little experimental evidence for genistein functioning as anything other than a mitosis-inducing oestrogen in this regard. Assuming that soy and/ or genistein contribute to low Asian incidence, it may be the lifetime exposure and its effects on mammary gland development that are most important.

The low affinities and exposure for xeno-oestrogens, and the lack of any clear and compelling epidemiological evidence, do not support a major affect on breast cancer risk. Despite their potentially greater availability and persistence, the concentrations of free compound likely to be accessible for ER binding are probably not sufficient to compete effectively with the concentrations of natural- and phyto-oestrogens. Thus, any effect of these compounds is unlikely to be related to their oestrogenicity.

Conclusions

There is a difference between risk and 'cause' in cancer. Oestrogens could be chemical carcinogens. Some natural oestrogen metabolites are highly chemically reactive and can damage DNA. However, it is not clear whether this damage alone is sufficient to cause cancer (Clarke *et al.*, 1992). It is most likely that the effects we have discussed reflect either promotional activities, *i.e.*, promoting the survival and proliferation of cancerous cells, or pre-initiation effects, *i.e.*, changes in the susceptibility of normal tissues to transformation.

We have suggested that the xeno-oestrogens contribute little to affecting breast cancer risk. We believe this to be a reasonable conclusion, given what we currently know about the pharmacology of these compounds. The phyto-oestrogens may contribute to breast cancer risk. However, whether the influence is protective or destructive will likely depend on the nature, timing and dose of exposure.

Oestrogenicity is not only associated with breast cancer risk: there is good evidence to suggest that oestrogen or soy intake may reduce the risk of cardiovascular disease; apparent reduction in sperm counts has been attributed to increased xeno-oestrogen exposure; and changes in the patterns of sexual differentiation of various reptiles may reflect contamination of the environment with oestrogenic compounds. Not surprisingly, there has been considerable

interest in hormonally active compounds. It seems likely that additional phyto- and xeno-oestrogens will be identified in the next few years. Understanding the precise importance of exposure to these agents may take a little longer.

Acknowledgements

This work was supported in part by US Public Health Service grants R01-CA58022 (to R. Clarke), P50-CA5815 (to R. Clarke, B. Trock) and the American Cancer Society CN-80420 and Cancer Research Foundation of America (to L A Hilakivi-Clarke).

References

Adlercreutz H (1990) Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scandinavian Journal of Clinical and Laboratory Investigation*, **201,** 3-23.

Adlercreutz H *et al.* (1992) Dietary phyto-oestrogens and cancer: *in vitro* and *in vivo* studies. *Journal of Steroid Biochemistry and Molecular Biology*, **41,** 331-337.

Clarke R *et al.* (1992) Hormonal aspects of breast cancer: growth factors, drugs and stromal interactions. *Critical Reviews in Oncology and Hematology*, **12,** 1-23.

Clarke R *et al.* (1996) Oestrogens, phyto-oestrogens and breast cancer. *Advances in Experimental Biology and Medicine*, **401,** 63-86.

Hilakivi-Clarke LA *et al.* (1997) A high fat diet during pregnancy increases mammary epithelial density and breast cancer risk among female rat offspring. *Proceedings of the National Academy of Sciences USA*, **94,** 9372-9377.

Hilakivi-Clarke LA *et al.* (1998) The oestrogenicity of selected nutrients, phytochemicals, pesticides and pollutants. In: *Breast Cancer: Contemporary Cancer Research*. Bowcock A (Ed) Clifton: Humana Press, 537-568.

Hilakivi-Clarke LA, *et al.* (1997) Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. *British Journal of Cancer*, **80,** 1682-88.

Hsieh C-Y, *et al.* (1998) Oestrogenic effects of genistein on the growth of oestrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. *Cancer Research*, **58,** 3833-3844.

Hulka BS and Stark AT (1995) Breast cancer: cause and prevention. *Lancet*, **346,** 883-887.

Murrill WB *et al.* (1996) Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis*, **17,** 1451-1457.

Potter SM (1995) Overview of proposed mechanisms for the hypocholesterolemic effect of soy. *Journal of Nutrition*, **125,** 606s-611s.

Setchell KD *et al.* (1997) Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet*, **350,** 23-27.

Telang NT *et al.* (1997) Inhibition of proliferation and modulation of estradiol metabolism: novel mechanism for breast cancer prevention by the phytochemical indole-3-carbinol. *Proceedings of the Society of Experimental Biology and Medicine*, **216,** 246-252.

Websites

http://dir.niehs.nih.gov/dirover/bcancer/bcancermain.htm
National Institute of Environmental Health Sciences – with summaries of NIEHS funded studies into the environmental causes of breast cancer.

http://www.som.tulane.edu/ecme/eehome/
Center for Bioenvironmental Research of Tulane and Xavier Universities – An educational service and an interactive forum where those interested in environmental estrogens and other environmental hormones can find accurate, timely information and can contribute to the ongoing public debate.

http://www.epa.gov/scipoly/oscpendo/index.htm
Environmental Protection Agency – the endocrine disruptor screening program web site. This web site provides information about the endocrine system and why certain chemicals can affect it, how the EPA Endocrine Disruptor Screening Program was developed, and the current status of EPA's implementation activities.

Robert Clarke, PhD DSc CBiol FIBiol CChem FRSC *(corresponding author) is Professor of Oncology and Physiology & Biophysics at The Vincent T Lombardi Cancer Center, Georgetown University School of Medicine. clarker@gunet.georgetown.edu*

Leena A Hilakivi-Clarke, PhD *is Associate Professor of Oncology and Psychiatry at The Vincent T Lombardi Cancer Center, Georgetown University School of Medicine*

Bruce Trock, PhD *is Associate Professor of Oncology and Biomathematics & Biostatistics, also at The Vincent T Lombardi Cancer Center, Georgetown University School of Medicine.*

0026-895X/01/5901-62–68$3.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 59:62–68, 2001

Vol. 59, No. 1
248/875317
Printed in U.S.A.

Competitive and Allosteric Interactions in Ligand Binding to P-glycoprotein as Observed on an Immobilized P-glycoprotein Liquid Chromatographic Stationary Phase

LILI LU, FABIO LEONESSA, ROBERT CLARKE, and IRVING W. WAINER

Department of Pharmacology and the Lombardi Cancer Center, Georgetown University School of Medicine, Washington, DC

Received June 8, 2000; accepted September 29, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

A liquid chromatographic stationary phase containing immobilized P-glycoprotein (Pgp) was synthesized using cell membranes obtained from Pgp-expressing cells. The resulting Pgp-stationary phase was used in frontal and zonal chromatographic studies to investigate the binding of vinblastine (VBL), doxorubicin (DOX), verapamil (VER), and cyclosporin A (CsA) to the immobilized Pgp. The compounds were added individually to the chromatographic system with or without ATP in the running buffer. Using this approach, dissociation constants were calculated for VBL (23.5 ± 7.8 nM), DOX (15.0 ± 3.2 μM), VER (54.2 ± 4.7 μM), and CsA [97.9 ± 19.4 nM (without ATP) and 62.5 ± 4.6 nM (with ATP)]. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that competitive interactions occurred between VBL and DOX, cooperative allosteric interactions occurred between VBL and CsA and ATP and CsA, and anticooperative allosteric interactions occurred between ATP and VBL and VER. The chromatographic studies indicate that the immobilized Pgp was modified by ligand and cofactor binding and that the stationary phase can be used to study drug-drug binding interactions on the Pgp molecule.

P-glycoprotein (Pgp) is a member of the ATP binding cassette (ABC) superfamily of transport proteins (Loe et al., 1996; Doyle et al., 1998). It is a 170-kDa cell membrane protein with two ATP binding sites and ATPase activity (Rosenberg et al., 1997). Pgp acts as an efflux drug transporter whose substrates include anthracycline antibiotics and Vinca alkaloids (Cordon-Cardo et al., 1989; Clarke et al., 1993; Clarke and Leonessa, 1994), steroids (Barnes et al., 1996), verapamil (VER) (Yusa and Tsuro, 1989), peptides (Foxwell et al., 1989), and quinolines (Kusuhara et al., 1997). Pgp is expressed in normal tissues and appears to be a major contributor to the blood-brain barrier (Cordon-Cardo et al., 1989; Tsuji et al., 1992). Expression also has been detected in breast cancer where it is associated with a poor clinical response (Trock et al., 1997).

Pgp's broad substrate specificity has not been definitively explained. Several indirect and direct models for Pgp activity have been proposed (Shapiro and Ling, 1994). The most popular model is the "membrane vacuum cleaner" mechanism in which Pgp binds its substrate from the inner leaflet of the plasma membrane and releases it into the extracellular fluid (Gottesman and Pastan, 1993). In a related mechanism, Pgp activity has been described as a "flippase" that transports its substrates from the inner to the outer leaflet of the plasma membrane (Raviv et al., 1990; Higgins and Gottesman, 1992).

The number of binding sites on the Pgp molecule has not been determined. There is evidence for the existence of multiple binding sites as some substrates bind to Pgp in a mutually noncompetitive manner (Raviv et al., 1990; Ferry et al., 1992, 1995). Other data suggesting multiple binding sites include synergistic activity on ATPase activation (Garrigos et al., 1997), substrate discriminating effect of specific Pgp mutations (Devine et al., 1992), and differential effect of chemosensitizers on the photoaffinity labeling at two different locations on the Pgp molecule (Dey et al., 1997).

One experimental approach to determine Pgp selectivity and transport mechanism has been the isolation of the transporter followed by purification using a combination of anion exchange and affinity chromatography (Shapiro and Ling, 1994; Sharom, 1995). The isolated protein was then reconstituted into proteoliposomes either by the detergent dilution method (Shapiro and Ling, 1994) or by detergent dialysis followed by Sephadex-G50 chromatography (Sharom, 1995). In the proteoliposomes prepared by either method, >90% of Pgp was reconstituted with an inside-out orientation, i.e., ATP-binding and cytoplasmic domains exposed to the ex-

This research was supported by National Institutes of Health Grant 2R42M56591-02 (I.W.W.).

ABBREVIATIONS: Pgp, P-glycoprotein; VBL, vinblastine; DOX, doxorubicin; VER, verapamil; CsA, cyclosporin A; IAM, Immobilized Artificial Membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; BCA, bicinchoninic acid.

travesicular medium (Sharom, 1995). The reconstituted Pgp could be used to study and characterize both drug-stimulated ATPase activity and ATP-dependent transport. Using this approach, the effect of verapamil and daunorubicin on [^{3}H]vinblastine ([^{3}H]VBL) accumulation in the proteoliposomes, a measure of transport, could be measured (Sharom, 1995). The effect of verapamil on the ATPase kinetics (K_m and V_{max}) also could be determined (Shapiro and Ling, 1994).

Another approach to the determination of the effect of compounds on Pgp transport used the transepithelial flux of digoxin across Caco-2 cells (Wandel et al., 1999). This method was used to determine the IC_{50} for digoxin transport for 14 compounds. An in vivo method for Pgp transport in tumors and the blood-brain barrier also has been reported (Hendrikse et al., 1999). This approach used [^{11}C]verapamil and [^{11}C]daunorubicin as the transport substrates and positron emission tomography as the detection method.

The binding of compounds to Pgp has been investigated by measuring the displacement of [^{3}H]vinblastine and [^{3}H]verapamil from human intestinal Caco-2 cells overexpressed with Pgp (Doppenschmitt et al., 1999). The assays were performed in 96-well plates, and the method was designed to be adapted to high-throughput screens. Using this method, K_m and IC_{50} values for nine compounds were determined.

An alternative experimental approach to the determination of binding affinities is affinity chromatography. We have previously reported the synthesis of a liquid chromatographic stationary phase containing immobilized Pgp and its use in the determination of Pgp binding affinities (Zhang et al., 2000). The present work expands the characterization of the Pgp-stationary phase and uses frontal and zonal chromatographic techniques to investigate the binding of vinblastine, doxorubicin, verapamil, and cyclosporin A (CsA) to the immobilized Pgp. The compounds were added individually to the chromatographic system with or without ATP in the running buffer. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that both competitive and allosteric interactions occurred during the chromatographic studies and that the binding affinities of immobilized Pgp are altered by the presence or absence of ATP.

Experimental Procedures

Materials. Immobilized Artificial Membrane (IAM) particles were obtained from Regis Chemical Co. (Morton Grove, IL). A glass column (HR5/5) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). [^{3}H]Vinblastine and [^{3}H]cyclosporin A were purchased from Amersham Life Science Products (Boston, MA). [^{3}H]Verapamil was from NEN Life Science Products, Inc. (Boston, MA). Vinblastine, verapamil, doxorubicin, cyclosporin, CHAPS, glycerol, benzamidine, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). GF/C glass microfiber filters were from Whatman (Ann Arbor, MI). Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT).

Preparation of Membranes. As previously described, the Pgp-positive MDA435/LCC6^{MDR1} cell line was obtained by transduction of Pgp-negative-expressing MDA435/LCC6 human breast cancer cells with a retroviral vector carrying MDR1 cDNA (Pgp) (Leonessa et al., 1996). Approximately 8×10^7 cells were harvested in 10 ml of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 μM leupeptin, 2 μM phenylmethanesulfonyl fluoride, and 4 μM pepstatin). The suspension of cells was homogenized twice for 30 s (with a cooling period in between) with a Brinkmann (Westbury, NY) Polytron homogenizer. The homogenized cells were centrifuged first at 1,000g for 10 min, the pellets were discarded, and the supernatant was collected and centrifuged at 150,000g for 30 min. The membrane pellets were collected.

Immobilization of Pgp on IAM Particles. The membrane pellets were resuspended in 6 ml of solubilization solution (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 15 mM CHAPS, 2 mM dithiothreitol, 10% glycerol) for 3 h at 4°C. This was mixed with 100 mg of dried IAM particles and stirred for 1 h at room temperature. The suspension of Pgp-IAM was then dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine) for 36 h at 4°C (1.5 liters for every 12 h).

Preparation of the Liquid Chromatographic Column. The IAM particles with immobilized Pgp were packed into a HR5/5 glass column (0.5 × 0.8 cm) after centrifugation three times at 350g for 3 min at 4°C. Then the column was equilibrated with buffer B (50 mM Tris-HCl, pH 7.4) at room temperature for 3 h.

Frontal Chromatographic Studies. The chromatographic system has been previously described (Zhang et al., 2000) and was primarily based upon the Pgp-IAM column connected on-line to a flow scintillation monitor (Radiometric FLO-ONE Beta 500 TR instrument; Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min.

The marker ligand, either [^{3}H]VBL (1.0 nM), [^{3}H]VER (0.3 nM), or [^{3}H]CsA (2.0 nM) were applied to the Pgp-IAM column in sample volumes of 25 to 50 ml. The solutions containing the marker ligands were supplemented with a range of concentrations of either cold VBL, VER, doxorubicin, or CsA. Elution profiles were obtained showing front and plateau regions as illustrated for [^{3}H]VER (Fig. 1). The observed elution volume data were used for calculation of ligand dissociation constants. The K_d values of VER and CsA were calculated by nonlinear regression using Prism (GraphPad Software, San Diego, CA) and a one-site binding (hyperbola) equation (1) (Klotz, 1983)

$$Y = B_{max} \cdot X/(K_d + X) \quad (1)$$

in which X is the concentration of VER or CsA; Y is equal to [verapamil] $(V - V_{min})$ or [CsA]$(V - V_{min})$, where V_{min} is the elution volume of VER or CsA under conditions where specific interactions

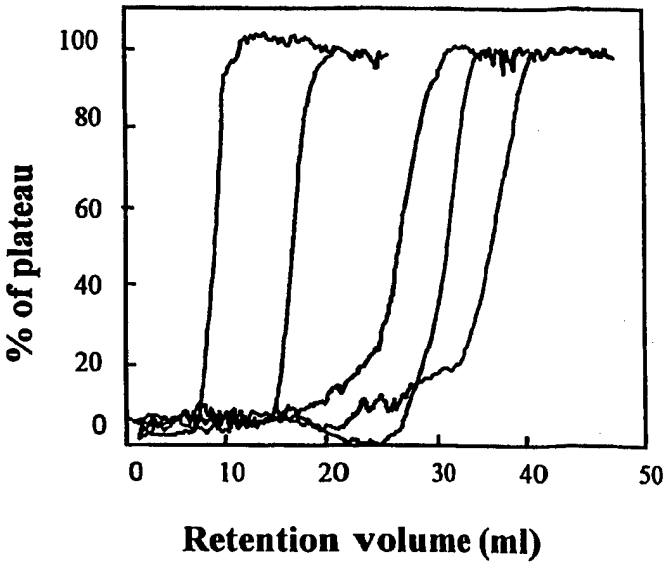


Fig. 1. Frontal analysis of interactions of Pgp with verapamil on an immobilized Pgp-IAM column (0.5 × 0.8 cm). The elution profiles of 1.0 nM [^{3}H]verapamil in solution with 10, 40, 60, 200, and 400 μM nonradioactive verapamil are shown (from right to left). Running buffer was 50 mM Tris-HCl, pH 7.4, at a flow rate of 0.5 ml/min.

are completely suppressed and V is the retention volume of VER or CsA at different concentrations (0.3–400 μM for VER and 2.5–100 nM for CsA).

Two series of runs were made to determine the K_d value for VBL and the K_d values for doxorubicin and CsA. One series was performed with cold VBL (3–100 nM) to displace [^{3}H]VBL, and the other was performed with cold doxorubicin (5–70 μM) or CsA (10–250 nM) with [^{3}H]VBL as the displaced ligand. The K_d value of VBL and the K_d values of doxorubicin and CsA were calculated using eqs. 2 and 3 (Winzor, 1985; Brekkan et al., 1996; Zhang et al., 1998).

$$(V_{max} - V)^{-1} = (1 + [VBL]K_{VBL}) \cdot (V_{min}[P]K_{VBL})^{-1} + (1 + [VBL]K_{VBL})^2 \cdot (V_{min}[P]K_{VBL}K_i)^{-1} \cdot [I]^{-1} \quad (2)$$

$$(V - V_{min}) - 1 = (V_{min}[P]K_{VBL})^{-1} + (V_{min}[P])^{-1}[VBL] \quad (3)$$

where I represents doxorubicin or CsA; $[P]$ represents the concentration of active receptor in the volume; V_{min} represents the elution volume of VBL under conditions where the specific interaction is completely suppressed; V_{max} is the elution volume obtained with 1.0 nM [^{3}H]VBL.

Control Experiments. Membranes from the Pgp-negative parental cell line MDA435/LCC6 (Leonessa et al., 1996) were prepared and immobilized on an IAM support as described above. Using the procedure described above, the Pgp-negative-IAM support was packed into a glass column (0.5 × 0.8 cm), and a second glass column (0.5 × 0.8 cm) was packed with untreated IAM support. The three columns, IAM support (negative control), Pgp-negative-IAM (positive control), and Pgp-IAM (experimental), were separately connected on-line to a flow scintillation monitor and used in zonal chromatographic experiments. In these studies, a mobile phase composed of Tris-HCl (50 mM, pH 7.4) was constantly pumped through the column at a flow rate of 0.5 ml/min. A single 100-μl injection of the marker ligand [^{3}H]VER (23.5 nM) was injected onto the column, and the radioactive signal (cpm) was recorded every 6 s. The chromatographic data was summed up in 0.5-min intervals and smoothed using the Microsoft Excel program with a 5-point moving average.

Membrane Binding Assays. The binding assays were accomplished using a previously described method (Ferry et al., 1995). Briefly, 50 μl of [^{3}H]VBL [3–100 nM with 2% ethanol (v/v)] was incubated with Pgp-containing or Pgp-negative membranes (150 μg in 50 μl) or bare IAM particles and 50 μl of cold VBL (12 μM) for 2 h at room temperature. Bound and free drug were separated by rapid filtration through Whatman GF/C filters that had been presoaked with 0.1% bovine serum albumin in Tris-HCl (50 mM, pH 7.4). The filters were then washed with 2 portions of 5 ml of ice-cold 20 mM Tris-HCl, 20 mM $MgCl_2$ buffer. The filters were dried, and retained radioactivity was quantitated by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding.

Protein Assay. The amount of membrane and the immobilized membrane were determined by bicinchoninic acid (BCA) protein assay. The sample was diluted with NaOH (0.1 M). A protein standard (0.3–37.5 μg in 50 μl) was prepared with albumin standard (Pierce, Rockford, IL). The measurement procedure followed the instruction in the Pierce BCA protein assay kit in which 20 ml of reagent A was mixed with 0.4 ml of reagent B. Aliquots (50 μl) of standards and samples were added in triplicate to a 96-well plate and 200 μl of BCA reagent (A + B) were added to each well. The standards and samples were incubated at room temperature for 3 h, and the resulting absorbance at λ = 570 nm was determined using a spectrophotometer. The amount of protein was calculated by using the Microsoft Excel program.

Results

Chromatographic Studies with Vinblastine and Doxorubicin. The dissociation constants (K_d) of VBL and doxorubicin were determined on the Pgp-IAM stationary phase using displacement chromatography with [^{3}H]VBL as the marker ligand (Table 1). The calculated K_d of VBL was 23.5 ± 7.8 nM, consistent with the previously reported values of 37.0 ± 10 nM (Ferry et al., 1995) and 36 ± 5 nM (Korzekwa et al., 1998). The K_d value of 15.0 ± 3.2 μM determined for doxorubicin was also consistent with the reported value of 31.0 ± 7.3 μM (Ferry et al., 1995).

The chromatographic results also were consistent with the results obtained from binding assays using the same membranes used in the construction of the Pgp-IAM stationary phase. In these studies, membrane extracts were prepared from the Pgp-expressing cell line MDA435/LCC6^{MDR1} and the Pgp-negative cell line MDA435/LCC6 (Hendrikse et al., 1999). VBL binding to the two membrane extracts and the IAM support was determined using a previously described rapid filtration method (Ferry et al., 1995). No specific binding was observed with the Pgp-negative cell membranes or the IAM particles, while a K_d value of 54.5 ± 40.8 nM was determined using the membranes from the Pgp-expressing cell line. The calculated affinity was consistent with the previously published value, 37 ± 10 nM, obtained using the same experimental approach (Ferry et al., 1995).

Chromatographic Studies with Verapamil and Vinblastine. When VER was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for VER was 54.2 ± 4.6 μM. This value was significantly higher than the previously reported values of 0.45 ± 0.05 μM (Ferry et al., 1995) and 0.6 ± 0.18 μM (Ferry et al., 1992). When the experimental conditions were reversed and [^{3}H]VER was the marker ligand and VBL the displacer, no displacement of [^{3}H]VER was observed when 50 and 100 nM concentrations of VBL were added to the mobile phase (Table 2).

The specificity of the chromatographic interactions of VER with the immobilized Pgp were investigated through the independent immobilization of membrane extracts from the Pgp-expressing cell line and the Pgp-negative cell line on the IAM support. Zonal chromatographic studies were conducted with columns containing either the Pgp-IAM, Pgp-negative-IAM, or IAM support. When a 100-μl sample of [^{3}H]VER was injected onto the columns containing either the Pgp-negative-IAM support or the IAM support alone, the retention volumes on both columns were less than 4 ml (Fig. 2, curves

TABLE 1
The K_d values calculated using frontal affinity chromatography on the immobilized Pgp-IAM stationary phase

Drugs	K_d[a]	K_d
Vinblastine	23.5 ± 7.8 nM	37.0 ± 10 nM[b]
		36.0 ± 5 nM[c]
Verapamil	54.2 ± 4.6 μM	0.45 ± 0.05 μM[b]
Doxorubicin	15.0 ± 3.2 μM[d]	31 ± 7.3 μM[b]
Cyclosporin A	62.5 ± 5.6 nM[e]	18 ± 3.6 nM[b]
	97.9 ± 19.4 nM[d]	

[a] These values were measured in the present work by frontal affinity chromatography with immobilized Pgp-IAM.
[b] These values are from Ferry et al. (1995).
[c] This value is from Callaghan et al. (1997).
[d] These values were obtained by displacing [^{3}H]vinblastine (see *Experimental Procedures*).
[e] This value was measured when 3 mM ATP was in the running buffer.

1 and 2). The volumes of these columns (as well as the Pgp-IAM column) are 0.5 ml, thus a retention of 4 ml indicates that it takes 8 column volumes to elute the [^{3}H]VER, indicating that an interaction occurred between the solute and both of the stationary phases. On the column containing the Pgp-IAM support, the retention volume of [^{3}H]VER was >20 ml (Fig. 2, curve 3).

Chromatographic retention on biopolymer containing stationary phases is a combination of nonspecific and specific interactions. The former interactions are due to the physicochemical properties of the solute and stationary phase, i.e., electrostatic and hydrophobic interactions, while the latter (specific) interactions are due to interactions between the solute and a specific binding site(s) on the biopolymer. The 5-fold increase in retention volume between the Pgp-IAM and both the Pgp-negative-IAM and IAM support alone indicates that specific binding interactions occur between [^{3}H]VER and the immobilized membrane extracts obtained from the Pgp-expressing cells.

Chromatographic Studies with Cyclosporin A and Vinblastine. When CsA was used as the displacer of the [^{3}H]VBL marker ligand, the calculated K_d value for CsA was 97.9 ± 19.4 nM, compared with the previously reported value of 18.0 ± 3.6 nM (Ferry et al., 1995) (Table 1). When [^{3}H]CsA was used as the marker ligand and migrated alone through the Pgp-IAM, the retention volume was 7.8 ml (Table 2), and no specific retention was observed (Fig. 3A). The addition of 50 nM VBL to the running buffer increased the retention volume of [^{3}H]CsA to 15.7 ml (Table 2) and produced the expected frontal chromatogram (Fig. 3B). When the VBL concentration was increased to 100 nM, the observed retention of the frontal chromatogram increased to 18.8 ml (Fig. 3D; Table 2).

Effect of ATP on the Chromatographic Properties of the Pgp-IAM. The addition of 3 mM ATP to the running buffer resulted in changes in the retention volumes of CsA, VBL, and VER. The concentration of ATP was selected based upon the previously reported studies of the secondary and tertiary structures of reconstituted Pgp (Sonveaux et al., 1996).

In the case of CsA, the addition of ATP increased the retention volume from 7.8 to 17.5 ml (Table 2). In addition to the change in elution volume, the observed chromatogram changed from a frontal curve indicative of nonspecific retention (Fig. 3A) to a frontal chromatogram characteristic of specific retention due to binding interactions between the CsA and the immobilized Pgp-IAM (Fig. 3C). With 3 mM ATP in the running buffer, [^{3}H]CsA was displaced from Pgp by the addition of unlabeled CsA. The results from the CsA displacement studies were used to calculate a K_d value of 62.5 nM for CsA binding to the immobilized Pgp.

When VBL was the marker ligand, the addition of 3 mM ATP decreased the retention volume from 32.1 to 8.4 ml (Table 2). The presence of ATP in the running buffer also changed the observed chromatograms from a frontal curve demonstrating specific retention (Fig. 4A) to a nonspecific curve (Fig. 4B). A similar effect was observed for VER as the addition of 3 mM ATP to the running buffer reduced the elution volume from 34.2 to 5.9 ml (Table 2) with a resulting loss in specific retention, as demonstrated by the shape of the frontal curve (data not shown).

Discussion

Quantitative affinity chromatography is an extensively studied and documented approach for the measurement of ligand-protein interactions (cf. Jaulmes and Vidal-Madjar, 1989). This technique uses both frontal and zonal chromatography to perform equilibrium, thermodynamic, and kinetic studies. In addition, displacement chromatographic techniques can be used to observe binding interactions between two or more ligands binding at the same or separate sites. In this manner, competitive and allosteric (cooperative or anticooperative) interactions can be readily identified.

In this study, both zonal and frontal chromatography were used to evaluate Pgp-ligand and ligand-ligand binding interactions. Using zonal chromatography, a comparison of the chromatographic retention of verapamil, a known Pgp substrate, on the native chromatographic support and the Pgp-positive and Pgp-negative supports (Fig. 2) demonstrated that, for Pgp substrates, the observed chromatographic retentions were a function of specific interactions between the substrate and the immobilized Pgp.

The relationship between chromatographic retention on the Pgp-IAM stationary phase and Pgp binding affinity was also illustrated by comparison of substrate affinities calculated using frontal chromatography on the Pgp-IAM column and the results from classical filtration binding assays (Table 1). The initial studies in this series were conducted using [^{3}H]VBL as the marker ligand and Tris buffer (50 mM, pH 7.4) as the running buffer. Under these conditions, CsA displaced [^{3}H]VBL, producing a calculated K_d value of 97.9 nM (Table 1), which is consistent with results from filtration binding assays (Ferry et al., 1992, 1995).

The displacement of [^{3}H]VBL by CsA indicated that CsA specifically and competitively binds to immobilized Pgp, but frontal chromatography with [^{3}H]CsA alone in the running buffer produced a low retention volume, 7.8 ml (Table 2), and no detectable specific retention (Fig. 3A). This indicates that under the experimental conditions, [^{3}H]CsA did not specifically bind to immobilized Pgp. However, the addition of 50 nM VBL to the running buffer produced a classical frontal

TABLE 2

Retention volumes of [^{3}H]vinblastine and [^{3}H]cyclosporin A were obtained when 1) no ATP was present in the running buffer, 2) 3 mM ATP was added in the running buffer, 3) 50 nM cold vinblastine was supplemented in the sample (no ATP in the buffer), and 4) 100 nM cold vinblastine was in the sample (no ATP in the buffer)

Drugs	Retention Volume (ml) at			
	No ATP	3 mM ATP	50 nM Vinblastine (No ATP)	100 nM Vinblastine (No ATP)
[^{3}H]Vinblastine	32.1	8.4	11.0	9.5
[^{3}H]Verapamil	34.2	5.9	34.1	34.0
[^{3}H]Cyclosporin A	7.8	17.5	15.7 (15.4)[a]	18.8

[a] 15.7 ml was measured at the condition of no ATP present in the running buffer, and 15.4 ml was obtained when 3 mM ATP was in the running buffer.

chromatogram for [^{3}H]CsA (Fig. 3B) and increased the retention volume to 15.7 ml (Table 2). When the VBL concentration was increased to 100 nM, the retention volume also increased to 18.8 ml (Table 2; Fig. 3D).

The results from the studies with [^{3}H]VBL and [^{3}H]CsA as the marker ligands indicate that the addition of VBL to the running buffer produced a cooperative allosteric interaction in the binding process between [^{3}H]CsA and the immobilized Pgp. This suggests that the binding of VBL to the immobilized Pgp alters the protein in such a manner that the site at which CsA binds is formed or made accessible to the ligand.

The data also indicated that once the VBL-induced change had occurred CsA bound to Pgp and displaced VBL through competitive and/or anticooperative allosteric interactions. The addition of CsA to the running buffer did not change the shape of the [^{3}H]VBL frontal chromatograms, demonstrating that the displacement was competitive in nature. One explanation of these results is that the VBL-induced CsA binding site is contiguous with or part of the VBL site. Thus, CsA binding to the induced site does not directly compete with VBL for the same site but inhibits VBL binding through steric interactions. Korzekwa et al. (1998) have proposed a similar model for enzymatic inhibition and activation of cytochrome P450 isoforms. In this model, the simultaneous but independent binding of two different substrates in the active site of the enzyme results in steric interactions that produce the displacement (inhibition) or reorientation (activation) of one of the substrates.

In these studies, the addition of increasing concentrations of VER to the running buffer reduced the retention volumes of [^{3}H]VBL without changing the shapes of the frontal chromatograms. This indicates that VER competitively displaced VBL from its binding to Pgp, although the calculated K_d value was significantly higher than previously reported values (Table 1). However, VBL was unable to displace [^{3}H]VER from the immobilized Pgp. These results suggest that VER binds to two or more distinct sites on the Pgp molecule including the site at which VBL binds. Furthermore, the site common to VBL and VER is not the primary, high-affinity VER binding site. Thus, the K_d value calculated from the frontal chromatographic studies (Table 1) appears to be the sum of VER binding affinities. It could not be determined from the experimental conditions used in this study whether the VER and VBL sites are allosterically linked. Further studies will be required to select specific markers for the high- and low-affinity VER binding sites.

The existence of multiple binding sites on the Pgp molecule has been previously proposed. Using classical filtration binding assays, Ferry et al. (1992) obtained evidence of nonoverlapping binding sites for Vinca alkaloids and dihydropyridine substrates and for Vinca alkaloids and doxorubicin. Also, distinct sites for steroids and Vinca alkaloids (Garrigos et al., 1997), steroids and VER (Orlowski et al., 1996), VER and dihydropyridines (Pascaud et al., 1998), and between differ-

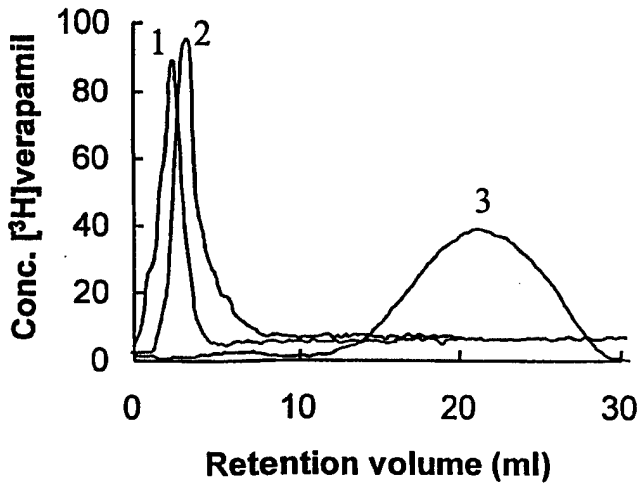


Fig. 2. Zonal affinity chromatographic profiles of 100 μl of 23.5 nM [^{3}H]verapamil at a flow rate of 0.5 ml/min with 50 mM Tris-HCl, pH 7.4, buffer. 1, from Pgp-negative-IAM column; 2, from IAM particles column; and 3, from Pgp-IAM column.

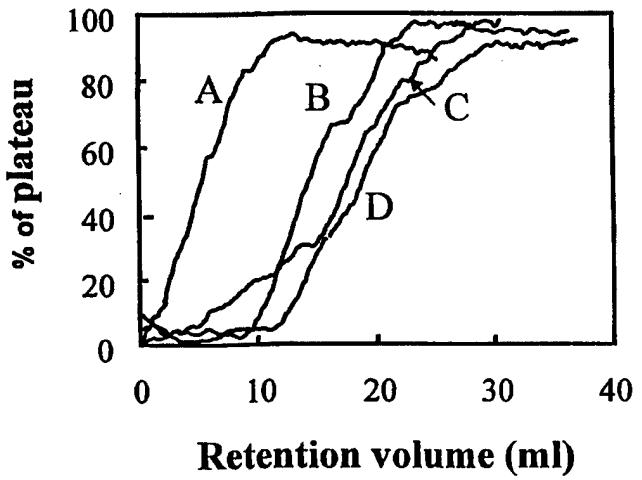


Fig. 3. Frontal affinity analysis of 1.0 nM [^{3}H]cyclosporin A. A, [^{3}H]cyclosporin A was in the sample alone; B, 50 nM cold vinblastine was supplemented in the sample; C, 3 mM ATP was in the sample and running buffer; D, 100 nM cold vinblastine was added in the sample. The running buffer was 50 mM Tris-HCl, pH 7.4.

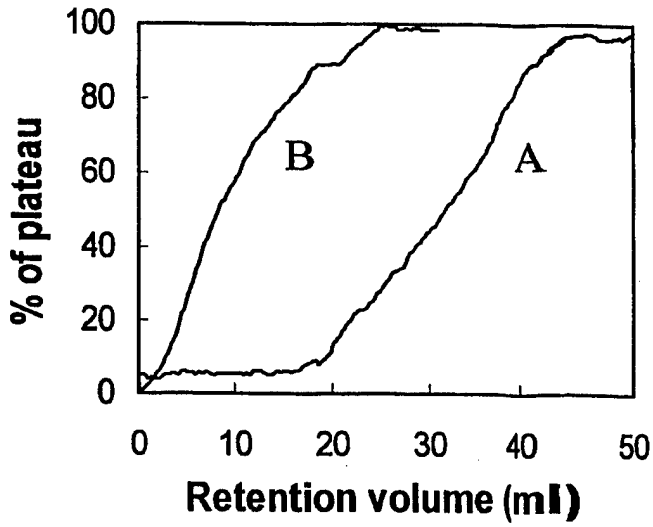


Fig. 4. Frontal affinity chromatographic analysis of 1 nM [^{3}H]vinblastine with Pgp-IAM on a column of 0.5 × 0.8 cm at a flow rate of 0.5 ml/min. A, 1.0 nM [^{3}H]vinblastine only; B, 1.0 nM [^{3}H]vinblastine supplemented with 3 mM ATP. The running buffer for both A and B was 50 mM Tris-HCl, pH 7.4, with 1.6% ethanol.

ent steroids (Orlowski et al., 1996) were supported by the results of studies using an ATPase activation endpoint. Moreover, separate binding sites have been suggested for VER and anthracyclines (Spoelstra et al., 1994; Litman et al., 1997), VER and colchicine (Korzekwa et al., 1998), and cyclosporins and dihydropyridines (Tamai and Safa, 1991).

Pgp contains two ATP binding sites (Rosenberg et al., 1997). A previous study has investigated the effect of ATP binding on the secondary and tertiary structures of Pgp using infrared attenuated total reflection spectroscopy (Sonveaux et al., 1996). In this work, purified Pgp was functionally reconstituted into liposomes, and the effect of ATP, ATP with VER, VER alone, and ADP on the structure of Pgp was investigated. No effects were observed with VER alone or with ADP. However, the addition of ATP induced a change in the tertiary structure of Pgp.

Sonveaux et al. (1996) used 3 mM ATP versus no ATP as the two experimental states for Pgp. In this study, we have used a running buffer without ATP and one to which we have added the same concentration of ATP (i.e., 3 mM). Thus, the chromatographic results with ATP in the running buffer should reflect the shift in Pgp tertiary structure indicated by Sonveaux et al. (1996). Indeed, the addition of 3 mM ATP to the running buffer increased the retention volume of [^{3}H]CsA from 7.8 to 17.5 ml (Table 2), produced a classical frontal chromatogram for [^{3}H]CsA (Fig. 3C), and permitted the calculation of a K_d value of 62.5 nM (Table 1). These results indicate that the addition of ATP to the running buffer produced a cooperative allosteric interaction that increased the binding affinity of Pgp for CsA. Similar results were obtained in the VBL-CsA binding interaction studies.

The presence of ATP in the running buffer produced the opposite effect on the retention volumes of [^{3}H]VBL and [^{3}H]VER. With [^{3}H]VBL, the addition of 3 mM ATP reduced the observed retention from 32.1 to 8.4 ml (Table 2; Fig. 3), and the retention volume for [^{3}H]VER was reduced from 34.2 to 5.9 ml, with the loss of specific retention in both cases. These results suggest an ATP-induced anticooperative allosteric interaction. Allosterically produced reductions in retention volume can be distinguished from competitive displacements as illustrated by the effect of the addition of VBL on the retention volume of [^{3}H]VBL (Table 2). In this case, the retention volume decreased, but the specific frontal chromatographic curves were retained (data not shown).

Thus, the addition of ATP to the running buffer produced changes in the chromatographic interactions between the ligands and the immobilized Pgp (i.e., specific to nonspecific and vice versa) that are consistent with the changes in the tertiary structure identified by Sonveaux et al. (1996). In this case, the consequence of the change in Pgp tertiary structure was the creation of a specific binding site for CsA. The same change that increased the binding affinity for CsA also altered the site at which VBL binds, decreasing the affinity of Pgp for VBL. The effect of VBL on CsA binding affinity and the effect of ATP on the binding affinities of both VBL and CsA indicate that separate, but closely linked, binding sites for CsA and VBL exist on the Pgp molecule.

The immobilized Pgp liquid chromatographic stationary phase described in this report appears to reproduce Pgp substrate binding as determined by classical filtration binding assays. The observed binding is Pgp-specific, is highly sensitive to changes in the protein's tertiary conformation caused by Pgp interactions with substrates and ATP, and reflects changes occurring in the functional cycle of Pgp. Thus, Pgp-affinity chromatography represents a promising tool for a quick and reproducible evaluation of potential Pgp substrates and/or inhibitors and a useful probe of the transport mechanism. The data obtained through this approach provide new information on Pgp's mechanism of action, including evidence of binding sites for verapamil and for cyclosporins distinct from the ones for Vinca alkaloids. The data directly support a model of Pgp's action where these substrates can bind to distinct, although often allosterically connected, regions.

Acknowledgment

We thank Dr. Yanxiao Zhang for useful discussion.

References

Barnes KM, Dickstein B, Cutler GB, Fojo T and Bates SE (1996) Steroid transport, accumulation and antagonism of P-glycoprotein in multidrug-resistant cells. *Biochemistry* **35**:4820–4827.

Brekkan E, Lundqvist A and Lundahl P (1996) Immobilized membrane vesicle or proteoliposome affinity chromatography. Frontal analysis of interactions of cytochalasin B and D-glucose with the human red cell glucose transporter. *Biochemistry* **35**:12141–12145.

Callaghan R, Berridge G, Ferry DR and Higgins CF (1997) The functional purification of P-glycoprotein is dependent on maintenance of a lipid-protein interface. *Biochim Biophys Acta* **1328**:109–124.

Clarke R and Leonessa F (1994) Cytotoxic drugs and hormones in breast cancer: Interactions at the cellular level, in *Drug and Hormonal Resistance in Breast Cancer: Cellular and Molecular Mechanisms* (Dickson RB and Lippman ME eds) pp 407–432, Ellis Horwood, Chichester, UK.

Clarke R, Thompson EW, Leonessa F, Lippman J, McGarvey M and Brunner N (1993) Hormone resistance, invasiveness and metastatic potential in human breast cancer. *Breast Cancer Res Treat* **24**:227–239.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR and Bertino JR (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* **86**:695–698

Devine SE, Ling V and Melera PW (1992) Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance. *Proc Natl Acad Sci USA* **89**:4564–4568.

Dey S, Ramachandra M, Pastan I, Gottesman MM and Ambudkar S (1997) Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proc Natl Acad Sci USA* **94**:10594–10599.

Doppenschmitt S, Langguth P, Regardh CG, Andersson TB, Hilgendorf C and Spahn-Langguth H (1999) Characterization of binding properties to human P-glycoprotein: Development of a [^{3}H]verapamil radioligand-binding assay. *J Pharmacol Exp Ther* **288**:348–357.

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK and Ross DD (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* **95**:15665–15670

Ferry DR, Malkandi PJ, Russell MA and Kerr DJ (1995) Allosteric regulation of [^{3}H] vinblastine binding to P-glycoprotein of MCF-7 ADR cells by dexniguldipine. *Biochem Pharmacol* **49**:1851–1861.

Ferry DR, Russel MA and Cullen MH (1992) P-glycoprotein possesses a 1,4-dihydropyridine-selective acceptor site which is allosterically coupled to a vinca-alkaloid-selective binding site. *Biochem Biophys Res Commun* **188**:440–445.

Foxwell BM, Mackie A, Ling V and Ryffel B (1989) Identification of the multidrug resistance-related P-glycoprotein as a cyclosporin binding protein. *Mol Pharmacol* **36**:543–546.

Garrigos M, Mir LM and Orlowski S (1997) Competitive and non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase. Further experimental evidence for a multisite model. *Eur J Biochem* **224**:664–673.

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann Rev Biochem* **62**:385–427.

Hendrikse NH, de Vries EGE, Eriks-Fluks L, van der Graaf TA, Hospers GAP, Willemsen ATM, Vaalburg W and Franssen EJF (1999) A new *in vivo* method to study P-glycoprotein transport in tumors and the blood-brain barrier. *Cancer Res* **59**:2411–2416.

Higgins CF and Gottesman MM (1992) Is the multidrug transporter a flippase? *Trends Biochem Sci* **17**:18–21.

Jaulmes A and Vidal-Madjar C (1989) Theoretical aspects of quantitative affinity chromatography: An overview, in *Advances in Chromatography, Volume 28* (Giddings JC, Grushka E and Brown PR eds) pp 1–64, Marcel Dekker, New York.

Klotz IM (1983) Ligand-receptor interactions: What we can and cannot learn from binding measurements. *Trends Pharmacol Sci* **4**:253–255.

Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: Evidence that multiple substrates can simultaneously bind to cytochrome P450. *Biochemistry* **37**:4137–4147.

Kusuhara H, Suzuki H, Terasaki T, Kakee A, Lamaire M and Sugiyama YP (1997) P-glycoprotein mediates the efflux of quinidine across the blood-brain barrier. *J Pharmacol Exp Ther* **283**:574–580.

Leonessa F, Green D, Licht T, Wright A, Wingate-Legette K, Lippman J, Gottesman

MM and Clarke R (1996) MDA435/LCC6 and MDA435/LCC6MDR1: Ascites models of human breast cancer. *Br J Cancer* **73**:154–161

Litman T, Zeuthen T, Skovgard T and Stein WT (1997) Competitive, non-competitive and cooperative interactions between substrates of P-glycoprotein as measured by its ATPase activity. *Biochim Biophys Acta* **1361**:169–176.

Loe DW, Deeley RG and Cole SPC (1996) Biology of the multidrug resistance-associated protein, MRP. *Eur J Cancer* **32A**:945–957.

Orlowski S, Mir IM, Belehradek J Jr and Garrigos M (1996) Effect of steroids and verapamil on P-glycoprotein ATPase activity: Progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators. *Biochem J* **317**:515–522.

Pascaud C, Garrigos M and Orlowski S (1998) Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents. *Biochem J* **333**:351–358.

Raviv Y, Pollard HB, Bruggemann EP, Pastan I and Gottesman MM (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. *J Biol Chem* **265**:3975–3980.

Rosenberg MF, Callaghan R, Ford RC and Higgins CF (1997) Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *J Biol Chem* **272**:10685–10694.

Shapiro AB and Ling L (1994) ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. *J Biol Chem* **269**:3745–3754.

Sharom FJ (1995) Characterization and functional reconstitution of the multidrug transporter. *J Bioenerg Biomembr* **27**:15–22.

Sonveaux N, Shapiro AB, Goormaghtigh E and Ling V (1996) Secondary and tertiary structure changes of reconstituted P-glycoprotein. *J Biol Chem* **271**:24617–24624.

Spoelstra EC, Westerhoff HV, Pinedo HM, Dekker H and Lankelma J (1994) The multidrug resistance-reverser verapamil interferes with cellular P-glycoprotein-mediated pumping of daunorubicin as a non-competing substrate. *Eur J Biochem* **221**:363–373.

Tamai I and Safa AR (1991) Azidopine noncompetitively interacts with vinblastine and cyclosporin binding to P-glycoprotein in multidrug resistant cells. *J Biol Chem* **266**:16796–16800.

Trock BJ, Leonessa F and Clarke R (1997) Multidrug resistance in breast cancer: A meta analysis of MDR1/gp170 expression and its possible functional significance. *J Natl Cancer Inst* **89**:917–931.

Tsuji A, Terasaki T, Takabatake Y, Tenda Y, Tamai I, Yamashima T, Moritani S, Tsuruo T and Yamashita J (1992) P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci* **51**:1427–1437.

Wandel C, Kim RB, Kajiji S, Guengerich FP, Wilkinson GR and Wood AJJ (1999) P-glycoprotein and cytochrome P-450 3A inhibition: Dissociation of inhibitory potencies. *Cancer Res* **59**:3944–3948.

Winzor DJ (1985) Quantitative characterization of interactions by affinity chromatography in affinity chromatography, in *A Practical Approach* (Dean PDG, Johnson WS and Middle FA eds) pp 149–168, IRL Press, Oxford.

Yusa K and Tsuro T (1989) Reversal mechanism of multidrug resistance by verapamil: Direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* **49**:5002–5006.

Zhang Y, Leonessa F, Clarke R and Wainer IW (2000) Development of an immobilized P-glycoprotein stationary phase for on-line liquid chromatographic determination of drug-binding affinities. *J Chromatogr B* **739**:33–37.

Zhang Y, Xiao Y, Kellar KJ and Wainer IW (1998) Immobilized nicotinic receptor stationary phase for on-line liquid chromatographic determination of drug-receptor affinities. *Anal Biochem* **264**:22–25.

Send reprint requests to: Dr. Irving W. Wainer, Department of Pharmacology, Georgetown University School of Medicine, Rm. C305, Medical Dental Bldg., 3900 Reservoir Rd., NW, Washington, DC 20007. E-mail: waineri@gunet.georgetown.edu

0031-6997/01/5301-25–71$3.00
PHARMACOLOGICAL REVIEWS

Pharmacol Rev 53:25–71, 2001

Vol. 53, No. 1
114/881408
Printed in U.S.A

Cellular and Molecular Pharmacology of Antiestrogen Action and Resistance

ROBERT CLARKE,[1] FABIO LEONESSA, JAMES N. WELCH, AND TODD C. SKAAR

Vincent T. Lombardi Cancer Center, Georgetown University School of Medicine, Washington, DC

This paper is available online at http://pharmrev.aspetjournals.org

[1] Address for correspondence: Robert Clarke, Ph.D., D.Sc., W405A Research Building, Vincent T. Lombardi Cancer Center, Georgetown University School of Medicine, 3970 Reservoir Rd., NW, Washington, DC 20007. E-mail: clarker@gunet.georgetown.edu

Abstract*——Antiestrogen therapy remains one of the most widely used and effective treatments for the management of endocrine responsive breast cancers. This reflects the ability of antiestrogens to compete with estrogens for binding to estrogen receptors. Whereas response rates of up to 70% are reported in patients with tumors expressing estrogen and progesterone receptors, most responsive tumors will eventually acquire resistance. The most important factor in de novo resistance is lack of expression of these receptors. However, the mechanisms driving resistance in tumors that express estrogen and/or progesterone receptors are unclear. A tamoxifen-stimulated phenotype has been described, but seems to occur only in a minority of patients. Most tumors (>80%) may become resistant through other, less well defined, resistance mechanisms. These may be multifactorial, including changes in immunity, host endocrinology, and drug pharmacokinetics. Significant changes within the tumor cells may also occur, including alterations in the ratio of the estrogen receptor α:β forms and/or other changes in estrogen receptor-driven transcription complex function. These may lead to perturbations in the gene network signaling downstream of estrogen receptors. Cells may also alter paracrine and autocrine growth factor interactions, potentially producing a ligand-independent activation of estrogen receptors by mitogen-activated protein kinases. Antiestrogens can affect the function of intracellular proteins and signaling that may, or may not, involve estrogen receptor-mediated events. These include changes in oxidative stress responses, specific protein kinase C isoform activation, calmodulin function, and cell membrane structure/function.

I. Introduction

Endocrine manipulations are among the most effective, and least toxic, of the systemic therapies currently available for the management of hormone-responsive breast cancers. Ovariectomy in premenopausal women is the oldest of these therapies (Beatson, 1896) and has long been known to produce benefit in approximately one-third of all patients (Boyd, 1900). Although ovariectomy is still an effective therapy, currently the administration of antiestrogenic drugs is the most widely applied endocrine manipulation. Antiestrogenic drugs are effective in both premenopausal and postmenopausal patients and in the metastatic, adjuvant, and chemopreventive settings. The drugs are well tolerated, the incidence of dose-limiting toxicities is low, and responses are seen in approximately 70% of patients selected on the basis of the steroid hormone receptor expression profile of their tumors (Clark and McGuire, 1988). Additional benefits associated with some antiestrogens likely include reductions in the risk and/or severity of osteoporosis. Evidence also supports a possible reduction in the risk of cardiovascular disease (McDonald et al., 1995), but this is not consistent across all studies (EBCTCG, 1998; Fisher et al., 1998). Whether the estrogenic effects of Tamoxifen (TAM[2]) are responsible for any reduction

[2] Abbreviations: TAM, Tamoxifen; AEBS, antiestrogen binding site; AP-1, activator protein-1; CMI, cell-mediated immunity; EGF, epidermal growth factor; EGF-R, epidermal growth factor-receptor; ER, estrogen receptor; ERE, estrogen-responsive element; FGF, fibroblast growth factor; GR, glucocorticoid receptor; HRT, hormone replacement therapy; 4-hydroxyTAM, 4-hydroxytamoxifen; IC_{50}, in-

in coronary heart disease has also become somewhat controversial, since the preventive effects of estrogenic hormone replacement therapy (HRT) on coronary heart disease have been questioned (Hulley et al., 1998).

Currently, the most widely used antiestrogen is the triphenylethylene TAM (ICI 46,477), which is administered orally as the citrate salt. Cole et al. (1971) described the first clinical study demonstrating TAM's efficacy. TAM was approved for use in advanced disease several years later. Clinical experience with this drug likely now exceeds 10 million patient years. Unfortunately, in most patients, cancers that initially respond to TAM will recur and require alternative systemic therapies. Despite extensive experience with this drug, the precise mechanisms that confer resistance remain unknown. This review will discuss evidence from recent clinical trials and experimental models that identify several possible mechanisms of resistance. Because the activity of antiestrogens is intimately involved with the role of estrogens and their receptors, a brief discussion of the role of estrogens and estrogen receptors (ERs) is included. Additional ER-independent events, which also may be important, are discussed.

A. Role of Estrogens in Affecting Breast Cancer Risk and Progression

The utility of antiestrogens as treatments and/or chemopreventives for breast cancer is closely associated with antagonizing the activity of estrogens. Estrogens have been widely implicated in affecting breast cancer risk in the postmenopause. Evidence includes the association of increased serum estrogens, or estrogen excretion, with postmenopausal breast cancer (Table 1) (see Thomas et al., 1997 for review). Prolonged HRT, which also elevates serum estrogen levels, can significantly increase breast cancer risk (CGHFBC, 1997), and the tumors arising tend to be primarily ER-positive (Lower et al., 1999). HRT is often prescribed to naturally perimenopausal or postmenopausal women, but may also be given to younger women with primary ovarian failure, or who have had their ovaries removed/irradiated.

The estrogenicity of HRTs can vary significantly, and dose is important, at least in some studies. For example, low potency oral and transdermal estrogens may not increase risk, whereas more potent estrogens significantly increase breast cancer risk (Magnusson et al., 1999). Serum estradiol concentrations can exceed 0.77 nM with some HRT regimens (Garnett et al., 1990). This concentration is almost 10-fold higher than that seen in untreated postmenopausal women and is comparable with that seen in the luteal phase of the menstrual cycle (Table 1). Recent evidence suggests that the greatest increase in breast cancer risk is associated with replacement therapies that combine estrogens and progestins (Schairer et al., 2000). Most studies observe the greatest risk in current/recent users, perhaps reflecting a promotional rather than initiating action of the estrogens.

Whereas HRT increases the risk of developing breast cancer, the resulting biology of the tumors may be different from those arising in the absence of HRT. Patients using HRT at the time of diagnosis have a reduced mortality from breast cancer (Schairer et al., 1999), perhaps reflecting a less aggressive biology (CGHFBC, 1997; Holli et al., 1997). Thus, the estrogenicity of HRT may have allowed the survival of less aggressive tumors. This is consistent with the observation that estrogen-dependent breast cancer cells selected in vivo for growth in a low estrogen environment, rather than in the presence of an adequate estrogenic stimulus, can acquire a more aggressive phenotype (Thompson et al., 1993).

Indirect evidence for a role for estrogens in affecting lifetime breast cancer risk is provided by several known risk factors. For example, breast cancer risk is increased in women who either began menstruating at a young age (<12 years) and/or ceased menstruating (menopause) at a late age (≤55 years) (Hulka and Stark, 1995). This would tend to increase the number of cycles and total lifetime exposure to ovarian estrogens. Postmenopausal obesity is also associated with increased breast cancer risk (Hulka and Stark, 1995). Peripheral adipose tissue is the primary source for the production of circulating estrogens in postmenopausal women, and serum estrogen concentrations are generally higher in obese postmenopausal women (Ingram et al., 1990; Madigan et al., 1998). There are also data implicating estrogenic exposure and risk of premenopausal breast cancer. Perhaps the most compelling evidence is the efficacy of ovariectomy and luteinizing hormone releasing hormone analogs in inducing responses in premenopausal patients (Crump et al., 1997).

Estrogens may affect carcinogenesis by acting either as initiators (i.e., directly damage DNA) or as promoters (i.e., promoting the growth and/or survival of initiated cells). For example, administration of estrogens alone can produce tumors in some rodents (Lacassagne, 1932). This may reflect an effect mediated through mouse mammary tumor virus, and/or activities of the more chemically reactive metabolites of 17β-estradiol. Reactive estrogen semiquinone/quinone intermediates are produced by the redox cycling of the hydroxylated estrogen metabolites. These can produce DNA adducts (initiation). This has been most closely associated with the 4-hydroxy (Liehr and Ricci, 1996) and 3,4-hydroxy metabolites, with a recent study strongly implicating the

hibitory concentration of 50%; IGF, insulin-like growth factor; IGF-BP, insulin-like growth factor-binding protein; IGF-I-R, insulin-like growth factor-I- receptor; IGF-II-R, insulin-like growth factor-II-receptor; JNK, c-Jun NH_2-terminal kinase; K_d, concentration of ligand yielding half-maximum binding; LAK, lymphokine-activated killer; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NPM, nucleophosmin; NSABP, National Surgical Adjuvant Breast and Bowel Project (P-1 Study); NK, natural killer; PgR, progesterone receptor; PKC, protein kinase C; SAPK, stress-activated protein kinase; TGF, transforming growth factor; TPA, triphenylethylene antiestrogen.

TABLE 1
Examples of the association of serum estrogens and HRTs with increasing risk of breast cancer in postmenopause

Serum Estrogen Levels (Postmenopausal), HRT, and Breast Cancer Risk			
Study	Healthy Controls	Breast Cancer	Significance
Berrino et al., 1996 (HC = 88; BC = 24)[a]	0.08 nM	0.09 nM	$p = 0.027$
Zeleniuch-Jacquotte et al., 1995 ER+ (HC = 101; BC = 53)	0.107 nM	0.133 nM	$p = 0.05$
ER− (HC = 45; BC = 23)	0.086 nM	0.110 nM	$p = 0.07$
ER unknown (HC = 102; BC = 54)	0.099 nM	0.121 nM	$p = 0.04$
Overall mean estimates	0.093 nM	0.114 nM	

Study	Free Serum Estradiol[b]	OR (Unadjusted)	OR (Adjusted)
Toniolo et al. (1995)	<1 pM	1.0	1.0
	1–1.7 pM	1.5 (0.8, 3.0)	1.4 (0.7, 2.8)
	1.7–2.4 pM	3.8 (1.95, 7.5)	3.0 (1.4, 6.3)
	>2.4 pM	3.9 (1.8, 8.1)	2.9 (1.3, 6.6)

HRT—Estrogens Alone[c]	
Study	Risk Estimates
CGHFBC (1997)[d]	RR = 1.14 ± 0.03 (p = 0.00001)
Schairer et al., 2000[e]	RR = 1.1 (1.0, 1.3)
Magnusson et al., 1999[f]	OR = 1.99 (1.67, 2.38)

Serum Estrogen Levels in Premenopausal Women[g]	
Follicular phase	≤0.28 nM
Luteal phase	≤1.1 nM
Pregnancy: 3rd trimester	≤150 nM

[a] HC = health control; BC = breast cancer patients. There were a total of 4,043 women enrolled in the Berrino et al. study and 7,063 women in the Zeleniuch-Jacquotte study.
[b] Quartiles (approximate) of serum estradiol concentrations and odds ratios for postmenopausal breast cancer. Data are adjusted for the Quetelet index (Toniolo et al., 1995).
[c] There are various differences in study design, population, and analysis. Nonetheless, these selected studies reflect the generally consistent association of increased breast cancer risk with estrogenic HRT use. Data are presented as provided in each publication. RR = relative risk; OR = odds ratio.
[d] CGHFBC = Collaborative Group on Hormonal Factors in Breast Cancer. Data for every use presented as relative risk ± S.E.
[e] Data for every use presented as relative risk and 95% confidence interval.
[f] Data for every use of medium-potency estrogens presented as odds ratio and 95% confidence interval.
[g] Estimated upper limits in normal women. These values are provided as a general reference, with there being considerable variability among women. The highest concentrations of estrogens are found during the third trimester of pregnancy.

catechol estrogen-3,4-quinones as initiators (Cavalieri et al., 1997). The production of these metabolites is a function of several cytochrome P-450 isoforms that are expressed in the breast, liver, and other tissues (Zhu and Conney, 1998).

The potential role of estrogens as promoters of carcinogenesis is more firmly established. Ovariectomy—whether chemical, surgical, or radiation-induced—remains a highly effective treatment (Crump et al., 1997). Indeed, surgical ovariectomy and the suppression of gonadotropin secretion by luteinizing hormone releasing hormone analogs are as effective as TAM in managing premenopausal breast cancer (Jonat, 1998). Chemically induced mammary adenocarcinomas in rats also require functional ovaries (Russo et al., 1990), probably reflecting promotion of the carcinogen-initiated cells. Several human breast cancer cell lines require estrogen for proliferation in vitro and in vivo (Clarke et al., 1996). This proliferation can be blocked by the administration of antiestrogens, consistent with the removal of a mitogenic effect. Although estrogens may function as both initiators and promoters of carcinogenesis, for the purposes of this review the promotional effects are most relevant.

B. Antiestrogens: Partial Agonists and Antagonists

Antiestrogens primarily function through their ability to compete with available estrogens for binding to ER. However, the consequences of occupying ER with an antiestrogen appear dependent upon the cellular context, which ER is occupied (ERα and/or ERβ), and the structure of the ligand. The most important biological consequence is whether the activated receptor complex induces an estrogenic or antiestrogenic response. This has significant implications. Producing an estrogenic response in bone and an antiestrogenic response in the breast would be highly beneficial. In contrast, the reverse pattern of response could stimulate the growth of an existing breast tumor and concurrently increase the risk of debilitating fractures.

TAM provides a good illustration of several of these points. TAM is a classical partial agonist and exhibits both species and tissues specificity for inducing either an agonist or antagonist response. In the mouse, TAM is an agonist. In rats and humans, it exhibits partial agonism (Jordan and Robinson, 1987) [e.g., producing antagonist effects in the breast, but agonist effects in the vagina and endometrium (Harper and Walpole, 1967;

Ferrazzi et al., 1977)]. Long-term TAM use is generally associated with a reduced incidence of contralateral breast cancer (antagonist), a reduced incidence of primary breast cancer in high-risk women (antagonist), maintenance of bone density (agonist), and increased risk of endometrial carcinomas (agonist) (Fisher et al., 1998).

The ability to generate these tissue-specific effects has lead to the search for other selective ER modulators, which will have the beneficial effects seen with TAM but without the increased risk of endometrial carcinoma. Several triphenylethelene variations on TAM are already available, including Toremifene (chloro-TAM) and Droloxifene (3-OH-TAM). Both drugs seem to be approximately equivalent to TAM in terms of their antitumor activities and toxicities; both drugs are partial agonists (Roos et al., 1983; Pyrhonen et al., 1999).

The clinical utility of several of these newer antiestrogens has recently been reviewed by others (Lien and Lonning, 2000), and an exhaustive review is beyond the scope of this article. Nonetheless, several of the newer compounds are notable. Many are not triphenylethylenes [e.g., Raloxifene is a benzothiophene (previously called keoxifene; LY 156,758)]. It is now available in the U.S. as a treatment for the prevention of osteoporosis in postmenopausal women. Evidence suggests that Raloxifene may not have the same uterotropic effects as TAM (Delmas et al., 1997) and that it may regulate gene expression through novel pathways (Yang et al., 1996). In the multiple outcomes of Raloxifene randomized trial, Raloxifene significantly reduced the number of breast cancer cases, from 27/2576 to 13/5129 (Cummings et al., 1999), but did not increase the incidence of endometrial cancers (Delmas et al., 1997; Cummings et al., 1999). It also produces beneficial effects comparable with TAM on other endpoints, including lowering levels of both total and low-density lipoprotein cholesterol (Delmas et al., 1997; Walsh et al., 1998) and increasing bone mineral density (Delmas et al., 1997). However, Raloxifene increases the incidence of hot flashes (Davies et al., 1999).

Other antiestrogens that have received attention are the steroidal compounds ICI 164,384 and ICI 182,780. Both ICI 164,384 and ICI 182,780 have high affinities for ER (Wakeling and Bowler, 1988). There may also be some preference for ERβ, since ICI 164,780's relative binding affinity for ERβ = 166%, but for ERα = 85% (Kuiper et al., 1997). Both ICI 164,384 and ICI 182,780 seem to be antagonists, being devoid of agonist activity in most experimental models. For example, ICI 164,384 does not exhibit agonist activity either in MCF-7 cells growing in the absence of estrogens (Clarke et al., 1989c; Thompson et al., 1989), or in the uterus or vagina of rats and mice (Wakeling and Bowler, 1988). ICI 164,384 can inhibit the agonist effects of both estrogen and TAM (Wakeling and Bowler, 1988). The estrogenic activities of TAM induce expression of a series of estrogen-regulated genes, including the progesterone receptor (PgR) and pS2. ICI 164,384 has no notable estrogenic effects on the regulation of these genes (Wiseman et al., 1989), other than a modest induction of PgR in endometrial cells (Jamil et al., 1991). However, there is evidence that ICI 182,780 can produce an estrogen-like effect in KPL-1 breast cancer cells (Kurebayashi et al., 1998). When ICI 182,780 is administered to pregnant rats, their female offspring exhibit changes in their mammary glands similar to those seen in offspring exposed to exogenous estradiol in utero (Hilakivi-Clarke et al., 1997). This could reflect primarily ERβ-mediated events, since ERβ is the predominant form at least in some normal human and rodent mammary tissues (Speirs et al., 1999b;Saji et al., 2000). Furthermore, ICI 182,7870 is an activator of transcription at AP-1 sites (Paech et al., 1997).

The steroidal antiestrogen ICI 182,780 retains its potency in vivo as determined by its ability to inhibit MCF-7 and Br10 tumors. This compound also exhibits substantial antiuterotrophic activity in the immature rat (de Launoit et al., 1991). ICI 182,780 (trade name: Faslodex) has already completed initial phase I clinical evaluation. The first study was performed on patients who had previously demonstrated a response to TAM, but recurred. The overall reported response rate of 69% (Howell et al., 1995) is substantially higher than the 5% objective response rate reported for crossover to another triphenylethylene (Toremiphene) following TAM failure (Vogel et al., 1993) and is more in line with responses to alternative second line endocrine therapies [e.g., aromatase inhibitors (Dowsett et al., 1995)]. This observation suggests that the steroidal antiestrogens affect breast cancer cells differently than the triphenylethylenes.

The partial agonist activities of TAM and Raloxifene are thought to be responsible for their beneficial effects on bone resorption. Pure antagonists like ICI 182,780 may further exacerbate bone loss, a concern that also applies to aromatase inhibitors (Dowsett, 1997). However, when combined with alternative therapies for osteoporosis, such as bisphosphonates, these drugs may have considerable potential as first-line endocrine therapies.

C. Response Rates to Tamoxifen and Expression of Steroid Hormone Receptors

Patients with ER-positive tumors have a significantly higher response rate to antiestrogens than patients with ER-poor/ER-negative tumors. This relationship holds whether ER is measured by ligand binding or immunohistochemistry, reflecting the high concordance seen with these different techniques (Molino et al., 1997). It also holds despite the range of cut-off values used for assessing ER positivity versus ER-poor/ER negativity. TAM also seems most effective in the suppression of ER-positive tumors in the chemopreventive setting (Fisher et al., 1998).

Expression of PgR also has been implicated as a predictor of response to TAM. Several studies have reported responses in patients with ER-negative but PgR-positive tumors. However, the number of tumors is small and could reflect false negative estimations of ER expression. Concurrent expression of both ER and PgR is often associated with a higher response rate than in ER-positive, but PgR-negative, tumors. In general, approximately 70% of patients with ER-positive/PgR-positive tumors will respond to TAM, whereas response rates of 45% are seen in patients with ER-negative, but PgR-positive tumors. A 34% response rate is seen in ER-positive, but PgR-negative, tumors (Honig, 1996). The predictive power of PgR expression is likely related to the ability of estrogens to induce its expression. Thus, the presence of both ER and PgR may reflect the existence of an at least partially functional ER signaling pathway (Horwitz et al., 1975).

The Early Breast Cancer Trialists Group's initial meta-analysis in 1992 reported both a significant reduction in recurrence or death, and a reduction in death from any cause, in patients with ER-poor tumors (Table 2). Their more recent meta-analysis found no significant reduction in recurrence rates in patients with ER-poor tumors. Indeed, a 3% (nonsignificant) increase in the risk of death from any cause was reported in women, receiving TAM, with ER-poor tumors (Table 2). These latter data do not strongly implicate ER-independent events in beneficial responses to TAM and possibly indicate an adverse effect in some women. What those adverse effects may be, whether they are real, and the extent to which they may be restricted to an undefined subset of patients, remain to be determined. It also may reflect the more aggressive biology of ER-negative tumors (Aamdal et al., 1984; Clark and McGuire, 1988). Whereas longer term TAM use (e.g., 10 yr) is less beneficial than 5 yr, it still produces an overall benefit (EBCTCG, 1992, 1998). Why the benefit should be lower with longer use is not known, but may also reflect an adverse effect in some women.

D. Overview of Antiestrogen Resistance Mechanisms

Antiestrogens clearly produce several beneficial effects in some patients, including improved disease-free survival and overall survival from breast cancer. However, most patients with initially responsive tumors will experience a recurrence, indicating acquired antiestrogen-resistant disease. There are several possible mechanisms that could influence response to antiestrogens and, when altered, contribute to resistance. These include changes in host immunity, host endocrinology, or antiestrogen pharmacokinetics. Competition with en-

TABLE 2

Treatment with TAM, its potential as a chemopreventive agent, and the potentially confounding effects of HRT on response to TAM

Early Breast Cancer Trialists Collaborative Group[a] (1992)				
Endpoint	TAM	Control	Reduction in Risk	Significance
Recurrence	2,852/15,027	4,387/15,054	16%	$p < 0.000001$
Contralateral breast cancers	122/9,128	184/9,135	39%	$p < 0.000001$
Mortality	5,052/15,027	6,043/15,054	25%	$p < 0.000001$
	Recurrence or Prior Death	Significance	Death Any Cause	Significance
ER+ (n = 14,972)	32%	$p = 0.00001$	21%	Significant
ER poor[b] (n = 5,366)	13%	$p = 0.001$	11%	$p = 0.02$
Early Breast Cancer Trialists Collaborative Group (EBCTCG, 1998)				
Endpoint	TAM ~5 yr	Control	Reduction ± S.D.	Significance
Recurrence (ER+)	692/2,966	1,110/2,903	50% ± 4	$p < 0.00001$
Recurrence (ER poor)	191/446	210/476	6% ± 11	N.S.
Death: any cause (ER+)	655/2,966	812/2,903	28% ± 5	Significant
Death: any cause (ER poor)	182/446	178/476	−3% ± 11	N.S.
Chemoprevention				
Study	Placebo	TAM	Reduction in Risk	Significance
U.K. (Powles et al., 1998) (n = 2,471)[c]	36	34	6%; 1.06 (0.7, 1.7)	$p = 0.8$
Italian (Veronesi et al., 1998) (n = 5,408)[d]	22	19	14%	$p = 0.6$
NSABP P-1 (Fisher et al., 1998) (n = 13,388)[e]			Relative risk	Significance
Invasive cancers	175	89	49%; 0.51 (0.39; 0.66)	$p < 0.00001$
Noninvasive cancers	69	35	50%; 0.50 (0.33, 0.77)	$p < 0.002$

[a] Data are adapted from each study. Significance estimates are from the appropriate study. In some cases, the precise p-values are not available. N.S. = not significant.
[b] ER poor is generally taken as <10 fmol/mg protein.
[c] 42% used HRT.
[d] TAM appears effective in 14% of women taking HRT (hazard ratio = 0.13; confidence interval = 0.02, 1.02).
[e] Subjects did not receive HRT.

dogenous ligands for binding to an antiestrogen's primary intracellular target(s), or altered function of its target(s), could also contribute to resistance (Fig. 1). The low rate of responses in ER-negative tumors is most consistent with antiestrogen action being primarily mediated through interactions with ER. However, antiestrogens, and TAM in particular, have been shown to bind intracellular proteins in addition to ER. It might be expected that, if these targets were critical for generating a response, many ER-negative tumors also would be responsive. Although such responses are not common, the ability of antiestrogens to influence the function of targets other than ER may still be important.

It is apparent that the cellular context (i.e., the gene/protein expression pattern in a cell) can affect how a cell responds to a specific stimulus (Clarke and Brünner, 1996). For example, ER's transcriptional activities can be influenced by phosphorylation events regulated by signaling, which activates mitogen-activated kinase (MAPK) (Kato et al., 1995). Downstream signaling from the ER also is likely to be complex and may interact/intersect with other (ER-independent) signaling pathways. Antiestrogens could influence the activities of these other pathways (e.g., through binding to non-ER proteins) and alter cellular context (Clarke and Brünner, 1996). Whereas such events are probably not sufficient to induce an antiestrogenic effect in most ER-negative cells, they may be necessary/permissive for signaling to a fully antiestrogenic effect in responsive cells. Thus, perturbations in the activity of some ER-independent effects could contribute to an acquired antiestrogen resistance. Both ER-mediated and ER-independent targets for antiestrogens are considered in this review.

II. Endogenous and Exogenous Estrogens in Antiestrogen Resistance

A. Origins of Intratumor Estrogens

In women, the biosynthesis of estrogens may arise from several sources. Ovarian production is the main source of circulating estrogens in premenopausal women, the primary estrogen being 17β-estradiol. The efficacy of ovariectomy and luteinizing hormone releasing hormone analogs in premenopausal women (Crump et al., 1997) strongly support a role for ovarian estrogen production in the breast cancers that arise in these women. Conversion of adrenal androgens in peripheral tissues is the predominant source of circulating estrogens in postmenopausal women. The primary estrogen produced in the postmenopause by the action of aromatase is the relatively weak estrone, which is generally present in serum as the inactive estrone sulfate. Breast cancer cells can release the biologically active estrone through the action of the steroid sulfatase enzyme (Pasqualini et al., 1988) and can further convert estrone to 17β-estradiol through the action of 17β-hydroxysteroid dehydrogenase type 1 (Brodie et al., 1997).

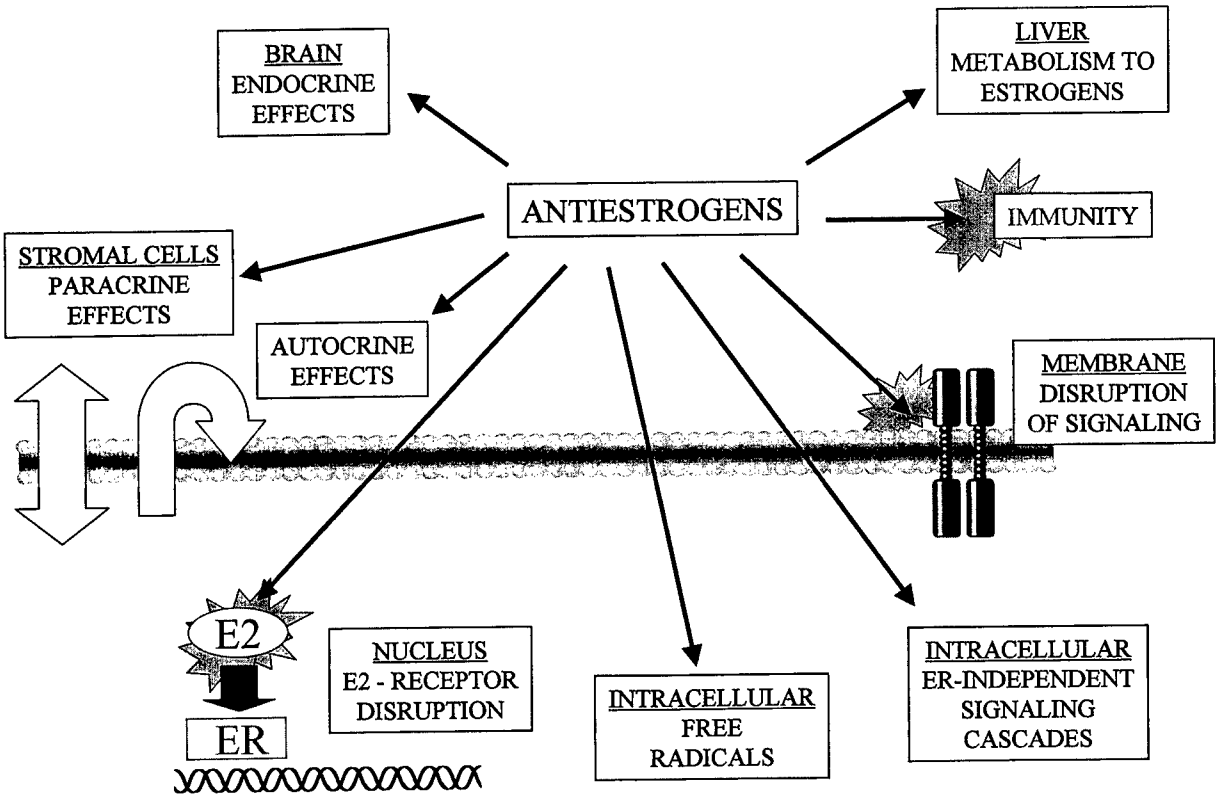


FIG. 1. Overview of the likely targets of antiestrogen action and resistance. E2, estradiol.

Mammary tissues accumulate serum estrogens to concentrations significantly higher than those present in serum (Masamura et al., 1997; Miller, 1997). However, breast tissues also synthesize estrogens through a pathway similar to that in peripheral adipose tissues. This biosynthesis can occur within the epithelial cells (Brodie et al., 1997), the associated breast adipose tissue (Bulun and Simpson, 1994), and in some infiltrating lymphoreticular cells (Mor et al., 1998).

The importance of the aromatase enzyme in generating biologically active estrogens is evidenced by the efficacy of aromatase inhibitors in inducing clinical responses in postmenopausal breast cancer patients. These drugs already are established as second-line endocrine therapies (Dowsett, 1997). Because they inhibit both peripheral and breast aromatase activities, it is often difficult to assess which site of synthesis predominates. Both peripheral and intratumor/stromal aromatase activities are likely to be important, with the relative contribution varying among tumors. Studies in experimental models suggest that local production may be more important (Santen et al., 1999). Although peripheral aromatization is reduced to comparable levels by both aminoglutethimide and testololactone in women, testololactone produces a much lower clinical response rate (Lonning et al., 1989a). However, aminoglutethimide significantly increases estrone sulfate clearance in addition to its inhibition of aromatase activity (Lonning et al., 1989b; Lonning et al., 1990). These data suggest that both serum estrogens and intratumor/stromal biosynthesis may contribute to intratumor estrogen concentrations.

B. Intratumor Estrogen Concentrations

High intratumor estrogen concentrations could prevent antiestrogens from blocking ER action and produce a resistant phenotype. Data in Table 3 show that normal, benign, and malignant breast tissues in postmenopausal women contain concentrations of 17β-estradiol up to 10-fold higher than those seen in serum. The range among tumors is considerable, from undetectable to over 5 μM 17β-estradiol, with these levels being essentially equivalent regardless of patients' menopausal status. The mean concentration estimated from these studies is 1.28 nM (Table 3). If this reflects the concentration in epithelial cells, and it is fully available for ER binding, there would be sufficient intratumor estradiol to produce a maximal stimulation of ER signaling. In serum, <5% of estrogens are "free" [i.e., not bound to serum proteins]. Using this as an estimate of intracellular availability within tumors, and with a K_d of approximately 0.1 nM in breast cancer and other cells (Bei et al., 1996), only 25% of ER would be occupied.

Generally, biological response is proportional to receptor occupancy. However, some cells up-regulate receptor expression, these "spare" receptors producing a left shift in the dose-response relationship (Ross, 1996). If this occurred in some breast tumors, they might exhibit a greater biological response than would be predicted by the proportion of occupied receptors. Consistent with the concept of spare receptors, MCF-7 cells respond to 17β-estradiol at concentrations well below its K_d for ER. Some MCF-7 cells selected in vitro for growth in the absence of estradiol further up-regulate ER expression

TABLE 3

17β-Estradiol concentrations in breast tumors, normal and benign breast tissues, and in sera

Study	Mean ± S.D./S.E. (nM)[b]	Range (nM)
Intratumor concentrations of 17β-estradiol[a]		
Bonney et al., 1983 (n = 13)	1.76 ± 0.3	
de Jong et al., 1997 (n = 9)	0.84 ± 0.58	0.148–1.77
Drafta et al., 1983 (n = 41)	ER+ = 1.58 ± 1.06; ER− = 0.56 ± 0.39[c]	
Edery et al., 1981 (n = 78)	ER+ = 2.92 ± 1.29; ER− = 0.94 ± 1.03[d]	
Fishman et al., 1977 (n = 129)	ER+ = 0.33 ± 0.21; ER− = 0.19 ± 0.14[e]	
Maynard et al., 1978	N.D.	ER+ = 0–1.1; ER− = 0–0.24
Mehta et al., 1987 (n = 65)	1.34 ± 0.13	
Millington, 1975 (n = 18)	3.1 ± 11.97	0.7 nM–5.5 μM
Mistry et al., 1986 (n = 16)	0.756 ± 0.49	
Pasqualini et al., 1996 (n = 34)	1.4 ± 0.7 (postmenopausal)	
Recchione et al., 1995 (n = 34)	0.169 (median value)	0.033–0.775
van Landeghem et al., 1985 (n = 105)	0.62 ± 0.39	0.02–1.52
Vermeulen et al., 1986 (n = 50)	1.64 ± 1.89	0.07–9.02
	Overall mean estimate = 1.28 nM	
Normal and benign breast tissues		
Kyo et al., 1999	0.625 ± 0.018 (adjacent normal tissue)	
Schaefer et al., 1995	N.D.	0.06–0.56
Mehta et al., 1987 (n = 61)	0.93 ± 0.10 (adjacent normal tissue)	
Pasqualini et al., 1997 (n = 15)	1.0 ± 0.25 (fibroadenoma)	
Szymczak et al., 1998 (n = 30)	0.203 ± 0.025 (adipose)	
Vermeulen et al., 1986 (n = 14)	1.05 ± 0.9 (glandular tissue)	0.15–2.76
	Overall mean estimate = 0.76 nM	

Where values are missing, they cannot be readily identified from the publication(s). N.D. = not detected.

[a] All values are nM unless otherwise indicated. Numbers in parentheses are the number of subjects in the study.

[b] Mean estimates are provided with either the standard deviation or standard error and are based on the data presented in the studies using the following conversions: (a) 1 g tissue weight ≅ 1 ml; and (b) 50 mg protein ≅ 1 g tissue weight.

[c] $p < 0.01$ for ER+ vs. ER− (Drafta et al., 1983).

[d] $p < 0.001$ for ER+ vs. ER− (Edery et al., 1981).

[e] $p < 0.02$ for ER+ vs. ER− (Fishman et al., 1977).

(Jeng et al., 1998). However, MCF-7 cells, which represent the most widely used endocrine responsive experimental model (Levinson and Jordan, 1997), have ER levels of ~400 fmol/mg protein (Martin et al., 1991). This is 40 times greater than the lower limit used to determine ER positivity in tumors. Relatively few breast tumors express these very high levels of ER, nor the levels seen in an estrogen supersensitive MCF-7 variant (Masamura et al., 1995).

In the absence of spare receptors, our estimate of 25% receptor occupancy would predict that many breast tumors exist in a weak estrogenic environment. Evidence of a suboptimal estrogenic environment being present in tumors is apparent from the associations of increased serum estrogens, HRT (Table 1), and oral contraceptive use (Hulka and Stark, 1995; CGHFBC, 1997) with increased breast cancer risk in some populations. Similarly, some metastatic tumors, which develop while a patient is taking HRT, regress upon withdrawal of HRT (Dhodapkar et al., 1995). Generally, the effects of HRT are not seen in heavier women (Magnusson et al., 1999; Schairer et al., 2000), probably reflecting the ability of higher serum estrogen levels, derived from peripheral adipose tissues, to compensate for a low intratumor estrogenic environment. In lean postmenopausal women, HRT could stimulate tumors with otherwise suboptimal intratumor estrogen concentrations.

Tumors arising in women exposed to HRT tend to be ER-positive (Lower et al., 1999). In one recent study, the mitogenic effects of HRT (high *S*-phase fraction) were seen only in ER-positive tumors (Cobleigh et al., 1999). ER-positive tumors often proliferate more slowly than ER-negative tumors (Wenger et al., 1993), which have no obvious need of estrogens for proliferation. This may reflect a suboptimal estrogenic/mitogenic environment, and could contribute to the different biologies apparent between ER-positive and ER-negative tumors.

Some tumors with no effective estrogenic stimulation could be driven by a ligand-independent activation of the ER signaling network (Tzukerman et al., 1990; Clarke and Brünner, 1996). Others with insufficient ligand may benefit from a concurrent ligand-independent activation of the remaining unoccupied ER. Generally, ligand independent activation is weaker than ligand activation. Both forms of activation can be blocked by antiestrogens (Clarke and Brünner, 1996; Tzukerman et al., 1990). Thus, tumors driven exclusively or partly by ligand-independent activation of ER should still exhibit responses to several endocrine therapies.

C. Does the Pituitary-Ovarian Axis Affect Response to Tamoxifen in Premenopausal Women?

The potential contribution of serum estrogens to intratumor estrogen concentrations implies that factors influencing serum estrogen concentrations might affect response to antiestrogens. Some early studies suggested that TAM is of greater benefit when administered to postmenopausal rather than premenopausal women. However, these data are not supported in the recent Breast Cancer Trialists Cooperative Group meta- analysis, where it is clear that TAM is equally effective in both postmenopausal and premenopausal patients (EBCTCG, 1998). This does not exclude possible important mechanistic differences concerning how tumors respond in premenopausal versus postmenopausal women. For example, the presence of functional ovaries, particularly if these provide a major component of intratumor estrogenicity, could affect responsiveness.

The release of estrogens from the ovaries is regulated by the pituitary-ovarian axis. Estrogens can regulate the release of gonadotropins at two levels: the release of gonadotropin releasing hormone from the hypothalamus and the release of gonadotropins from the anterior pituitary. If TAM effectively blocks the ER in both the hypothalamus and anterior pituitary, this would disrupt the negative feedback on gonadotropin releasing hormone, ultimately producing a "hyperstimulation" of the ovaries. This might partly explain how TAM increases the circulating levels of estrogens in some premenopausal women (Ravdin et al., 1988; Szamel et al., 1994). Other studies have not reported an ability of TAM to affect circulating estrogen levels. However, ovariectomy and aromatase inhibitors can induce remissions in premenopausal women who initially responded to TAM but eventually recurred. This suggests that TAM produced an incomplete antiestrogen action, possibly as a result of increased circulating estrogens.

TAM can affect gonadotropin levels in premenopausal women, but its ability to do so in postmenopausal women is not so clear (Lien and Lonning, 2000). Small increases in serum dehydroepiandrosterone, estrone, and estradiol levels are also produced by antiestrogens in postmenopausal women (Szamel et al., 1994; Pommier et al., 1999). This probably reflects an effect mediated either through the release of adrenal androgens and/or increases in adrenal estrogen production in postmenopausal women (Pommier et al., 1999).

Where serum estrogens are increased, a consequent elevation in intratumor 17β-estradiol concentrations would be predicted, reflecting the ability of tumors to accumulate serum estrogens. Such an effect might compromise response to TAM by increasing intratumor estrogen competition for binding to ER. Whether this occurs to an extent sufficient to affect the response to TAM is unclear. Response rates to TAM are comparable in premenopausal and postmenopausal women, but serum estrogen levels are higher in premenopausal women. A clearer understanding of the role of serum estrogens in influencing TAM response will probably await data from appropriately designed clinical trials. Nonetheless, it is evident that estrogens can readily reverse the inhibitory effects of antiestrogens in experimental models in vitro and in vivo. Since the primary estrogen produced in premenopausal women in response to TAM is also the

most potent (17β-estradiol), and tumors can significantly accumulate estrogens to levels in excess of that seen in serum (Masamura et al., 1997; Miller, 1997), changes in serum estrogens could affect TAM responsiveness in some individual tumors.

D. Can Endogenous Estrogens or Hormone Replacement Therapies Produce Antiestrogen Resistance?

Antiestrogens can block both ligand-dependent and ligand-independent ER activation (Tzukerman et al., 1990; Clarke and Brünner, 1996). Thus, the precise origin of the ligand, and whether or not it is required for receptor activation, is less important than the potential of available intratumor estrogens to prevent antiestrogen action. Free intracellular estrogens could compete with antiestrogens for binding to ER, reducing their ability to block ligand dependent receptor activations.

The mean intratumor concentration (1.28 nM from Table 3) would probably not be sufficient to fully compete with TAM and its metabolites. This is consistent with evidence from experimental models suggesting that combinations of an antiestrogen and an aromatase inhibitor is no better than either drug alone (Lu et al., 1999). However, where reduced intratumor TAM accumulation also occurs (Johnston et al., 1993), the higher intratumor estradiol concentrations in some tumors might overcome TAM's antiestrogenic activities. Very high intratumor estrogen levels (up to 5 μM) are only occasionally observed, but would provide sufficient estrogenicity to compete with the mean intratumor concentrations of triphenylethylene antiestrogens (3.4 μM; see *Section III.A.*). Assuming that both estrogens and antiestrogens have equivalent intracellular availability for binding ER, it is theoretically possible for some tumors to acquire sufficient intratumor estrogen concentrations to either eliminate or reduce the inhibitory effects of TAM and its major metabolites.

Although this is a reasonable hypothesis, it has been inadequately addressed in clinical trials. It is evident that approximately 30% of tumors that acquire TAM resistance will respond to a second-line aromatase inhibitor. The proportion may be higher in selected populations (Dowsett et al., 1995). This response pattern is consistent with an important role for estrogen biosynthesis in acquired TAM resistance. It implies that the responding tumors have retained both a functional ER signaling network and a dependence upon that network's estrogenic activation/regulation for continued survival/proliferation. In some of these tumors, the levels of intratumor estrogens may reach sufficient levels to overcome any antiestrogenic activities of TAM and support an estrogen-dependent proliferation.

Currently, determining the possible contribution of HRT to antiestrogen resistance can also be done only indirectly. The National Surgical Adjuvant Breast and Bowel Project (NSABP)-P1 TAM chemoprevention trial precluded women who were receiving HRT, but found a significant reduction in the incidence of invasive breast cancers (Fisher et al., 1998). The apparent lack of a chemopreventive effect of TAM in the Italian (Veronesi et al., 1998) and United Kingdom studies (Powles et al., 1998) has been partly attributed to their inclusion of women receiving HRT. This explanation for the failure of these studies remains somewhat controversial. For example, it is not clear that many HRTs, particularly those using low-dose/potency estrogens, would produce an environment any more estrogenic than that occurring naturally in TAM-responsive premenopausal women. Tumors in premenopausal patients have a response rate comparable with those arising in postmenopausal women (EBCTCG, 1998). Other differences in the chemoprevention trials probably account for the lack of activity in the European studies. These may include differences in the patient populations and the greater statistical power of the NSABP study (Pritchard, 2000).

The timing of TAM treatment relative to any HRT may affect clinical outcome. Initiation of HRT during TAM may have a greater inhibitory effect on TAM's ability to affect serum lipid profiles than initiation of TAM in current HRT users (Decensi et al., 1998). Since these are agonist cardiovascular endpoints rather than antagonist cancer endpoints, extrapolation to the antiestrogenic effects of TAM in breast cancer is difficult. Nonetheless, data raise the possibility that the timing of HRT may affect TAM's antineoplastic activity in these patients. Additional studies are required to definitively answer the possible contribution of HRT to TAM resistance. The limited information available does not provide strong evidence for an effect of HRT on TAM responsiveness, which, if it occurs, may be restricted to specific HRT formulations and/or specific populations.

III. Pharmacokinetics in Resistance to Tamoxifen

There are several pharmacologic properties of TAM that directly influence its biological activity and that, when significantly altered, could contribute to the emergence of an antiestrogen resistant phenotype. These include the classical pharmacokinetic parameters of absorption, distribution, biotransformation, and elimination. The intracellular availability of TAM will determine the concentration free to interact with ER. This could be affected by changes in TAM accumulation in tumors. There are several likely major intracellular binding compartments for TAM that could limit intracellular availability. These include binding to antiestrogen binding sites (AEBSs) and other intracellular proteins, and partition into the lipophilic domains of cellular membranes. Such interactions could effectively sequester active TAM and its metabolites to produce the resistance phenotype. Since TAM is extensively metabolized in humans, and several metabolites are agonists,

a resistance phenotype could also be conferred by a switch to the generation of predominantly estrogenic metabolites.

A. Basic Pharmacology of Tamoxifen

Steady-state serum concentrations of TAM are generally achieved after approximately 4 weeks with the conventional dosing regimen of 20 mg TAM daily (Buckely and Goa, 1989; Etienne et al., 1989). Following administration of 30 mg/day, the mean steady-state plasma concentrations of parent drug and major metabolites can be up to 1.1 μM (Etienne et al., 1989). High-dose TAM, 150 mg/m^2 twice daily following a loading dose of 400 mg/m^2, produces plasma concentrations of 4 μM TAM and 6 μM *N*-desmethyl TAM (Trump et al., 1992). In most studies, clinical response does not seem to correlate with TAM plasma levels (Bratherton et al., 1984; Clarke and Lippman, 1992).

Greater than 98% of TAM and its major metabolites are bound to serum proteins. Most of this appears to reflect binding to serum albumin, which can bind drugs in a ratio of 1:1 (Lien et al., 1989). The extensive degree of association with albumin (Lien et al., 1989), peripheral tissues (Daniel et al., 1981; Lien et al., 1989) and cellular membranes (Clarke et al., 1990), and its large volume of distribution (Herrlinger et al., 1992) may contribute to TAM's long terminal elimination phase. The relatively low affinity binding to serum albumin might facilitate transport to tissues, where dissociation may occur to allow for tissue accumulation. This role for albumin as a transporter has been described for estrogens, with albumin-bound estrogens often being considered within the available component (Moore et al., 1986; Jones et al., 1987).

Despite the low free concentrations in serum, TAM concentrations of 5 to 110 ng/mg protein (25 $\pm$ 27 ng/mg protein; mean $\pm$ S.D.) have been reported in the breast tumors of women receiving 40 mg TAM/day (Daniel et al., 1981). This would approximate 0.67 to 14 μM (3.36 $\pm$ 3.63 μM; mean $\pm$ S.D.) using the conversions in the legend to Table 3. Similar intratumor concentrations have been described for brain metastases, with mean concentrations of TAM $\simeq$ 4 μM, 4-hydroxytamoxifen (4-hydroxyTAM) $\simeq$ 0.13 μM, and *N*-desmethyl TAM $\simeq$ 8 μM (approximate values derived from the published data) detected in a small study of patients receiving 30 to 50 mg TAM/day (Lien et al., 1991). Thus, as with estrogens, there is clear evidence of intratumor accumulation of TAM and its major metabolites to concentrations significantly in excess of that seen in serum (MacCallum et al., 2000).

When compared with the mean intratumor 17β-estradiol concentration ($\simeq$1.28 nM; Table 3), and assuming approximately equivalent intratissue availability, it is apparent that there should be sufficient TAM present to effectively compete with most concentrations of intratumor estrogens. This would be the case even if all the drug was present as either the relatively weak parent or the *N*-desmethyl TAM metabolite. The latter is present at concentrations of approximately 7 $\pm$ 8 μM (estimated from the values of Daniel et al., 1981). However, a significant proportion of the antiestrogenic activity will be provided by the 4-hydroxyTAM metabolite (77 $\pm$ 64 nM estimated from the values of Daniel et al., 1981), which has an affinity for ER $\geq$ 17β-estradiol (Kuiper et al., 1997). Although these estimates were obtained several years ago, a more recent study by MacCallum et al. (2000) obtained mean intratumor concentrations of TAM and its major metabolites (4-hydroxyTAM = 0.18 μM; *N*-desmethyl TAM = 0.61 μM; TAM = 0.32 μM) within the range of these prior studies.

The potentially significant intratumor excess of antiestrogenicity over estrogenicity (>10-fold for 4-hydroxyTAM) explains, in part, why TAM is an effective therapy in many patients with ER-positive tumors. This likely also contributes significantly to the apparent lack of a strong dose-related response rate in clinical trials. Many of the lower doses studied could still produce antiestrogen concentrations in excess of any intratumor estrogens.

B. Intracellular Antiestrogen Binding Sites

Several intracellular binding proteins have been identified for estradiol (Anderson et al., 1986; Takahashi and Breitman, 1989; Masamura et al., 1997), and it would be remarkable if none of these also bound TAM. Indeed, it is likely that there are several such proteins that can sequester TAM and reduce its intracellular availability. One intracellular binding component, at least for the triphenylethylenes, is the AEBS protein. AEBS seems to be predominately microsomal (Katzenellenbogen et al., 1985) and may represent a novel histamine receptor (Clemmons et al., 1990). More recent data imply a protein complex containing the microsomal epoxide hydrolase as one of the subunits (Mésange et al., 1998). This is a type II detoxification enzyme involved in the hydrolysis of aliphatic and aromatic electrophilic epoxides. TAM-AEBS interactions could contribute to the putative mutagenicity of TAM in some species (Greaves et al., 1993; Mésange et al., 1998). Whereas TAM induces expression of the epoxide hydrolase mRNA (Nuwaysir et al., 1995), it is an inhibitor of the enzyme's catalytic activity (Mésange et al., 1998). Such an inhibition could leave reactive epoxide metabolites of TAM, or other electrophilic epoxides, available to induce DNA damage (Mésange et al., 1998). TAM-induced hepatocellular carcinomas have been reported in rats (Greaves et al., 1993), but the incidence of these tumors is not increased in humans (Muhlemann et al., 1994). Any role for the epoxide hydrolase-TAM interactions may be tissue- and species-specific.

A basic alkylether side chain, as occurs in many of the nonsteroidal antiestrogens, seems important for recognition of AEBSs by triphenylethylenes (Murphy and

Sutherland, 1985). AEBSs do not bind either the natural estrogens or the steroidal antiestrogens with high affinity (Pavlik et al., 1992) and will not interfere with intratumor estrogen activation of ER. Thus, overexpression of AEBSs could contribute to TAM resistance in the presence of continued ER expression. The antiestrogen-resistant LY2 cells (Bronzert et al., 1985; Clarke et al., 1989c) overexpress AEBSs relative to ER, as do a significant proportion of human breast (Pavlik et al., 1992) and ovarian carcinomas (Batra and Iosif, 1996). The affinity of TAM for AEBSs in ovarian cells is estimated <1 nM (Batra and Iosif, 1996) significantly greater than its affinity for ER. This implies a preferential binding of TAM to AEBSs relative to ER. Where TAM inhibits the epoxide hydrolase activity of AEBSs allowing reactive metabolites to persist, this could increase the genetic instability of some tumors. One consequence could be an increased potential to induce mutations in genes required for TAM function, with a subsequent increased risk of producing mutations that produce antiestrogen resistance.

The biological potency of antiestrogens does not correlate with their affinity for AEBSs (Katzenellenbogen et al., 1985). Although it has generally been assumed that the primary function of AEBSs has been to sequester drugs, several studies imply otherwise. Lymphoid cells that express AEBSs, but not ERs, are growth inhibited by antiestrogens (Tang et al., 1989; Hoh et al., 1990; Teo et al., 1992). The compound *N,N*-diethyl-2-(4 phenyl-methyl)-phenoxy ethamine HCl binds AEBSs, but not ERs, and is growth inhibitory in MCF-7 cells (Brandes, 1984). A TAM-resistant MCF-7 variant (RTx_6) does not express AEBSs (Faye et al., 1983) and is not inhibited by either benzylphenoxy ethanamine derivatives (Poirot et al., 1990) or other selective ligands for AEBSs (Fargin et al., 1988; Teo et al., 1992). Parental MCF-7 cells are growth inhibited by these compounds.

Polyunsaturated fatty acids can block TAM binding to AEBSs (Hoh et al., 1990). Cholesterol and lipoproteins can reverse the inhibitory effects of antiestrogens in an ER-negative lymphoid cell line (Tang et al., 1989). The antiproliferative activities of oxygenated sterols may be mediated by AEBSs. Ligand binding to AEBSs also affects cholesterol metabolism. Benzofurans can inhibit de novo cholesterol metabolism in ER-negative cells that express AEBSs (Teo et al., 1992). This raises the possibility that the hypocholesterolemic effects of some antiestrogens may be related to effects mediated by binding AEBSs.

Whereas AEBSs can sequester TAM, the extent to which antiestrogen-mediated activation of any AEBS function contributes to the antiproliferative effects of antiestrogens is unclear. If sufficient alone to confer responsiveness, the response rate to antiestrogens would be expected to be high in ER-negative tumors. However, responses in ER-negative tumors are infrequent (EBCTCG, 1998). The relationship between AEBS affinity and the IC_{50} for antiproliferative effects is also of concern. The affinities of the antiestrogens TAM and clomiphene for AEBSs are two to three orders of magnitude greater than their respective antiproliferative IC_{50}s (Lin and Hwang, 1991). Whatever the role of AEBSs, these sites cannot affect the activities of the steroidal antiestrogens because steroids do not bind AEBSs (Pavlik et al., 1992).

C. Binding to Plasma Membranes

Many lipophilic compounds are sequestered within plasma membranes and other intracellular bilipid membranes. This is probably a relatively nonspecific phenomenon, reflecting their physicochemical properties. Compounds with a high degree of lipophilicity would be expected to preferentially partition into lipophilic domains in cellular membranes. This has been widely reported for steroids (Duval et al., 1983). We have previously shown that both TAM and estradiol can affect membrane structure in breast cancer cells in vitro (Clarke et al., 1990). Sequestration of TAM in a cell's plasma membrane, and potentially within other intracellular bilipid membranes, could significantly reduce intracellular availability for binding to ERs. Some breast tumors exhibit a marked desmoplastic response, associated with the presence of fibroblastic and myofibroblastic cells, and/or significant infiltration of lymphoreticular cells (Clarke et al., 1992b). Thus, TAM could be further sequestered within the membranes of infiltrating cells and adjacent adipose tissue.

D. Altered Drug Accumulation / Transport and P-glycoprotein (mdr1)

The precise mechanism for intracellular uptake of TAM is not known. Passive diffusion, as probably occurs for steroids, seems most likely. Although tumors can concentrate TAM relative to its levels in serum (Fromson and Sharp, 1974; Daniel et al., 1981; Lien et al., 1989), intracellular sequestration could produce a relatively low concentration of unbound TAM, favoring its diffusion from extracellular sources. Some tumors may appear to have high TAM concentrations, but respond poorly because of low intracellular drug availability.

Reduced uptake of TAM from extracellular sources could confer resistance, provided the intracellular levels of available drug/metabolites fell below those required to effectively compete with any intratumor estrogens. Lower intratumor levels of TAM have been reported in some resistant versus sensitive tumors (Osborne et al., 1991, 1992; Johnston et al., 1993) and in some cell lines (Kellen et al., 1986). However, data are inconsistent. In a recent study, tumor concentrations of TAM, 4-hydroxy-TAM, and *N*-desmethyl TAM did not correlate with responsiveness or resistance. Indeed, the serum concentrations of 4-hydroxyTAM and *N*-desmethyl TAM were significantly higher among nonresponding patients

(MacCallum et al., 2000). The sources of inconsistency require further study but one source may be related to the ER content of the tumors in the study population. For example, the subgroup of patients with ER-poor tumors seem to have lower serum levels of antiestrogens, and their tumors have a low response rate to TAM (MacCallum et al., 2000). Future studies may need to carefully control for the ER content of tumors in their study populations.

TAM is antiangiogenic (Haran et al., 1994; Lindner and Borden, 1997) and reduces tumor vascularization, leading to decreased tumor perfusion and TAM delivery. However, this could not explain the reduced accumulation of TAM in some cells growing in vitro (Kellen et al., 1986). If accumulation is dependent on the expression of intracellular binding proteins, altered expression of these could affect accumulation. Altered TAM levels are not seen in one TAM-stimulated MCF-7 xenograft model (Maenpaa et al., 1994). We also have not found any significant difference in accumulation of [^{3}H]TAM among TAM-resistant and TAM-responsive breast cancer cells growing in vitro (unpublished results).

TAM's ability to diffuse into cells could be related to specific plasma membrane domains into which it initially partitions (Clarke et al., 1990). The structure of these domains might depend on critical membrane-associated proteins or lipids, the altered expression of which could contribute to reduced diffusion/uptake. A simple reduction in the number of such putative domains also could reduce accumulation. These comments are speculative; further studies are required to determine the extent to which TAM's association with, and diffusion through, the plasma membrane is dependent upon definable membrane domains and/or functions.

The mechanism for TAM efflux also is not known, although a passive diffusion again seems most likely. We and others (Ramu et al., 1984; Leonessa et al., 1994) have described the ability of TAM to interact with the P-glycoprotein (also known as MDR1, gp170, and PGP) efflux pump, the product of the *mdr1* (multidrug resistance 1) gene. P-glycoprotein is widely expressed in human breast tumors and is associated with a worse than partial response to cytotoxic chemotherapy (Trock et al., 1997). To determine the ability of P-glycoprotein to alter response to TAM, the MDR1 gene was overexpressed in MCF-7 cells. TAM can compete with azidopine for binding to P-glycoprotein and reverse the multidrug resistance phenotype in the transfectants (Leonessa et al., 1994). However, the transfectants' response to TAM is unaffected (Clarke et al., 1992a), and TAM accumulation is equivalent to wild-type cells (Clarke and Lippman, 1996). Thus, TAM is an inhibitor but not a substrate for this efflux pump, and expression of P-glycoprotein is probably not a contributor to TAM resistance.

E. Metabolism and Resistance

TAM is subject to extensive hepatic metabolism. Not surprisingly, several of the metabolites are predominately estrogenic, rather than antiestrogenic. Differences in TAM metabolism among mice, rats, and humans probably contribute to its species-specific agonist versus partial agonist properties (Jordan and Robinson, 1987).

The most relevant metabolites will be discussed only briefly, since the metabolism of TAM has been extensively reviewed elsewhere (Buckely and Goa, 1989; Lonning et al., 1992b). Demethylation of the aminoethoxy side chain produces *N*-desmethyl TAM, with further *N*-demethylation producing the primary amine (*N*-didesmethyl TAM). Deamination of the primary amine produces the primary alcohol (Kemp et al., 1983). Metabolite E is generated when the aminoethane side chain is removed. Hydroxylation of the parent drug produces the two more polar metabolites 4-hydroxyTAM and 3,4-dihydroxyTAM. Loss of the aminoethane side chain and hydroxylation at position 4 produces the bisphenol. Metabolite E and the bisphenol are estrogens and exhibit a lower affinity for ER than TAM (Jordan and Robinson, 1987). The other metabolites (B, D, X, Y, and Z) are partial agonists. The relative affinities for ERs are 4-hydroxyTAM $\geq$ 17β-estradiol $>$ TAM $>$ *N*-desmethyl TAM $>$ metabolite Y (Jordan et al., 1983; Katzenellenbogen et al., 1984).

Increased isomerization of TAM to estrogenic metabolites is observed in some TAM-resistant breast tumors (Osborne et al., 1991, 1992). A preferential generation of estrogenic metabolites could compete with the antiestrogenic metabolites for binding to ERs, perhaps interacting additively with existing intratumor estrogens to block antiestrogen action. It also would reduce the concentrations of antiestrogenic metabolites, potentially shifting the ratio of estrogenic:antiestrogenic metabolites in an unfavorable direction.

Evidence firmly establishing altered metabolism as a clinically relevant event remains elusive. Data from one animal model of TAM-stimulated growth, a phenotype that could reflect the preferential intracellular generation of estrogenic metabolites, clearly excluded the generation of such metabolites in this phenotype (Wolf et al., 1993). A series of elegant studies were performed using nonisomerizable TAM. These could not be metabolized to estrogenic metabolites, but the tumors still exhibited a mitogenic response to these derivatives (Wolf et al., 1993). Subsequent studies implicated a mutant ER protein in conferring the phenotype (Jiang et al., 1992). In a similar model from Dr. Osborne's laboratory (Baylor College of Medicine, Houston, TX), nonisomerizable TAM analogs also produced a stimulation of tumorigenesis. These data imply that the TAM-stimulated phenotype, at least in these models, is unlikely to

be explained by the significant conversion of parent drug to estrogenic metabolites (Osborne et al., 1994).

F. Comments

Altered intracellular availability could be a key event in affecting response and may account for a proportion of those ER-positive tumors that fail to respond to TAM. Ultimately, the ability of intracellular binding sites to affect TAM's availability will reflect both the relative affinities of each site for TAM versus ER and their intracellular localization. For example, binding proteins in the cytosol may sequester TAM such that it never reaches the nuclear ER. Clearly, it will be important to determine the relevance and relative importance of intracellular availability. Identifying additional intracellular binding proteins may provide useful intermediate biomarkers for identifying those patients with ER-positive tumors that will fail to respond to TAM.

The importance of reduced TAM accumulation also requires further study. It is unlikely that P-glycoprotein contributes to lower intratumor TAM levels. However, we have preliminary data suggesting that P-glycoprotein may confer resistance to steroidal antiestrogens (Leonessa et al., 1998). The role of other membrane transporters has not been well defined.

The extent to which metabolism of TAM to estrogenic metabolites confers resistance remains to be clearly established. TAM-stimulated growth, the predicted response to this mechanism, can arise from mutations in ER and may not require estrogenic metabolites (Jiang et al., 1992). Nonetheless, it may be premature to entirely exclude the generation of estrogenic metabolites as a possible contributing resistance mechanism in some breast tumors.

IV. Cell Culture Models of Antiestrogen Responsiveness and Resistance

The study of acquired resistance has been greatly facilitated by the generation of several series of resistant variants. Most have been obtained by in vitro selection of the MCF-7 human breast cancer cell line. Almost all of these variants retain ER expression and show various patterns of resistance and cross-resistance. Resistant variants of other estrogen-responsive cell lines also have been reported. Although not a full listing, Table 4 describes several antiestrogen-resistant models. This section will focus primarily on those models of apparent pharmacological resistance (i.e., cells that do not exhibit a growth response to specific antiestrogens). Models that are growth stimulated by TAM are discussed in *Section V*. The models presented are selected to reflect the most widely used models and the diversity of phenotypes.

A. R27 and LY2

These were among the first stable antiestrogen-resistant variants reported. R27 cells were obtained following anchorage-independent cloning of MCF-7 cells in the presence of TAM. The cells retain an attenuated response to estradiol and are resistant to the growth inhibitory activities of TAM (Nawata et al., 1981). The LY2 cells were generated by a stepwise selection against the benzothiophene antiestrogen LY 117,018 (Bronzert et al., 1985). While retaining some responsiveness to estrogens, LY2 cells are cross-resistant to 4-hydroxy-TAM (Bronzert et al., 1985; Clarke et al., 1989c) and ICI 164,384 (Clarke et al., 1989c). Unfortunately, LY2 cells are nontumorigenic, restricting their use to in vitro studies (Clarke et al., 1989c). The tumorigenicity of R27 cells is not reported.

B. MCF-7RR

The MCF-7RR subline was obtained by selecting MCF-7 cells for their ability to grow in medium supplemented with 2% calf serum and 1 μM TAM (Butler et al., 1986). The cells exhibit an altered chromatin structure and chromatin acceptor sites for the antiestrogen 4-(*N,N*-diethylaminoethoxy)-4′methoxy-α)-(*p*-hydroxyphenyl)α-ethylstilbene (Singh et al., 1986). Of interest is MCF-7RR cells' retinoic acid cross-resistance (Butler and Fontana, 1992), which has not been fully studied in many other antiestrogen-resistant variants. Whereas the cross-resistance pattern among other antiestrogens is not reported for MCF-7RR, these cells provide a novel model for studying the relationships among responsive-

TABLE 4
Representative antiestrogen-resistant human breast cancer variants derived from ER+/PgR+ parental cells

Parental	Variant	ER/PgR[a]	Phenotype[b]
MCF-7	LY2	+/−	E2-independent; TAM and ICI 164,384 cross-resistant
MCF-7	R27	+/?	TAM-resistant
MCF-7	RR	+/?	E2-independent; TAM-resistant
MCF-7	MCF7/LCC1	+/+	E2-independent; antiestrogen-responsive
MCF7/LCC1	MCF7/LCC2	+/+	E2-independent; TAM-resistant/ICI 182,780-responsive
MCF7/LCC1	MCF7/LCC9	+/+	E2-independent; TAM and ICI 182,780 cross-resistant
MCF-7	MCF-WES	+/+	E2-independent; TAM-stimulated, ICI 182,780-resistant
ZR-75-1	ZR75/LCC3	−/−	E2-independent; TAM and ICI 182,780 cross-resistant
ZR-75-1	ZR-75-9a1	−/−	E2-independent; TAM and ICI 182,780 cross-resistant
T47D	T47Dco	−/+	E2-independent; TAM and ICI 182,780 cross-resistant

? = unknown or unclear.
[a] ER/PgR expression in variants.
[b] Citations for the cells and their phenotypes can be found in the text.

ness and resistance to both antiestrogens and retinoids. Another MCF-7 variant selected against 4-hydroxyTAM (MCF/TOT) has also been shown to exhibit cross-resistance to retinoic acid (Herman and Katzenellenbogen, 1996).

C. The LCC Series

This series was established to facilitate a further evaluation of cross-resistance phenotypes and to identify underlying molecular mechanisms. LCC variants were established from an estrogen-independent variant of MCF-7 cells (MCF7/MIII), initially selected for growth in vivo in ovariectomized nude mice (Clarke et al., 1989b). Circulating estrogen concentrations in these mice are similar to those found in postmenopausal women (Seibert et al., 1983), and the parent MCF-7 cells were derived from a postmenopausal patient (Soule et al., 1973). MCF7/MIII cells form proliferating tumors in these mice, but their growth is further increased upon estrogen supplementation. The cells retain ER expression and are growth inhibited by antiestrogens (Clarke et al., 1989b). A further in vivo selection produced the MCF7/LCC1 variant (Brünner et al., 1993a). These cells are similar to the MCF7/MIII, but tend to produce tumors more rapidly in ovariectomized nude mice. MCF7/LCC1 cells also retain ER expression, are estrogen-independent for growth, and are inhibited by triphenylethylene and steroidal antiestrogens (Brünner et al., 1993a; Brünner et al., 1997).

To generate antiestrogen-resistant variants, MCF7/LCC1 cells were stepwise selected against increasing concentrations of either 4-hydroxyTAM or ICI 182,780. Cells selected against the TAM metabolite produced stable, TAM-resistant cells (MCF7/LCC2), which also retain estrogen-independent growth in vitro and in vivo (Brünner et al., 1993b; Coopman et al., 1994). However, the MCF7/LCC2 cells are not cross-resistant to ICI 182,780. This predicts that tumors that responded and then failed TAM might show a strong response to a steroidal antiestrogen (Brünner et al., 1997). This prediction has now been confirmed in the clinic. The first trial of ICI 182,780 was performed in TAM responders who subsequently recurred. Consistent with the MCF7/LCC2 phenotype, the overall response rate to ICI 182,780 (69%) was substantially higher than would be predicted if the patients had been treated with another triphenylethylene (Howell et al., 1995). Using similar approaches, others have reported a MCF-7 variant (MCF-7/TAM^R-1) expressing a phenotype similar to MCF7/LCC2 (Lykkesfeldt et al., 1994).

Cells resistant to ICI 182,780 (MCF7/LCC9) were generated by selecting the MCF7/LCC1 variant against ICI 182,780. The resulting phenotype is clearly ER-positive, ICI 182,780-resistant, estrogen-independent, and TAM-crossresistant. Indeed, TAM cross-resistance emerges at early passages during the selection, arising before stable ICI 182,780 resistance is apparent (Brünner et al., 1997). The cross-resistance pattern may reflect the greater potency of ICI 182,780 relative to TAM and/or the differences in its interactions with ER (Fawell et al., 1990; Dauvois et al., 1992), which may have more substantial effects on ER functioning/signaling. Others have selected MCF-7 cells against ICI 182,780, but have not seen TAM cross-resistance (Jensen et al., 1999). The clinical relevance of these diverse phenotypes remains to be established.

D. ZR-75-9a1

ZR-75–1 cells are another of the relatively few, well established, estrogen-responsive human breast cancer cell lines. They were established from an ascites that developed in a 63-yr-old woman with an infiltrating ductal breast carcinoma (Engel et al., 1978). The patient had been receiving TAM for 3 months before the time when cells were removed to establish the ZR-75–1 cell line (Engel et al., 1978). ZR-75–1 cells are ER-positive and PgR-positive (Engel et al., 1978; van den Berg et al., 1987) and are growth stimulated by estrogens and inhibited by antiestrogens in vitro (Engel et al., 1978; van den Berg et al., 1989). However, the patient did not respond to TAM (Engel et al., 1978). A stepwise selection of the ZR-75–1 cells produced a resistant variant (ZR-75–9a1) that is not growth inhibited or stimulated by TAM (van den Berg et al., 1989). Unlike the MCF-7 TAM- resistant variants, the ZR-75–9a1 variant has lost expression of both ERs and PgRs. The cells remain stably resistant and receptor negative for only 3 months in the absence of selective pressure (van den Berg et al., 1989). Thus, ZR-75–9a1 cells are a useful model for studying initial acquired receptor negativity as an antiestrogen resistance phenotype.

E. Resistance Phenotypes Implied by Cell Culture Models

Some tumors with little or no effective estrogenic stimulation could be driven by a ligand-independent activation of the ER signaling network. This type of activation has been clearly described in vitro (Clarke and Brünner, 1996). Although independent of estrogens, antiestrogens are able to inhibit, and estrogens can further increase this ER activation. Consistent with these observations, cells acquiring estrogen independence retain responsiveness to antiestrogens and are growth stimulated by estrogens in vivo (e.g., MCF-7/MIII and MCF7/LCC1 phenotypes). Thus, proliferation of some estrogen-independent cells, which continue to express ERs, may be primarily maintained by ligand-independent ER signaling. This also suggests that available intracellular estrogens may not be required for some tumors to exhibit an ER-positive, antiestrogen responsive phenotype. It is also apparent that estrogen independence and antiestrogen resistance are independent phenotypes (Clarke et al., 1989c).

Together, these observations suggest the existence of at least three ER-positive phenotypes: 1) estrogen-dependent (requires an adequate estrogenic stimulus for proliferation); 2) estrogen-independent, but responsive (does not require, but may be stimulated by, available intracellular estrogens); and 3) estrogen-independent and unresponsive (does not require, and will not respond to, available intracellular estrogenic stimuli even if estrogens are present). Phenotype (1) would be responsive to both antiestrogens and aromatase inhibitors, whereas phenotype (3) would be cross-resistant to these therapies. Phenotype (2) would be antiestrogen responsive and also might exhibit responses to aromatase inhibitors. For example, removal of the estrogenic stimulation by the aromatase inhibitors would leave the cells reliant on the less potent ligand-independent ER-activated signaling. Estrogen-independent, but responsive, cells would either grow more slowly, or undergo growth arrest but perhaps not die, in response to an effective aromatase inhibitor. TAM-stimulated growth might be seen in both phenotypes (1) and (2). Since breast tumors are highly heterogenous, the overall clinical response would partly reflect the relative proportions of the responsive phenotypes within the tumor.

V. Tamoxifen-Stimulated Proliferation as a Resistance Mechanism

TAM-stimulated growth is one possible mechanism for clinical resistance, a response not unusual in some normal tissues. For example, TAM stimulation of uterine proliferation (estrogenic/agonist effect) has been known for many years (Harper and Walpole, 1967). Switching to a TAM-stimulated phenotype can arise in MCF-7 cells following in vivo selection against TAM, spontaneously in estrogen-deprived cells, and after transfection with members of the fibroblast growth factor (FGF) family of proteins. There also is limited evidence suggesting that TAM-stimulated tumor growth may occur in a minority of breast cancer patients (see *Section V.E.*).

A. In Vivo Selection against Tamoxifen or ICI 182,780

Perhaps the most consistent models of TAM-stimulated growth are generated by in vivo selection of established MCF-7 xenografts against TAM (Osborne et al., 1987; Gottardis et al., 1989). Since MCF-7 tumors require estrogens for growth in vivo, tumors are first established in the presence of estradiol, which is then replaced with TAM. Tumors initially stop proliferating or regress, but prolonged therapy produces re-emergent tumors. These appear to be TAM-stimulated because they subsequently regress upon removal of TAM (Osborne et al., 1987; Gottardis et al., 1989). The TAM-stimulated tumors are not cross-resistant to the steroidal antiestrogens (Osborne et al., 1995), consistent with the cells now selectively perceiving TAM as an agonist. MCF-7 tumors also have been selected in vivo for resistance to ICI 182,780. ICI 182,780 resistance arises, but takes longer than does the development of TAM resistance (Osborne et al., 1995), perhaps reflecting the greater potency of ICI 182,780 relative to TAM (Brünner et al., 1993b).

B. MCF-WES and MCF/TOT

Although most in vitro selection models have identified phenotypes that are no longer growth inhibited by antiestrogens, the MCF-WES cells are growth stimulated by TAM (Dumont et al., 1996). MCF-WES was obtained from a MCF-7 tumor growing in an ovariectomized nude mouse. The cells are estrogen-independent, but respond mitogenically to estrogens. While being growth stimulated by TAM, MCF-WES cells are cross-resistant to ICI 182,780 [i.e., treatment with the steroidal antiestrogens does not affect growth rate (Dumont et al., 1996)]. The ability of these cells to grow both in vitro and in vivo provides a novel model to study TAM-stimulated proliferation. A MCF-7 cell population that is stimulated by 4-hydroxyTAM (MCF/TOT) has also been obtained by long-term exposure to 4-hydroxyTAM in vitro (Herman and Katzenellenbogen, 1996) and may be derived from a subpopulation similar to that which produced MCF-WES cells. These cells appear to have a TAM-responsive phenotype broadly comparable with the MCF/WES cells, but the cells do not exhibit cross-resistance to ICI 164,384 (Herman and Katzenellenbogen, 1996).

C. Fibroblast Growth Factor-Transfected MCF-7 Variants and Their Role(s) in Antiestrogen Resistance

The expression of several growth factors have been implicated in estrogen independence and antiestrogen resistance. Several angiogenic growth factors, most notably members of the FGF family, have recently been evaluated for their ability to produce antiestrogen resistance. Overexpression of FGF-1 by transfection into MCF-7 cells produces cells that generate highly vascularized, estrogen-independent, metastatic tumors (Zhang et al., 1997). Estrogen-independent growth is not affected by 4-hydroxyTAM, indicating the ability of FGF-1 overexpression to confer TAM resistance. When FGF-4 is overexpressed, the cells become TAM-stimulated in vivo (Kurebayashi et al., 1993; Zhang et al., 1997), a response similar to that seen in the MCF-WES cells and some in vivo TAM-selected models (see above). FGF-1 and FGF-4 transfected MCF-7 cells are still growth inhibited by ICI 182,780 in vitro, but exhibit some reduction in responsiveness compared with controls (McLeskey et al., 1998). Thus, overexpression of these FGFs is sufficient to confer TAM resistance, but not full cross-resistance to ICI 182,780.

The ability of overexpression of FGFs to produce these phenotypes may reflect the induction of both mitogenic and growth inhibitory effects in breast cancer cells

(Fenig et al., 1997; Wang et al., 1997). The apoptosis induced by FGF-2 (Wang et al., 1998) may suggest an additive growth inhibitory effect, since triphenylethylenes also induce apoptosis (Kyprianou et al., 1991; Huovinen et al., 1993). Nonetheless, FGF transfected cells provide a unique series in which to study the role of FGFs and compare the biologies of antiestrogen resistance, angiogenesis, and increased metastatic potential.

D. Angiogenesis and Tamoxifen Resistance

Data from the FGF transfected cell lines imply a role for angiogenesis in TAM resistance. Limited evidence from studies in humans also suggests that more angiogenic tumors have a poor response to antiestrogens. In node-positive patients, those with ER-positive and poorly vascularized tumors have the best prognosis in response to TAM therapy (Gasparini et al., 1996). Antiestrogens are antiangiogenic in some experimental models (Gagliardi and Collins, 1993). Thus, an antiangiogenic effect could contribute to good TAM responses, or conversely, highly angiogenic tumors may respond poorly to TAM.

Angiogenesis will increase tumor perfusion and might increase TAM accumulation. This could increase the number of cells to which TAM is delivered and perhaps increase the intracellular concentrations of TAM in previously poorly vascularized regions. Such an effect might be expected to increase responsiveness rather that induce resistance. However, increased angiogenesis will also increase intratumor concentrations of estradiol precursors, improve perfusion of oxygen and nutrients, and improve removal of cellular waste and dead/dying cells. These events would be expected to improve the overall "health" of tumor cells. However, the simplest explanation might be that highly angiogenic tumors may have a higher metastatic potential. This could produce an effect independent of ER expression, as seen in the study by Gasparini et al. (1996).

Signaling through receptors for angiogenic growth factors could also contribute to cellular resistance by changing the activation of cell signaling pathways within the cell. This seems most likely in some models, since the cells are resistant in vitro where the angiogenic effects are irrelevant. Zhang et al. (1998) have used a dominant negative FGF-receptor to assess the relative importance of both autocrine and angiogenic responses. In an elegant approach, these investigators generated cells that overexpress FGF-1, but cannot respond to autocrine stimulation because of the coexpression of a dominant negative FGF receptor. Importantly, xenografts from these cells require either estrogen or TAM. This indicates that the tumors can be driven by TAM, and that the paracrine and/or angiogenic effects of FGF-1 are important for this TAM-stimulated growth.

E. Tamoxifen Stimulation as a Resistance Phenotype in Patients and Tamoxifen Flare

If the TAM-stimulated phenotype arose in a patient, the tumor would be considered resistant. Thus, TAM-stimulated growth can be considered a resistance mechanism in the broadest sense. However, the tumor is clearly not resistant in the pharmacologic sense. Superficially, this resistance phenotype looks like TAM-induced tumor flare, which occurs when patients respond by a temporary worsening of their disease shortly after initiation of TAM treatment. This response is often accompanied by increased pain, hypercalcemia, and progression of metastatic disease (Plotkin et al., 1978). Many patients who initially exhibit TAM flare obtain a beneficial clinical response if treatment is continued. This is quite different from recurrence on TAM, where continued treatment provides little benefit.

Flare probably reflects TAM's pharmacology. Steady-state levels of TAM in patient sera are not reached for up to 4 weeks (Buckely and Goa, 1989; Etienne et al., 1989). In cell culture, low concentrations of TAM can be mitogenic (Clarke et al., 1989c). Thus, the low TAM serum/tissue concentrations at the initiation of treatment in patients may be mitogenic, producing the flare response. Once the elevated steady-state levels are reached in patients, the antagonist properties of TAM could predominate, accounting for the subsequent remissions. Another possibility is a TAM-induced increase in serum dehydroepiandrosterone (estrogen precursor), estrone, and estradiol concentrations (Pommier et al., 1999). These hormones could stimulate proliferation until the levels of TAM become sufficient to overcome this effect. It is possible that both the direct (low concentrations of TAM perceived as an estrogen) and indirect effects (increased estrogen production) contribute to TAM flare.

Since we can delineate TAM flare from a TAM-stimulated resistance phenotype, it is important to estimate the frequency of the latter. The precise frequency of the TAM-stimulated phenotype is difficult to assess in patients. One approach is the measurement of clinical withdrawal responses (i.e., where the patient obtains a beneficial response upon cessation of treatment). Unfortunately, the number of TAM withdrawal cases may be underdocumented. Table 5 shows those identified using a proven literature retrieval approach (Trock et al., 1997). Despite approximately 10 million patient years of experience, only 16 cases of partial and complete responses were found in five relatively small studies. The few other reports were identified as individual case reports. When combined, data suggest significant withdrawal responses in approximately 7% of patients. When disease stabilization is included, the estimate of the incidence of putative TAM withdrawal clinical responses approaches 20%.

Nomura et al. (1990) measured the ability of TAM to increase the proliferation ($\geq$150%) of breast tumor biop-

TABLE 5
Evidence of TAM-stimulated growth in breast tumors and biopsies

Patients	Worse than PR[a]	PR	CR	PR + CR/Duration (Range)	Citation
TAM Withdrawal Responses					
Advanced disease	19/19	0/19	0/19	0%	Beex et al., 1981
Postmenopausal with metastatic disease[b]	6/9	1/9	2/9	22%/10–14 months	Rudolph, 1986
Postmenopausal with metastatic disease	84/87	3/87	0/87	3%/9–10.3 months	Taylor et al., 1986
Postmenopausal	56/61[c]	4/61	1/61	8%/3–10 months	Canney et al., 1989
Advanced disease	60/65	5/65	0/65	8%/3–40 months	Howell et al., 1992
Mean (PR+CR)	225/241	13/241	3/241	6.6%	
Overall (PR+CR+DS)		19.5% (47/241)[d]			

ER Status	Response	*n* (%)
TAM Stimulation of Primary Breast Tumors In Vitro[e]		
ER-positive	TAM-stimulated	11/153 (7)
ER-negative	TAM-stimulated	1/71 (1.4)
ER-positive	Estradiol-stimulated	47/153 (31)
ER-negative	Estradiol-stimulated	10/71 (14)
ER-positive	TAM-stimulated and estradiol-stimulated	6/153 (4)
ER-negative	TAM-stimulated and estradiol-stimulated	0/71 (0)

[a] PR, partial response; CR, complete response.
[b] All patients were selected on the basis of having experienced a response to TAM.
[c] All responses were seen in the group of 28 patients who had originally responded to TAM (18% of initial responders).
[d] DS = disease stabilization.
[e] Data adapted from Fig. 1 in Nomura et al., 1990.

sies in short-term culture in vitro (data adapted in Table 5). Approximately 7% of ER-positive biopsies exhibit a mitogenic response to TAM. The biopsies appear to have been collected from previously untreated patients. Thus, at the time of diagnosis, a small proportion of tumors may already contain cells that will perceive TAM as an estrogen.

Half of the TAM-stimulated tumor biopsies did not respond to estradiol (Table 5), suggesting that the true proportion perceiving TAM as an estrogen could be as low as 4% of all ER-positive tumors. This raises the possibility that some tumors might be TAM-stimulated through other mechanisms. For example, TAM can sensitize cells to the proliferative activities of IGF-1 (Wiseman et al., 1993b). This would still require ER expression, and is consistent with the low frequency of TAM-stimulated, ER-negative, breast biopsies in the data adapted in Table 5. Data from the TAM withdrawal responses clearly implicate TAM stimulation in about 7% of recurrences, equivalent to the estimated proportion of TAM-stimulated biopsies from naive patients (Nomura et al., 1990). TAM treatment would tend to select for these cells, which would be predicted to have a clear proliferative advantage over other cell populations within the tumor, ultimately producing a TAM-stimulated tumor.

Data in Table 5 are consistent with acquired TAM stimulation being one of several mechanisms that contribute to clinical resistance. However, it is not entirely clear that this phenotype exclusively reflects cells that perceive TAM as an estrogen. Since >80% of tumors probably do not use this mechanism to acquire resistance, it may not be the primary resistance mechanism in most breast tumors.

VI. Estrogen Receptors, Mutant Receptors, Coregulators, and Gene Networks

Two ER proteins exist (ERα, ERβ), each being the product of different genes on separate chromosomes. Both proteins have similar functional domains including ligand binding, DNA binding, and two transcriptional activating domains (AF-1; AF-2). These have been extensively discussed and reviewed by others (Kumar et al., 1987; Enmark and Gustafsson, 1998). ERs function as nuclear transcription factors and regulate the expression of a considerable number of different genes. The patterns of gene regulation probably differ across cell types and can be thought of as regulating a series of different gene networks. These networks may be independent, interdependent, and/or intersecting (Clarke and Brünner, 1995, 1996; Clarke and Lippman, 1996).

ER proteins adopt various conformations when occupied by different ligands (Brzozowski et al., 1997; Grese et al., 1997) and may recruit different proteins into the transcription complexes being formed at the promoters of target genes (Shiau et al., 1999). The potency and direction of transcriptional regulation (induction or repression) are strongly affected by the ligand and receptor. For example, ICI 182,780 inhibits ERα-mediated transcription, but activates ERβ transcriptional activities at an AP-1 site (Paech et al., 1997). The mix of coregulators recruited (coactivators or corepressors) (Clarke and Brünner, 1996; Horwitz et al., 1996) and probably the phosphorylation status of the receptor (Arnold et al., 1995; Kato et al., 1995; Notides et al., 1997) are also important components that can affect transcription.

Since most antiestrogen-resistant tumors retain ER expression (Johnston et al., 1995), continued signaling through ER may be required for cell proliferation. This is probably the case in those tumors that remain responsive to other antiestrogens or aromatase inhibitors, but may also apply to other phenotypes. If sufficient ERs remain occupied by antiestrogens, either the cells have eliminated the antiestrogenic signaling, changed how this signaling is perceived by the cell, and/or altered the expression of other genes that counteract any remaining antiestrogenic signals. Such effects could be produced by changes in receptor function, perhaps through the emergence of either mutant receptors, perturbations in post-translational receptor modifications (e.g., phosphorylation patterns), and/or other changes in the cellular context (e.g., coregulator expression/availability; changes in the regulation of intersecting/interdependent signaling pathways).

Membrane-associated ERs have been reported for many years (Nelson et al., 1987) and are also present on human breast cancer cells (Nelson et al., 1987; Watson et al., 1999). These membrane-associated ERs were generally considered experimental artifacts once the predominately nuclear localization was reported (Welshons et al., 1984). More recently, proteins derived from both the ERα and ERβ genes have been identified in the cell membranes of Chinese hamster ovary cells transfected with the respective cDNAs (Razandi et al., 1999). Moreover, there is an increasing body of evidence suggesting that membrane-associated ERs are functional. For example, estrogens that cannot enter cells induce critical biological events in pituitary tumor cells (Watson et al., 1999), human sperm (Luconi et al., 1999), rat hypothalamic cells (Prevot et al., 1999), and human neuroblastoma cells (Watters et al., 1997). In some (Prevot et al., 1999), but not all, instances (Watters et al., 1997), these estrogenic effects can be blocked by antiestrogens. Some investigators used high concentrations of ligands, and these can produce nonspecific effects. However, the ability of antiestrogens to block the estrogenic activities of membrane receptors implies a signaling similar to that of nuclear ERs. Clearly, additional studies on the role and function of membrane ERs are required.

A. Wild-Type and Mutant Estrogen Receptor-α and Estrogen Receptor-β

Since the ERβ gene was cloned in 1996 (Kuiper et al., 1996; Mosselman et al., 1996), and ERβ-selective reagents have only recently been reported (Sun et al., 1999), most studies have focused on the role of ERα. The importance of ERα expression in predicting response to antiestrogens was described in *Section I.C.*

ERβ mRNA has been detected by polymerase chain reaction in breast tumors (Leygue et al., 1998; Dotzlaw et al., 1999; Speirs et al., 1999b), but ERα may be the predominant species in many ER-positive breast tumors (Leygue et al., 1998; Speirs et al., 1999b). This reflects an apparent increase in ERα expression in neoplastic versus normal mammary tissues (Leygue et al., 1998). When present in tumors, ERβ is associated with a poorer prognosis, absence of PgR, and lymph node involvement (Dotzlaw et al., 1999; Speirs et al., 1999b). Thus, it may be important to separate any effects on response to antiestrogens from an association of ERβ expression with this more progressed phenotype. In contrast, ERα expression is generally associated with a better prognosis.

The relative binding affinities of ERα and ERβ for 17β-estradiol are comparable. Similar effects are seen in the regulation of transcription in simple promoter (estrogen- responsive element; ERE)-reporter assays (Kuiper et al., 1997). However, there are notable differences in the molecular pharmacology of these two receptors. Agonists and antagonists exhibit opposite effects on ERα- versus ERβ-mediated transcription at AP-1 sites in a promoter-reporter assay (Paech et al., 1997). The ability of ERβ to activate the retinoic acid receptor promoter is driven by antiestrogens. Estradiol alone is inactive, but can block the activities of antiestrogens. The effect of 4-hydroxyTAM appears to be mediated through SP1 sites in the retinoic acid receptor promoter and is conferred by the 3′ region of ERβ [i.e., independent of the two transactivating domains (Zou et al., 1999)].

Compounds that are antagonist for ERα may be agonists for ERβ, at least at AP-1 and SP-1 sites (Paech et al., 1997; Zou et al., 1999). An increase in ERβ expression, acting through genes with AP-1 and/or SP-1 sites in their promoters, could produce the TAM-stimulated phenotype seen in some MCF-7 xenografts and cell lines. Binding ICI 182,780 targets ERα for degradation (Dauvois et al., 1992). Since it is transcriptionally activated upon binding ICI 182,780 (Paech et al., 1997), ERβ may not be so targeted. ERβ's transcriptional activation could contribute to the apparent agonist-like effects of ICI 182,780 seen in some tissues (Paech et al., 1997).

The ratio of ERα:ERβ also may be important in predicting response, particularly in those tumors that express ER, but do not respond to antiestrogens. When both receptors are present, transcriptionally active heterodimers can be formed (Pettersson et al., 1997). 4-HydroxyTAM can act as an agonist through ERα/ERβ heterodimers, but the effect is promoter- and cell context-dependent (Tremblay et al., 1999). Although the effects on proliferation were not evaluated, these agonist effects on transcription could affect the expression of genes induced by estrogens and responsible for proliferation. Thus, in breast cancer cells where adequate concentrations of functionally active ERα and ERβ proteins are present, TAM could induce, rather than inhibit, cell proliferation. This could explain some of the endogenous and acquired resistance seen in ER-positive breast tumors. Generally, the agonist effects of TAM are cell- and promoter context-dependent and related to the ER sub-

types expressed in the target cells (Clarke and Brünner, 1996; Watanabe et al., 1997).

Data from clinical material are still somewhat limited and the role of ERβ in antiestrogen-resistant and responsiveness requires further study. One small study of nine TAM-resistant and eight responsive tumors found 2-fold higher median levels of ERβ versus ERα mRNA expression by polymerase chain reaction in the TAM-resistant biopsies (Speirs et al., 1999a). However, protein levels were not reported. The association with TAM resistance may reflect the poor prognosis associated with ERβ expression (Speirs et al., 1999a).

The role of ER mutants has been most widely studied for ERα. Several mutant ERα genes have been reported, but the consequence of this expression is unclear. For example, it is often not known whether the mutant mRNA is translated, although some mutant ER proteins clearly are produced (Murphy et al., 1998). Most tumors that express mutant ER concurrently express the wild-type receptor, with the mutant representing a relatively small proportion of total ER proteins. Thus, only dominant negative mutants have a substantial chance of affecting transcription. A mutant ER that perceives TAM as an agonist has been described in some MCF-7 cell variants (Jiang et al., 1992). It is not clear whether this, or functionally similar mutant proteins, occur in breast tumors in patients.

At least five isoforms of ERβ have been identified, with three full-length isoforms exhibiting the ability to bind DNA as homodimers and heterodimers with ERα (Moore et al., 1998). A tyrosine mutant of ERβ has been reported, but is sensitive to the actions of antiestrogens and is likely not involved in antiestrogen resistance (Tremblay et al., 1998). An exon 5 deletion mutant of ERβ also has been reported (Vladusic et al., 1999). Whether this mRNA is translated, and its likely role in antiestrogen resistance, remain to be elucidated.

There is little compelling evidence that ER mutant proteins directly confer resistance in a significant proportion of breast tumors (Karnik et al., 1994). However, it would be premature to exclude the possibility that mutated ER confer resistance in some breast cancers. It is likely that a better understanding of the role of such ER mutants, whether these be of the ERα and/or ERβ genes, will likely emerge in the relatively near future.

B. Coregulators of Estrogen Receptor Action

Recently, several investigators have identified coregulator proteins that can significantly influence ER-mediated transcription; for an excellent recent review, see McKenna et al. (1999). These can be most easily thought of as being either coactivators (increase transcription, e.g., SRC-1) (Xu et al., 1998) or corepressors (inhibit transcription, e.g., N-CoR, SMRT) (Jackson et al., 1997; Soderstrom et al., 1997). Binding of the SRC family of proteins is mediated by a conserved LXXLL motif that facilitates interactions with ligand-occupied ER (Ding et al., 1998). One likely consequence of receptor-coactivator binding is the activation of SRC-1's histone acetyltransferase activity (Spencer et al., 1997), which would be expected to unwind and expose the adjacent promoter DNA. This should facilitate the binding of additional transcription factors and the initiation of transcription. In contrast, complexes containing corepressors such as N-CoR can exhibit deacetylase activity (Heinzel et al., 1997; Spencer et al., 1997), which would be expected to inhibit transcription (Pazin and Kadonaga, 1997). Whereas most studies of coregulator action have been done with ERα, ERβ function also appears to be affected by coregulators (Tremblay et al., 1997).

The ability of a liganded receptor to recruit coregulators is at least partly dependent on its conformation. Shiau et al. (1999) have recently shown that 4-hydroxyTAM induces a conformation that blocks the coactivator recognition groove in ER. The consequences of coregulator binding can be complex (McKenna et al., 1999). SRC-1 inactivates ER bound to pure antagonists, enhances the agonist activity of partial agonists like 4-hydroxyTAM, is involved in a ligand-independent activation, and interacts synergistically with cAMP response element-binding protein in regulating ER-mediated transcription (Smith et al., 1996, 1997; Jackson et al., 1997). The corepressor SMRT binds ER, inhibits the agonist activity of 4-hydroxyTAM, and blocks the agonist activity of 4-hydroxyTAM induced by SRC-1 (Smith et al., 1997). N-CoR binds TAM-occupied, but not ICI 182,780-occupied ER (Jackson et al., 1997).

These observations suggest that changes in coregulator expression or recruitment into an ER-antiestrogen–driven transcription complex could produce a resistance phenotype (Clarke and Brünner, 1996; Horwitz et al., 1996; Smith et al., 1997). However, mice lacking SRC-1 exhibit only partial hormone resistance (Xu et al., 1998). Overexpression of SRC-1 in MCF-7 cells may not significantly alter response to 4-hydroxyTAM (Tai et al., 2000), although data presented in this study are somewhat limited in this regard. The partial agonist (estrogenic) properties of 4-hydroxyTAM are increased by the coregulator L7/SPA (Jackson et al., 1997). In contrast, TAM's estrogenic activity is inhibited when SMRT is recruited into an ER-TAM complex (Smith et al., 1997). Thus, an increase in L7/SPA concurrent with reduced SMRT expression could generate a TAM-stimulated phenotype. A change in antiestrogen-ER complex conformation (e.g., through mutation or posttranslational modification) could either eliminate recruitment of corepressors and/or allow a preferential recruitment of coactivators. Either could contribute to antiestrogen resistance by influencing the regulation of ER-regulated gene networks that alter signaling to proliferation/differentiation/cell death.

Whether such effects occur and are biologically relevant clearly requires further study. MCF-7 xenografts that are TAM-stimulated express lower levels of N-CoR

(Lavinsky et al., 1998). However, a recent report failed to find any significant changes in the expression of the coactivators TIF-1, RIP140, or the corepressor SMRT in either a series of TAM-resistant cells, or in a cohort of 19 TAM-resistant human breast tumors. These investigators did not see any change in expression of the coactivator SUG-1 in the cell lines, but reported lower levels of expression in some TAM-resistant tumors (Chan et al., 1999).

Given the number and potential complexity of coregulator interactions, and the evidence of likely redundancy (McKenna et al., 1999), it is unclear whether measuring or affecting changes in the expression/function of any single coregulator will prove clinically useful. For example, SRC-1 and GRIP-1 appear to have overlapping nuclear receptor binding sites, and SRC-1 null mice exhibit only blunted responses to estrogens (Xu et al., 1998). Attempting to affect resistance by modifying the expression of any single coregulator could be confounded by compensatory responses in other coregulators, as likely happens in the SRC-1 null mice (Xu et al., 1998). Alternatively, it may be the balance of coactivators and coregulators that determines activity (Szapary et al., 1999).

C. Estrogenic and Antiestrogenic Regulation of Mitogen-Activated Protein Kinase

Estrogens can activate, rapidly, specifically, and at physiological concentrations, several well characterized signaling molecules/pathways, including intracellular Ca^{2+} (Mermelstein et al., 1996; Picotto et al., 1996), cAMP (Farhat et al., 1996; Picotto et al., 1996; Schaffer and Weber, 1999), protein kinase C (PKC) (Kelley et al., 1999), and MAPK (Migliaccio et al., 1996; Nuedling et al., 1999; Singh et al., 1999). Some of these activities are interrelated [e.g., intracellular Ca^{2+} (Burgering et al., 1993; Albert et al., 1997; Improta-Brears et al., 1999), PKC (Kazlauskas and Cooper, 1988; L'Allemain et al., 1991), and cAMP can each affect MAPK activation (Qian et al., 1995; D'Angelo et al., 1997)]. Thus, an estrogenic and/or growth factor activation of MAPKs could play a key role in ER-mediated signaling.

MAPK signaling is generally through one or more of the three MAPK modules (Fig. 2), each comprising one or more MEK kinases (activate MEK), a MEK (activates MAPK), and a MAPK (Cobb and Goldsmith, 1995; Marshall, 1995). Two additional, but less well defined, modules also exist; one where the MAPK is ERK3 and the other using ERK5 as the MAPK (Schaffer and Weber, 1999). The first of the three defined MAPK modules is dependent upon *ras/raf* activation, which regulates MEK1,2 activity, with the subsequent activation of ERK1,2 (Cobb and Goldsmith, 1995). This module is often associated with differentiation/proliferation and can be activated by receptor tyrosine kinases. The second module [stress-activated protein kinase (SAPK) module] is *ras*-independent and is primarily regulated

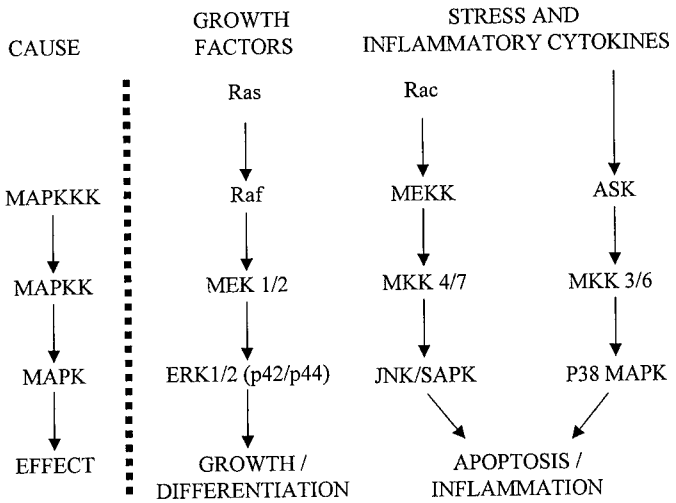


FIG. 2. MAPK modules and their role(s) in signaling to proliferation/apoptosis.

by *rac* (Lopez-Ilasaca, 1998; Vojtek and Cooper, 1999), *rac* being overexpressed in many breast cancers (Fritz et al., 1999). Subsequently, JNKK/SEK/MKK4 activates JNK/SAPK (Cobb and Goldsmith, 1995). The third module activates the p38/HOG1 MAPK and is associated with phosphorylation of HSP27 (Pelech and Charest, 1995). The latter two modules are often associated with signals arising from exposure to stressors and cytokines (Marshall, 1995; Woodgett et al., 1996; Vojtek and Cooper, 1999). Despite the complexity of cellular consequences of MAPK activation (see Schaffer and Weber, 1999, for recent review), cross-talk among modules can be effectively regulated. Activation of one module could produce contrasting effects in diverse cell types, or in the same cell type under different conditions.

MEK1,2 activities are increased in up to half of all breast cancers (Sahl et al., 1999). There also is evidence for a preferential activation of ERK1/MAPK (Xing and Imagawa, 1999). ERK/MAPK activities are elevated in experimental mammary tumor models driven by *c-myc*, *c-erb-B2,* and *v-Ha-ras,* but not those driven by either transforming growth factor (TGF)-α or heregulin (Amundadottir and Leder, 1998). Overexpression of *raf* can induce an estrogen-independent phenotype in MCF-7 breast cancer cells (El-Ashry et al., 1997).

Estrogen increases MAPK activity in some MCF-7 cells (Migliaccio et al., 1996; Improta-Brears et al., 1999), with this activity being constitutively elevated in estrogen-independent cells (Coutts and Murphy, 1998). Estrogenic activation of MAPK apparently signals through activation of *src* and *ras*. Blockade of MAPK activation eliminates estrogen signaling in primary cortical neurons (Singer et al., 1999). The rapidity and nonantiestrogen reversibility in some models are consistent with the widely reported nongenomic effects of steroids. Where antiestrogens reverse the effects of estrogens, the ER may be required. Thus, the ability of estrogens to activate MAPKs is probably multifactorial,

with both ER-dependent and ER-independent events occurring.

Determining the precise contribution of signaling through the MAPKs is complex. For example, FGF-2 inhibits breast cancer cell growth, but induces both ERK1 and ERK2, which are generally associated with mitogenic signals (Fenig et al., 1997). TAM can inhibit MAPK activation, an effect that may be related to TAM's ability to influence PKCϵ (Luo et al., 1997). However, TAM can increase both ERK2 activity and activate JNK1 (Duh et al., 1997). In rat cardiomyocytes, TAM activates ERK1/ERK2, but not p38 MAPK (Nuedling et al., 1999). The ability to concurrently activate both the MAPK and SAPK signaling modules could contribute to TAM's tissue-specific partial agonism. The importance of cellular context for downstream signaling from MAPKs is well established (Day et al., 1999b; Schaffer and Weber, 1999). In tissues where TAM initiates signaling only through the MAPK module, TAM might function as a partial agonist. Where only the SAPK module is activated, or where this activation predominates over any potentially mitogenic signaling from the MAPK module, TAM's apoptosis/growth inhibition-inducing properties could predominate (Fig. 3).

The ability of some cells to perceive TAM as an agonist (TAM-stimulated phenotype) may reflect a preferential activation/predominance of signaling through the MAPK module. Other resistant cells may no longer be able to either activate a SAPK pathway, change the way in which MAPK/SAPK signaling is perceived (e.g., by modifying expression of downstream signaling targets), and/or switch to alternative pathways to maintain cell proliferation/survival.

Ultimately, the role of MAPKs may be determined by the balance between their activation and inactivation. For example, PP2A is a major phosphatase for the deactivation of protein kinases (Millward et al., 1999), and inhibition of PP2A blocks the decay of epidermal growth factor-stimulated MAPK activity (Flury et al., 1997). PP2A activity is higher in estrogen-dependent, compared with estrogen-independent, breast cancer cell lines. Furthermore, it is induced by estrogens in a manner that is blocked by antiestrogens (Gopalakrishna et al., 1999). These effects are most consistent with the endocrine control of PP2A activity being required to regulate mitogenic signaling [e.g., to prevent an excessive or prolonged activation of MAPKs (Fig. 4)]. Since PP2A expression is lower in ER-negative cells (Gopalakrishna et al., 1999), estrogen-independent growth and/or an antiestrogen-resistant phenotype might require lower PP2A levels.

D. Regulation of Gene Networks by Receptor Cross-Talk: Mitogen-Activated Protein Kinase Activation and Estrogen Receptor Function

Inhibition of breast cancer cell proliferation by either antiestrogens or estrogen withdrawal produces cell cycle arrest in G_0/G_1. Cells that are resistant to these endocrine manipulations are no longer subject to the late G_1 restriction, a cell cycle check point that can be overcome by estrogens and/or several mitogenic growth factors alone or in combination. These growth factors can produce estrogenic effects in ER-positive cells in the absence of estrogenic stimuli (Bunone et al., 1996; Curtis et al., 1996; El Tanani and Green, 1996). Thus, signaling from growth factor receptors may play a critical role in regulating the proliferative response of some breast cancer cells to estrogens and antiestrogens. Perhaps the most widely studied signal cascade is the ability of growth factor receptor tyrosine kinases to activate MAPKs (Fig. 2).

MAPK activity is induced downstream of the receptor in an epidermal growth factor-receptor (EGF-R) signaling pathway (Tari et al., 1999; Xing and Imagawa, 1999). Blockade of MAPK activation can reduce EGF-induced mitogenesis (Reddy et al., 1999). The estrogenic effects of EGF are lost in ERα knockout mice (Curtis et al., 1996), suggesting that ERα but not ERβ is required. EGF-stimulated cell proliferation, in the absence of estrogen, is inhibited by TAM (Vignon et al., 1987). ICI 182,780 can block the abilities of EGF and TGF-α to increase expression of the otherwise estrogen-regulated pS2 mRNA (El-Tanani and Green, 1997).

The ability of EGF to induce estrogenic effects is dependent on the AF-1 (ligand independent), but not AF-2 domain of ERα, and is closely associated with EGF's

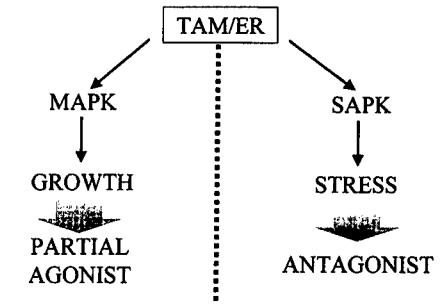


FIG. 3. Putative role of MAPKs in TAM/ER-mediated signaling. The tissue specificity for agonist/antagonist activities may reflect the specific MAPKs activated, their respective levels of activation, and/or the availability of their downstream substrates.

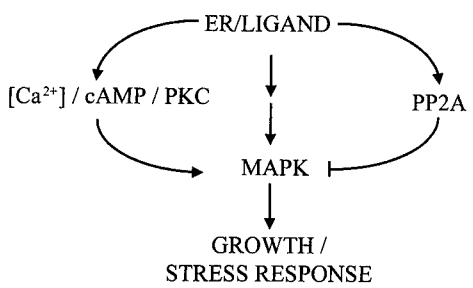


FIG. 4. Potential regulation of MAPK activation by ER. This is a general representation; the MAPKs activated and their levels of activation will reflect the cellular context, the balance of kinases/phosphatases, and/or the availability of their downstream substrates.

activation of MAPK and ultimate alteration of the ER's phosphorylation state (Bunone et al., 1996; El-Tanani and Green, 1998). ER phosphorylation occurs on both Ser^{118} (Bunone et al., 1996) and, as a consequence of pp90rsk1 (ribosomal S6 kinase), on Ser^{167} (Joel et al., 1998a), consistent with the abilities of EGF to induce ERK1,2 in breast cancer cells (Xing and Imagawa, 1999). As with Ser^{118}, phosphorylation of Ser^{167} is associated with ER's transcriptional activation (Castano et al., 1997). Whereas EGF partially reverses the growth inhibitory effects of antiestrogens (Koga and Sutherland, 1987), the mechanism(s) producing EGF's and TGF-α's mitogenic effects in breast cancer cells are not identical to that of estrogen (Novak-Hofer et al., 1987).

Activation of MAPK can phosphorylate ER on Ser^{118}, a phosphorylation that is required for activation of ER's AF-1 (Kato et al., 1995). The extent to which such cross-talk occurs is difficult to assess because others have reported Ser^{118} phosphorylation independent of ERK1,2 (Joel et al., 1998b). It seems likely that MAPK is not the only kinase capable of phosphorylating ER on this serine. However, MAPK appears important in the ability of growth factor receptor signaling to lead to ER phosphorylation, an event that may require *ras* (Patrone et al., 1998). Furthermore, when MAPK does phosphorylate this residue, it produces a sufficiently active conformation to initiate transcription (Kato et al., 1995). Thus, external stimuli that signal to an activation of MAPK, or that phosphorylate ER at Ser^{118} through their activation of other kinases, could produce a ligand-independent activation of ER-mediated transcription. Growth factor cross-talk with the ER will occur when these intracellular signals are initiated by their receptor tyrosine kinases (see Fig. 6).

Several other intracellular messenger systems can affect MAPK activation and ER function. For example, the intracellular concentration of cAMP affects MAPK activity (Qian et al., 1995; D'Angelo et al., 1997) and may determine isoform specificity in signaling to mitogenesis (Schaffer and Weber, 1999). The transcriptional activities of ER are also affected by cAMP (Aronica and Katzenellenbogen, 1993; Ince et al., 1994), an effect that may be primarily confined to the ligand-dependent AF-2 transactivation domain (El-Tanani and Green, 1998). Estradiol and TAM can increase cAMP levels in some cells (Ince et al., 1994; Picotto et al., 1996), although compounds that increase intracellular cAMP levels are generally growth inhibitory toward breast cancer cells (Fontana et al., 1987). The ability of estrogens to increase cAMP levels seems to be primarily nongenomic in several systems (Farhat et al., 1996; Gu et al., 1999). ER is an estrogen-regulated gene (Saceda et al., 1988), and cAMP produces a biphasic effect on ER mRNA expression (Ree et al., 1990). Together, these observations implicate changes in cAMP occurring in response to estrogens/antiestrogens. The consequences potentially include cAMP-driven perturbations in ER function and the expression of ER-specific estrogen-regulated genes. If these are primarily restricted to AF-2 activities, antiestrogen resistance could accompany changes in the cAMP/ER interactions that eliminate TAM's antiproliferative signals and/or cAMP-mediated changes in the function of a TAM/ER complex.

E. Mitogen-Activated Protein Kinases in Mediating the Effects of Estrogens and Conferring Antiestrogen Resistance

Many estrogen-regulated growth factors, including members of the EGF, FGF, IGF, and TGF-β families, activate tyrosine kinase receptors that are directly linked to activation of MAPK signaling. Consequently, activation of one or more of the MAPK signaling modules (Fig. 2) could provide a common integration point for signaling from both ER and growth factor receptors. Since MAPK can activate ER (Kato et al., 1995), a possible perpetual cycle between ligand independently activated ER and growth factor signaling could arise (see Fig. 6). Some of the inhibitory effects of antiestrogens could be derived from their abilities to either disrupt, or redirect, the downstream signaling from this MAPK-centered cycle.

Whether ligand-independent activation of ER AF-1 functions contribute to antiestrogen resistance is unknown. This activation does not produce a fully estrogenic response, in that not all estrogen-regulated genes are induced (Clarke and Brünner, 1996). This "weaker" estrogenicity may reflect the effects of ligand activation on the association of coregulators with ER (Parker, 1998). Estrogen-independent growth can be induced in breast cancer cells by selection either in vitro or in vivo in a low estrogen environment (Katzenellenbogen et al., 1987; Clarke et al., 1989b). It seems likely that this estrogen independence is associated with increased MAPK activity in some cells (Shim et al., 2000). However, many estrogen-independent cells retain a fully antiestrogen-responsive phenotype (Katzenellenbogen et al., 1987; Clarke et al., 1989c; Brünner et al., 1993a) and TAM can inhibit MAPK activation (Luo et al., 1997). In most experimental systems where ligand-independent ER activation occurs, antiestrogens block this activity. This is not surprising for the steroidal antiestrogens, since a major consequence of their interaction with ER is to down-regulate ERα expression. The ability of antiestrogens to block growth factor-induced mitogenesis is also predictable because ER expression appears essential for EGF to induce its estrogenic effects (Fig. 5). However, the ability of some growth factors to induce mitogenic signals through MAPK modules, in a manner independent of ER/antiestrogen signaling, could contribute to antiestrogen resistance. This might explain how some growth factors overcome the antiproliferative effects of antiestrogens.

Events apparently regulated by MAPKs are reversed/prevented by antiestrogens in some, but not all, studies.

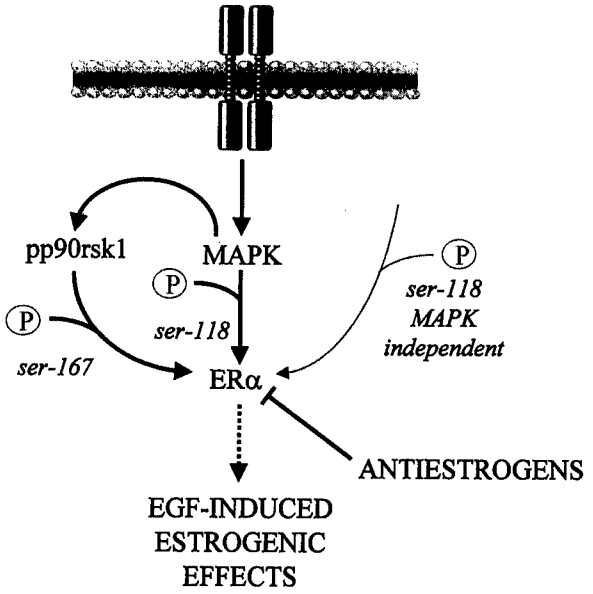


FIG. 5. Role of ER in mediating the estrogenic effects of EGF. Other growth factors may use similar mechanisms to activate/phosphorylate ER. The extent to which growth factor receptors affect ER function may be related to the level of MAPK activation and/or the MAPKs activated, since activation of some MAPKs can down-regulate ER expression.

FGFs inhibit MCF-7 cell proliferation despite activation of MAPK (Johnson et al., 1998; Liu et al., 1998) and the potential for a ligand-independent activation of ER with a consequent induction of ER-mediated transcription (Kato et al., 1995). However, FGF overexpressing cells do not increase transcription of an ERE-reporter construct (McLeskey et al., 1998). Similar evidence is apparent from studies of TGF-β signaling. TGF-β secretion is induced by antiestrogens, producing a potentially inhibitory autocrine loop (Clarke et al., 1992b). Generally, treatment with exogenous TGF-β inhibits breast cancer cell proliferation (Knabbe et al., 1987), but activates MAPK (Frey and Mulder, 1997a,b; Visser and Themmen, 1998). The apoptosis-inducing effects of TGF-β cannot be blocked by activation of the *ras*/MAPK pathway (Chen et al., 1998). Melatonin also inhibits MCF-7 cell proliferation, although it can cooperate with EGF to activate MAPK, phosphorylate ER, and activate ER's transcriptional regulatory functions (Ram et al., 1998).

Overexpression of a constitutive *raf-1* kinase or activated *c-erbB2* would be expected to activate MAPK. However, these transfectants significantly down-regulate ER expression. Thus, high levels of MAPK activation may be sufficient to fully produce estrogen-independent and antiestrogen resistant growth (Liu et al., 1995; El-Ashry et al., 1997). Whether activation of MAPKs produce a ligand-independent activation of ER or down-regulate ER expression, may be related to the level of MAPK activation and/or the MAPKs activated.

These observations suggest that the activation of MAPK alone is not sufficient to determine/predict the full nature of the cellular response to estrogens or antiestrogens. A necessary, but not sufficient, role for MAPK activation in signaling to mitogenesis could include its ability to phosphorylate/activate ER (Fig. 6). However, the direction/outcome of other downstream signaling also appears critical (i.e., cellular context). Unfortunately, cellular context is highly plastic and readily affected by many external signals (e.g., autocrine, paracrine, endocrine, and immunologic). Modifications in adjacent stromal populations and the tumor matrix are also likely to affect signaling within the tumor cells (Clarke et al., 1992b; Ronnov-Jessen et al., 1996; Cunha, 1999). These observations raise the possibility that individual cells or subpopulations within a single tumor may respond differently under various conditions. Thus, cells may exhibit cyclic changes in their responses to antiestrogens, perhaps reverting to responsiveness after a period of resistance.

Measuring the activity of ER, MAPK, or any other protein in isolation, as a means to assess its contribution to antiestrogen responsiveness or resistance, may be suboptimal. For example, measuring a combination of ER and PgR fails to predict response in approximately 30% of breast cancers that express these proteins. For MAPK studies, the situation may be complicated by the association of its activation with such divergent processes as initiation of mitogenesis, cell death, differentiation, activation of proto-oncogene expression (Hafner et al., 1996; Bornfeldt et al., 1997; Johnson et al., 1998) and both activation and repression of ER function (Kato et al., 1995; El-Ashry et al., 1997). The importance of cellular context to ER function (Clarke and Brünner, 1996) and MAPK signaling (Cobb and Goldsmith, 1995; Day et al., 1999b; Schaffer and Weber, 1999) are now becoming more clear. One of the challenges in the future will be to better understand the regulation of cellular context and how this can be manipulated to affect signaling through the ER and MAPKs. An understanding

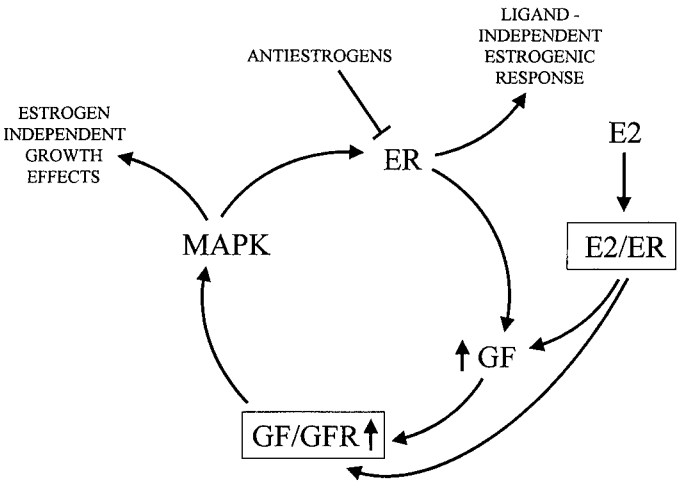


FIG. 6. Possible cyclic effect of growth factor activation of ER (ligand-independent). For some growth factor pathways, estrogens increase expression of both the growth factor and its ligand(s) [e.g., EGF and EGF-R are both induced by estrogens in MCF-7 cells. GF, growth factor; GFR, growth factor receptor.

of these interactions may lead to novel approaches for the modification of responsiveness and resistance to antiestrogens.

F. Estrogen Receptor Signaling through AP-1 and Antiestrogen Resistance

AP-1 is a transcription complex comprising either c-*jun* homodimers, c-*jun*/c-*fos* heterodimers, or heterodimers among other members of these families (Angel and Karin, 1991). Expression and activation of AP-1 are regulated by many extracellular signals, including those initiated by growth factors and steroid hormones, and in response to oxidative stress (Schultze-Osthoff et al., 1995; Xanthoudakis and Curran, 1996). Intracellular signals that result in the activation of AP-1 include those initiated by PKC, cAMP, calmodulin kinase (Angel and Karin, 1991), MAPK, and Janus kinases (Karin, 1995). However, the consequences of AP-1 activation appear cell context- dependent. AP-1 is induced by TGF-β in cells that are growth inhibited or stimulated by this growth factor (Angel and Karin, 1991). AP-1 expression has also been implicated in the induction of programmed cell death (Colotta et al., 1992; Smeyne et al., 1993). These differential responses to AP-1 activation likely reflect, at least partly, the composition of the AP-1 complex and other differences in cellular context.

We will consider three interactions between AP-1 and steroid hormone receptors. First, we described the ability of estrogens to regulate the expression of AP-1 components. This may affect AP-1 function by influencing composition of the AP-1 complex (e.g., altering the relative abundance of specific members of c-*jun*/c-*fos* family members). Second, we will consider the effects of AP-1 activation on ER expression/function. Finally, we will discuss recent evidence suggests that ER can signal through direct ER/AP-1 interactions to affect transcriptional regulation regulated by AP-1 response elements.

Data clearly demonstrate the ability of estrogens to up-regulate expression of c-*jun*/c-*fos* family members (Chiappetta et al., 1992). In ERβ-transduced Chinese hamster ovary cells, estradiol induces c-*jun* N-terminal kinase activity. This activity is inhibited when cells are transduced with ERα (Razandi et al., 1999). The c-*fos* protein is readily detected in breast tumors, but its role is unclear. Some investigators describe estradiol activation of AP-1–mediated transcriptional events in breast cancer cells (Chen et al., 1996). Antisense-mediated inhibition of c-*fos* expression can inhibit MCF-7 tumorigenicity (Arteaga and Holt, 1996). Since MCF-7 growth in nude mice requires estrogenic supplementation (Clarke et al., 1989b), inhibition of c-*fos* may block estradiol-ER signaling in vivo. TAM can activate an ER/AP-1 pathway in uterine cells, which are generally growth stimulated by the antiestrogen. In MCF-WES cells, TAM-stimulated growth is associated with increased AP-1 activity (Dumont et al., 1996). However, van der Burg et al. (1995) found AP-1 activity to be significantly reduced after 1 to 4 days of TAM treatment, and Webb et al. (1995) found no AP-1 regulation by TAM. These data suggest that not all MCF-7 cells may respond to TAM by affecting AP-1 expression/activity.

An enhancer element in the ER promoter has been described that requires AP-1 and might be expected to increase ER transcription (Tang et al., 1997). However, several ER-negative cell lines exhibit higher levels of AP-1/DNA binding than MCF-7 cells (van der Burg et al., 1995). Activation of AP-1 results in a down-regulation of ER expression (Martin et al., 1995), and might be expected to antagonize ER function and produce antiestrogen resistance. These latter observations may partly explain the associations of an up-regulation of AP-1, a down-regulation of ER, and the TAM-stimulated, but ICI 182,780, cross-resistant phenotype of the MCF-WES cells (Dumont et al., 1996). Overexpression of c-*jun* or c-*fos*, but not *jun*-D, inhibits ER activity in MCF-7 cells (Doucas et al., 1991). Consistent with these observations is the ability of transfection with *c-jun* to down-regulate ER, producing the consequent TAM-resistant phenotype (Smith et al., 1999).

Steroid hormone receptors can directly interact with AP-1 and affect its function (Ponta et al., 1992; for reviews, see Pfahl, 1993). The consequences of these interactions are strongly receptor, promoter, and cell-type specific (Shemshedini et al., 1991). The most widely reported interaction is the ability of the glucocorticoid receptor (GR) to antagonize the activities of AP-1. This appears to be the result of GR/AP-1 protein-protein interactions (Pfahl, 1993). AP-1/ER interactions also occur. The model described for the ER/AP-1 interactions (Webb et al., 1995), in which AP-1 is bound to both its response element and ER protein, is equivalent to those previously proposed by both Pfahl (1993) and Miner et al. (1991) to explain the GR/AP-1 interactions. The transcriptional response from an ER/AP-1 complex is dependent on the ER and its ligand. Estradiol induces transcription through AP-1/ERα, but inhibits transcription through AP-1/ERβ. In general, ligands elicit opposing effects through AP-1/ERβ, compared with AP-1/ERα (Paech et al., 1997).

These studies were performed using promoter/reporter constructs, and AP-1 activity is known to be highly context sensitive (Angel and Karin, 1991; Shemshedini et al., 1991). It remains unclear how many endogenous promoters are estrogen-regulated through this mechanism. ICI 164,384 is as potent a transcription inducer through AP-1/ERβ in Ishikawa cells (endometrial carcinoma) as are both TAM and Raloxifene (Paech et al., 1997). However, only TAM is believed to have a significant mitogenic effect in the endometrium. In one study, TAM could not active AP-1 in breast cancer cells (Webb et al., 1995), despite other evidence of a TAM-stimulated phenotype associated with increased AP-1 expression (Dumont et al., 1996). Nonetheless, the apparently estrogenic effects of ICI 182,780 on mouse

mammary gland development (Hilakivi-Clarke et al., 1997) and KPL-1 human breast cancer cell proliferation in vivo (Kurebayashi et al., 1998) might reflect activation of genes through an ERβ/AP-1 interaction.

One problem in evaluating the role of AP-1 in antiestrogen resistance is that, in many cell systems, AP-1 protein expression and DNA binding activity are poor predictors of its transcriptional activity. For example, phorbol esters can increase AP-1 binding, but not transactivation of AP-1/reporter constructs in ER-negative cell lines (van der Burg et al., 1995). Thus, directly establishing the functional relevance of altered AP-1 expression/DNA binding in patients' tumors is difficult. One study could not correlate c-*fos* expression with either proliferation or differentiation (Walker and Cowl, 1991), whereas another found a significant association with proliferation, but not differentiation (Gee et al., 1995). A more recent study by the latter group reports reduced *fos* expression in the tumors of TAM responders and increased expression in proliferating and de novo-resistant tumors (Gee et al., 1999).

A borderline association ($p = 0.09$) of higher phosphorylated *c-jun* expression is seen in patients with ER-positive tumors that exhibited progressive disease versus CR+PR+stable disease (Gee et al., 2000). The duration of responses is significantly shorter in tumors with high *c-jun* expression, but no association with the expression of known estrogen-regulated genes is observed. Thus, the association does not seem to be related to ER-mediated events (Gee et al., 2000). In another study, AP-1 DNA binding activity correlated with acquired TAM resistance in ER-positive tumors (Johnston et al., 1999). In neither study was it clear that this association reflected transcriptionally active AP-1, although the studies measured active (Ser^{63} phosphorylated) *c-jun*. These studies also did not clearly exclude the possibility that the associations identified reflect the high incidence of metastatic disease from tumors with high AP-1 activity (Gee et al., 2000). Other phosphorylation sites on *c-jun* can inhibit its activity and could be concurrently present with phosphorylation of the Ser^{63} site (Gee et al., 2000). *Jun-jun* homodimers may be the prevalent AP-1 complex in breast tumors, and these are 25-fold less active in regulating transcription (Gee et al., 2000).

Although certainly encouraging, further studies are clearly warranted to better define the role of AP-1 in TAM responsiveness/resistance. Some observations are potentially confounded by the importance of cell context on outcome, and the often poor abilities of AP-1's protein expression and DNA binding activities to consistently reflect its transcriptional regulatory effects. In future studies, it will be important to establish that any altered AP-1 expression/DNA binding is reflecting altered transcriptional activity. Perhaps it will be necessary to correlate changes in AP-1 expression/DNA binding with the regulation of several downstream target genes and response to antiestrogens. However, it is unclear which targets are appropriate, since many target genes can be regulated by factors independently of AP-1. Adjusting for the possibility that tumors with high AP-1 activity can be more aggressive, also may be necessary.

AP-1 is an important molecule in signaling to both proliferation and apoptosis, and it is likely that perturbations in its gene regulation activities may explain some antiestrogen resistant phenotypes. One possible mechanism is through AP-1's inhibition of ER expression (Doucas et al., 1991; Martin et al., 1995). However, several other mechanisms also can reduce/eliminate ER expression, including growth factors (Stoica et al., 2000) and methylation of the ER gene (Ferguson et al., 1995; Iwase et al., 1999). Conversion to ER negativity is not a particularly common form of acquired resistance (Johnston et al., 1995). Nonetheless, lack of ER expression is clearly a major de novo resistance mechanism. Perhaps the most important contribution of AP-1 is as one of the mechanisms that either initiate and/or maintain the de novo, ER-negative, resistance phenotype. A possible contribution to resistance in some ER-positive tumors also seems likely but remains to be established.

G. Signaling to Mitogenesis or Apoptosis in Antiestrogen Resistance

The consequences of affecting ER signaling in responsive cells is to alter the cell's choice to proliferate, differentiate, or die. The survival benefit some patients derive from antiestrogens implies that, in some cells, these drugs are cytotoxic. Whereas antiestrogens certainly reduce the rate of proliferation (cytostasis), it is likely that their cytotoxicity is at least partly a consequence of an increased rate of apoptosis (Zhang et al., 1999). Thus, altered signaling to apoptosis is one potential mechanism of resistance.

Proving cause and effect is often difficult. For example, cells that are resistant to the induction of apoptosis may already have changed the regulation of key effector molecules in the apoptotic signaling cascade. This may be a direct effect on specific genes in the cascade or altered signaling that ultimately could initiate the cascade at any one of several points. Since additional responses to other endocrine and cytotoxic therapies are common, a total loss of apoptotic signaling is most unlikely. Rather, cells seem to have considerable plasticity in adapting to selective pressures, and there is some redundancy in apoptotic signaling.

Several studies have focused on alterations in signaling through the *bcl-2* family. TAM can down-regulate *bcl-2*, but not *bax*, *bcl-X_L*, or p53 (Zhang et al., 1999). The down-regulation of *bcl-2* seems to reflect the relative potency of antiestrogens (Diel et al., 1999) and may be mediated through multiple enhancer elements in the *bcl-2* promoter. Direct binding of ER is not required (Dong et al., 1999). It might be expected that down-regulation of *bcl-2*'s antiapoptotic activities would be

associated with response to TAM. However, several studies have reported that a down-regulation or loss of *bcl-2* expression is associated with a poor response to TAM (Gasparini et al., 1995; Silvestrini et al., 1996; Daidone et al., 2000). This somewhat unexpected association may more closely reflect the ability of *bcl-2* expression to allow the survival of better differentiated cells, producing a selection for a less aggressive resistant phenotype (Daidone et al., 2000). Similarly, associations of p53 expression and poor response to antiestrogens have been attributed to p53's association with a more aggressive and undifferentiated phenotype (Daidone et al., 2000). However, a more recent study suggests that, after 3 months of TAM therapy, *bcl-2* levels are reduced in responders, but not nonresponders. The changes in *bcl-2* levels also are associated with changes in apoptotic index (Cameron et al., 2000).

The clinical studies with p53 and *bcl-2* demonstrate some of the difficulties in clearly attributing clinical observations to biological function and cell signaling. Nonetheless, it seems likely that several forms of antiestrogen resistance are closely linked to the altered regulation of the gene networks that control signaling to proliferation, differentiation and apoptosis. Precisely which networks are involved may well be first identified using experimental models.

VII. Growth Factors as Mediators of Antiestrogen Resistance

A. Gene Networks: Growth Factors, Their Receptors, and Cellular Signaling

The role of growth factors in the biology of the normal and neoplastic breast has been widely reviewed (Clarke et al., 1992b; Dickson and Lippman, 1995). Thus, this text will focus primarily on the potential role for growth factors in affecting ER function and as candidate components in a broad ER-regulated gene network associated with estrogen responsiveness and antiestrogen resistance.

De Larco and Todaro (1978) initially suggested that some tumor cells may produce the factors they require for continued proliferation. These factors could subsequently function in an autostimulatory or "autocrine" manner. Thus, cells would secrete ligands that then bind to their receptors on the surface of the same cell from which they were secreted. Internal autocrine stimulation may also result from ligand-receptor interactions that occur intracellularly, perhaps at the endoplasmic reticulum-Golgi complexes or within secretory vesicles (Browder et al., 1989).

Expression of several growth factors and their receptors is regulated by estrogens (Clarke et al., 1992b). These are prime candidates for inclusion in a key ER-driven gene network. Estrogen-dependent breast cancer cells might be expected to secrete increased levels of mitogenic growth factors, and lower levels of inhibitory growth factors, in response to estrogenic stimuli (Lippman et al., 1986). Furthermore, additional cross-talk may arise from the ability of signaling downstream of growth factor receptors to influence ER activation [e.g., through changes in MAPK activity (Kato et al., 1995)]. Antiestrogens should increase the production of inhibitory factors, concurrently decreasing the production of mitogens. Antiestrogen-resistant cells would be expected to produce an estrogenic pattern of gene expression, with its regulation perhaps uncoupled from antiestrogenic signaling from the ER. However, estrogenic signaling pathways from the ER could remain intact in resistant cells.

B. Epidermal Growth Factor, Transforming Growth Factor-α, and Other Family Members

The EGF family of proteins contains several structurally and functionally related molecules, including EGF, TGF-α, amphiregulin, and cripto. All four can bind EGF-R, are coexpressed with this receptor (LeJeune et al., 1993; Ma et al., 1998; Niemeyer et al., 1998), and are implicated in the control of normal breast development and in the maintenance of malignant phenotype (Clarke et al., 1989a; Niemeyer et al., 1998). TGF-α seems important in the formation of the terminal-end bud structures in rodent mammary glands (Hilakivi-Clarke et al., 1997; Tsunoda et al., 1997), where it can mimic some of the effects induced by estradiol (Hilakivi-Clarke et al., 1997). TGF-α transgenic mice develop mammary adenomas and adenocarcinomas (Matsui et al., 1990).

TGF-α secretion is induced by estradiol in most estrogen-dependent human breast cancer cell lines (Bates et al., 1988). TGF-α is constitutively expressed in many estrogen-independent cells (Perroteau et al., 1986; Bates et al., 1988), and EGF can induce the estrogen-dependent MCF-7 human breast cancer cells to form small transient tumors in ovariectomized nude mice (Dickson et al., 1987). Similarly, administration of EGF to castrate female mice produces estrogenic effects in the normal uterus (Ignar-Trowbridge et al., 1992). EGF-stimulated cell proliferation, in the absence of estrogen, is inhibited by TAM (Vignon et al., 1987). EGF, TGF-α, and IGF-I increase pS2 mRNA expression, which can be blocked by ICI 182,780 (El-Tanani and Green, 1997) and partially reverse the growth inhibitory effects of antiestrogens (Koga and Sutherland, 1987). Antisense TGF-α sequences reduce the estrogenic response in MCF-7, ZR-75–1 (Kenney et al., 1993), and T47D cells (Reddy et al., 1994). Together, these data are consistent with a contribution of EGF family members to estrogenic signaling and imply an ability of growth factors to initiate estrogenic signaling in the absence of estrogens. One possible pathway is through activation of MAPK activity (Fig. 5), which appears to be downstream of the receptor in an EGF-R signaling pathway (Tari et al., 1999; Xing and Imagawa, 1999).

To more directly address the role of TGF-α in estrogen independence and antiestrogen resistance, MCF-7 cells were transfected with the TGF-α cDNA. Transfectants secrete sufficient TGF-α to down-regulate EGF-R, but retain a fully estrogen-dependent and antiestrogen-responsive phenotype (Clarke et al., 1989a). These data suggest that the estrogenic regulation of TGF-α may be necessary, but is not sufficient, to produce a full estrogenic response in some estrogen-dependent cells. This interpretation is consistent with the observations that estradiol and EGF interact synergistically in stimulating the proliferation of human breast epithelial cells in primary culture (Gabelman and Emerman, 1992), that the effects of TGF-α in the mammary gland are similar but not identical to those induced by estradiol (Hilakivi-Clarke et al., 1997), and that blockade of either ligand (Kenney et al., 1993) or receptor (Arteaga et al., 1988) is not sufficient to consistently and fully eliminate the estrogen-induced growth of estrogen-dependent cells in vitro.

C. Epidermal Growth Factor-Receptor and c-erb-B2

Although the effects of the EGF family of ligands are mediated by their receptors, studies of the receptors alone have also shown association with both response and resistance to antiestrogens. EGF-R and *c-erb-B2* are estrogen regulated, and both are implicated in morphogenesis of the mammary ducts during development. This role appears to involve EGF-R heterodimerization with *c-erb-B2* in the mammary stroma (Sebastian et al., 1998). In neoplastic cells, estrogen produces opposing effects on the regulation of EGF-R and *c-erb-B2* expression. EGF-R expression is induced (Yarden et al., 1996), whereas *c-erb-B2* expression is inhibited (Dati et al., 1990).

In addition to its ligands, the EGF-R also is hormone regulated. Both estrogens and progestins increase EGF-R expression in hormone-responsive tissues (Leake et al., 1988; Lingham et al., 1988). Estrogen-independent breast cancer cell lines express high levels of EGF-R relative to hormone-dependent cells (Fitzpatrick et al., 1984; Davidson et al., 1987). Antisense to EGF-R reduces the tumorigenicity of three breast tumor models (Ma et al., 1998). Since estrogens increase the levels of both secreted ligand and receptor in breast cancer cells, the contribution of any estrogenic signaling mediated by EGF-R may only be sufficient where there are adequate levels of both EGF-R and its ligand(s).

A consistent inverse relationship between ER and EGF-R expression has been widely reported in breast cancer cell lines and tumors. Primary breast tumors that have either low ER content, or lost the ability to express ER, frequently express high levels of EGF-R (Davidson et al., 1987; Cattoretti et al., 1988). This partly explains the association of high EGF-R expression and poor response to TAM. However, there is some evidence that a poor response rate to TAM is seen in ER-positive tumors that also express EGF-R (Nicholson et al., 1994).

c-erb-B2 is a member of the EGF-R gene family, but no specific ligand has been identified. Signaling from *c-erb-B2* may be a consequence of heterodimerization with other liganded members of the family (Chang et al., 1997). Amplification of the *c-erb-B2* gene is detected in approximately 25% of human breast tumors (Revillion et al., 1998). High levels of protein may be expressed in up to 70% of tumors with an amplified gene (de Cremoux et al., 1999). However, active signaling by this receptor, as determined by the use of an activation-state specific monoclonal antibody, may only occur in one-third of invasive tumors that overexpress *c-erb-B2* (DiGiovanni et al., 1996). In univariate analyses, *c-erb-B2* expression is associated with a more aggressive phenotype, a high rate of cellular proliferation, ER negativity and worse histological grade, nuclear grade, and prognosis. Its prognostic significance is less clear in multivariate analyses because of *c-erb-B2*'s association with several other strong prognostic indicators (see Revillion et al., 1998, for a recent review).

In vitro, antiestrogen-responsive cells transfected with the *c-erb-B2* gene exhibit estrogen-independent growth and reduced responsiveness to TAM (Benz et al., 1993; Liu et al., 1995; Pietras et al., 1995). This effect may be related to the ability of *c-erb-B2* to up-regulate Bcl-2 and Bcl-X_L, and suppress TAM-induced apoptopsis in MCF-7 cells (Kumar et al., 1996). Addition of a *c-erb-B2* blocking antibody increases the antiproliferative effects of TAM in BT474 human breast cancer cells (Witters et al., 1997). Paradoxically, TAM increases (Antoniotti et al., 1992) and estrogens decrease (Dati et al., 1990) *c-erb-B2* expression, despite this gene's expression being associated with a poor prognosis and increased proliferation (Revillion et al., 1998). These effects might be expected to reduce TAM's antiproliferative activity. In transfection studies, down-regulation of ER expression, which would be expected to confer some degree of antiestrogen resistance, is seen inconsistently. Reduced ER expression occurs in some *c-erb-B2* transfectants (Pietras et al., 1995), not in others (Benz et al., 1993), and both increases and decreases in ER expression have been described in different clones from the same transfection (Liu et al., 1995).

Although data from in vitro studies provide some evidence for an association of *c-erb-B2* expression and resistance to TAM, the levels of overexpression in transfectants are generally higher than that seen in patients' tumors. Data from clinical studies provide a less clear indication of the putative role of *c-erb-B2* in conferring antiestrogen resistance. Several studies suggest a poorer response rate to TAM in patients with *c-erb-B2* expressing tumors (Wright et al., 1992; Borg et al., 1994; Carlomagno et al., 1996; Yamauchi et al., 1997). However, other studies have not confirmed this association (Archer et al., 1995; Elledge et al., 1998). Since ER-

negative tumors exhibit little response to TAM but are more frequently *c-erb-B2* positive, a major problem with many of these studies is the small number of *c-erb-B2*-positive/ER-positive tumors. In one of the largest studies of ER-positive tumors (Elledge et al., 1998), no significant association between *c-erb-B2* positivity and either TAM response rate, time to treatment failure, or survival was found. Furthermore, when (Newby et al., 1997) *c-erb-B2* expression was measured before TAM treatment and at recurrence, they found no change in *c-erb-B2* expression, regardless of whether the tumors responded or were resistant. Overall, current data are inconclusive, providing little in the way of compelling evidence of a strong association of *c-erb-B2* expression and TAM resistance.

D. Tranforming Growth Factor-β Family

There has been considerable interest in the possible role of the TGF-βs in antiestrogen responsiveness and resistance since the first report of the ability of estrogens and antiestrogens to differentially regulate TGF-β secretion in breast cancer cells (Knabbe et al., 1987). Both 4-hydroxyTAM and ICI 182,780 increase the secretion of TGF-β_2 by human breast cancer cells (Koli et al., 1997; Muller et al., 1998). In one small study, 11 of 15 breast tumors responding to TAM exhibited increased TGF-β_2 mRNA expression (MacCallum et al., 1996). Serum TGF-β_2 levels also are higher in TAM responders (Kopp et al., 1995). Although some cells exhibit resistance to both TAM and TGF-β (Herman and Katzenellenbogen, 1996), several MCF-7 cell lines that are resistant to TGF-β are not resistant to antiestrogens (Kalkhoven et al., 1996; Koli et al., 1997). Cells that are resistant to TAM often overexpress TGF-β (Herman and Katzenellenbogen, 1996; Arteaga et al., 1999), but their antiestrogen responsiveness cannot be restored in vitro by inhibiting TGF-β function with blocking antibodies (Arteaga et al., 1999). In responsive cells, the growth inhibitory effects of antiestrogens are not consistently blocked by the addition of anti-TGF-β antibodies (Koli et al., 1997).

In patients who do not respond to TAM, TGF-β_2 levels increase before clinical evidence of disease progression (Kopp et al., 1995). This implies that the tumor cells have become resistant to any possible growth inhibitory effects of TGF-β_2 and may even obtain an advantage from this increased expression. Overexpression of TGF-β_2 can suppress natural killer (NK) cell function. Inhibition of TGF-β_2 activity restores both NK cell function and response to TAM in vivo (Arteaga et al., 1999). Thus, some of the effects of TGF-β may be immunologic.

Clearly, the involvement of TGF-β_2 in antiestrogen-mediated signaling is complex. The ability of TGF-β to inhibit the proliferation of some breast cancer cells, and to be induced by antiestrogens but inhibited by estrogens, suggests that some breast tumors may initially respond through an autocrine inhibitory pathway. This may occur early in treatment, consistent with the increased tumor TGF-β mRNA expression and TGF-β_2 serum levels seen in some responders. If this is a direct autocrine effect on the cancer cells, any reduced immunosurveillance would have little effect. However, once the tumor cells become resistant to TAM/TGF-β, the TGF-β–induced immunosuppression could predominate. This changing response pattern would be consistent with the initial reduction in TGF-β_2 serum levels, followed by an increase before clinically detected recurrence, seen in TAM nonresponders (Kopp et al., 1995). Other TGF-β response patterns probably also occur, because not all responding tumors exhibit increased TGF-β_2 expression (MacCallum et al., 1996), and the antiestrogenic responsiveness of some cells is not directly associated with their sensitivity to TGF-β_2 (Koli et al., 1997).

E. Insulin-Like Growth Factors, Their Receptors, and Binding Proteins

IGF-I is a 70 amino acid polypeptide and IGF-II a 67 amino acid polypeptide, both proteins sharing structural and functional homologies with insulin. IGF-I increases the rate of proliferation of some breast cancer cells (Furlanetto and DiCarlo, 1984; Mayal et al., 1984; Leake et al., 1988) and can induce the transient formation of estrogen-independent MCF-7 tumors in ovariectomized athymic nude mice (Dickson et al., 1987). Although some breast cancer cell lines produce an estrogen-regulated IGF-like material (Huff et al., 1988), this does not appear to be authentic IGF-I (Yee et al., 1989b). IGF-II mRNA or protein has been observed in breast cancers (Peres et al., 1987), and this can be induced by estrogen in some cells (Parisot et al., 1999). Generally, the proportion of human breast cancer cell lines and tumor cells that express IGF-I and/or IGF-II mRNA appears to be small (Travers et al., 1988; Yee et al., 1989b). In contrast, significant IGF-I and IGF-II mRNA expression is observed in the stromal components of a number of breast tumors, implying a potential paracrine role for the IGFs (Yee et al., 1989b).

Several investigators have shown that the serum levels of IGF-I are moderately reduced in patients receiving TAM (Lonning et al., 1992a; Ho et al., 1998; Pollack, 1998). This may primarily reflect an effect of TAM on hepatic IGF secretion. Nonetheless, lower serum levels, and any reduction in local stromal production, could result in lower intratumor levels of the IGFs. This would reduce the ability of these proteins to induce/maintain tumor proliferation. Some, but not all, studies report a concurrent increase in the levels of IGF-II in antiestrogen-treated patients (Helle et al., 1996b; Ho et al., 1998). Increases in either the serum and/or stromal production of mitogenic IGFs could significantly impair the action of antiestrogens and produce an apparent resistance.

Determining the precise role of the IGFs is complicated by apparently concurrent changes in the levels of

several IGF-binding proteins (IGF-BPs) and the two IGF receptors. Both IGF-I receptors (IGF-I-Rs) and IGF-II receptors (IGF-II-Rs) are expressed in breast tumors (Papa et al., 1993; Zhoa et al., 1993). Of these, IGFs' activities are primarily mediated through IGF-I-Rs. The IGF-II-R is the mannose-6-phosphate receptor, which is also involved in the activation of the TGF-βs (Dennis and Rifkin, 1991). There are no direct intracellular signaling consequences for ligand binding to the IGF-II-R, which is primarily an extracellularly exposed membrane protein.

In the context of antiestrogen action and resistance, most interest has focused on the IGF-I-R. Growth of the estrogen-unresponsive MDA-MB-231 human breast cancer cells, both in vivo and in vitro, is partly inhibited by an antibody that blocks ligand binding to the IGF-I-R (Rohlik et al., 1987; Arteaga and Osborne, 1989). This antibody also inhibits proliferation of a number of other human breast cancer cell lines in vitro (Arteaga and Osborne, 1989). Growth of estrogen-dependent MCF-7 cells is inhibited in vitro, but not in vivo (Rohlik et al., 1987; Arteaga et al., 1989). Several groups have shown the ability of activation of the IGF-I-R to regulate the expression of otherwise estrogen-regulated genes (Hafner et al., 1996; Lee et al., 1997). These data imply cross-talk between the IGF-I-R and ER, and are consistent with the ability of ICI 182,780 to decrease the rate of IGF-I-R transcription (Hunyh et al., 1996a), and of estrogen to induce IGF-I-R expression (van den Berg et al., 1996; Parisot et al., 1999). TAM inhibits IGF-I's ability to phosphorylate the insulin receptor substrate-1 of the IGF-I-R in some studies (Guvakova and Surmacz, 1997), but not in others (Lee et al., 1997). Nonetheless, estrogen withdrawal produces a reduction in insulin receptor substrate-1 expression in MCF-7 xenografts (Lee et al., 1999; Salerno et al., 1999). Thus, either overexpression (Salerno et al., 1999), and/or a constitutive activation of insulin receptor substrate-1, could contribute to cross-talk with ER-mediated signaling to produce antiestrogen resistance.

There are several IGF-BPs that exhibit a high affinity for both IGF-I and IGF-II and generally inhibit IGF function. Breast cancer cell lines secrete significant levels of these IGF-BPs (Yee et al., 1989a; Adamo et al., 1992). Addition of IGF-BPs to cell culture media can inhibit the mitogenic effects of IGFs in human breast cancer cells (van der Burg et al., 1990). Since breast cancer cells secrete multiple IGF-BPs (Clemmons et al., 1990), it seems likely that the cumulative effect of IGF-BP secretion is to partly antagonize the mitogenic effects of IGFs in breast cancer cell growth. Both IGF-BP-3 (Nickerson et al., 1997) and IGF-BP-5 (Hunyh et al., 1996b) are induced by ICI 182,780. IGF-BP-3 alone can induce apoptosis, perhaps by sequestering IGF-I-R ligands (Nickerson et al., 1997). TAM-resistant cells secrete lower levels of IGF-BP-2 and IGF-BP-4 (Maxwell and van den Berg, 1999). In patients, triphenylethylene therapy is associated with increased levels of IGF-BP-1 (Helle et al., 1996a; Ho et al., 1998) and IGF-BP-3 (Helle et al., 1996a). However, there is no clear association between plasma sex steroids and either IGF-I or IGF-BP-1 levels (Lonning et al., 1995).

Cumulatively, these observations are consistent with a reduction in the secretion of IGF-I and a possible increase in secretion of selected IGF-BPs, within the tumor or from other sources, as being associated with antiestrogen treatment. Antiestrogen resistance could be produced by changes in IGF-I-R signaling, either directly or through downstream interactions with ER function, by changes in systemic IGF/IGF-BP secretion, and/or by autocrine/paracrine interactions mediated by IGFs. In addition, or alternatively, cells could become resistant to the loss of IGF-induced mitogenesis by becoming more dependent on the proliferative activities of other growth factors or mitogenic signaling pathways.

VIII. Estrogen Receptor-Independent Targets for Mediating Antiestrogen Action and Resistance

Several ER-independent targets have been described for TAM. These are often called nongenomic because they do not require interaction of TAM with ER and/or do not directly affect the transcriptional regulatory activities of ER. These targets have received considerable attention, primarily in an attempt to explain the apparent clinical responses occasionally seen in some patients with ER-negative tumors. However, the nongenomic (ER-independent) activities of antiestrogens may also be important in ER-positive tumors. For example, these may be necessary, but not sufficient, to induce a growth inhibitory effect in response to antiestrogen exposure. Although an initial interaction may be independent of ER, the downstream consequences of this could affect ER expression and/or function by altering cellular context. Some ER-independent interactions have already been discussed (e.g., binding to AEBS). Other targets may involve both direct ER interactions and nongenomic effects. For example, AP-1's transcriptional activity can be directly influenced by an occupied ER (direct genomic effect), whereas AP-1 activity can also be regulated downstream of an oxidative stress and/or cytokine/growth factor signaling that regulates Jun N-terminal kinases (ER-independent; nongenomic for ER involvement). The following sections focus on the more widely studied of the ER-independent targets for TAM.

A. Oxidative Stress

The generation of an excess of reactive oxygen species has been implicated in many diseases, including cancer. The mutagenic properties of these species is primarily associated with the production of DNA strand breaks, base modification, and DNA-protein cross-linkages (Toyokuni et al., 1995). However, the generation of an oxidative stress also has significant effects on the regu-

lation of several genes (Morel and Barouki, 1999), and can, therefore, substantially alter the cellular context of affected cells. The ability of reactive oxygen species to regulate gene expression is likely multifactorial. The promoter of some genes contain an electrophile response element that is sensitive to changes in redox state. Many of these genes are associated with a potentially general stress response, encoding proteins associated with cellular detoxification [e.g., glutathione-*S*-transferase, quinone reductase (Montano and Katzenellenbogen, 1997)].

TAM has been widely implicated as an antioxidant, potentially consistent with its ability to influence plasma membrane structure and function (Garcia et al., 1998). However, such activities, might also initiate an antioxidant cascade (Gundimeda et al., 1996). 4-HydroxyTAM is a scavenger of peroxyl radicals in several cells and experimental systems. For example, 4-hydroxyTAM inhibits lipid peroxidation in sarcoplasmic reticulum membranes (Custodio et al., 1994) and Fe(III)-ascorbate-induced lipid peroxidation in rat liver microsomes (Wiseman, 1994). Endogenous and UV light-induced oxidative damage to DNA, protein, and lipids is inhibited by TAM in mouse epidermis (Wei et al., 1998). In human neutrophils, TAM inhibits hydrogen peroxide formation in response to treatment with triphenylethylene antiestrogen (TPA) (Lim et al., 1992). The ability of TAM and 4-hydroxyTAM to inhibit Cu^{2+}-induced peroxidation of low-density lipoprotein has been suggested to contribute to the putative cardioprotective effects of these antiestrogens (Wiseman et al., 1993a).

Paradoxically, whereas both estradiol and TAM can act as antioxidants (Garcia et al., 1998; Schor et al., 1999), there is clear evidence that TAM is associated with intracellular oxidative stress. The membrane association of PKC induced by TAM appears to reflect its ability to partition into membranes and initiate an oxidative stress. This effect is largely eliminated upon administration of antioxidants (Gundimeda et al., 1996). TAM-induced lipid peroxidation has been described in which the generation of superoxide is implicated (Duthie et al., 1995). Both TAM and 4-hydroxyTAM can induce 8-hydroxy-2′-deoxyguanosine formation in rat liver microsomes (Ye and Bodell, 1996), potentially through changes in redox cycling (Okubo et al., 1998). In marked contrast, TAM inhibited the formation of this intermediate in HeLa cells treated with TPA (Bhimani et al., 1993). More recently, TAM has been shown to induce oxidative stress in ovarian and T-leukemic cells (Ferlini et al., 1999). TAM also induces TPA-induced AP-1 activity (van der Burg et al., 1995), NFκB (Ferlini et al., 1999), quinone reductase (Montano and Katzenellenbogen, 1997), and other genes associated with oxidative stress. These data clearly suggest that, despite its antioxidant properties, some cells respond to TAM as they would to an oxidative stressor.

Why should there be this apparent contradiction in pro-oxidative versus antioxidative activities is unclear. It is possible that, like many other events, cellular context is critical in determining response. The ability of TAM and its metabolites to generate an oxidative stress is likely related, at least partly, to their intracellular metabolism to species that can generate reactive intermediates. Day et al. (1999a) compared the one-electron activation of 4-hydroxyTAM and 3-hydroxyTAM by several enzymes. Although generation of the phenoxyl radical by myeloperoxidase was weak, several other enzymes effectively generated the species. The substrate specificity of the (myelo)peroxidases determined whether a phenolic substrate generated a reactive phenoxyl radical or an antioxidant. Thus, the ability of TAM to generate either a pro-oxidant or antioxidant response may depend on the levels and activities of activating enzymes in the target cells.

Another possibility is that TAM has antioxidant properties at the cell's surface, but acts as a pro-oxidant when metabolically activated within the cell, or when partitioned into specific membrane domains. This would appear consistent with antioxidant effects on some membrane lipids, but pro-oxidant effects on gene transcription. Although this might occur in the short term, intracellular activation could produce sufficient concentrations of reactive intermediates that even some membrane lipids and phospholipids eventually become peroxidated.

It is also possible that the oxidative stress is a result of TAM's effects on cellular metabolism. Preliminary data from our laboratory has implicated altered cytochrome C oxidase and NFκB activity with antiestrogen resistance. These changes could reflect differences in mitochondrial function and oxidative metabolism, the consequences of which could lead to free oxygen radical production, in excess of cells' abilities to scavenge these reactive metabolites.

B. Perturbations in Membrane Structure/Function

It is clear from their structures that most of the TPAs are relatively lipophilic and would be predicted to partition predominately into the hydrophobic domains of cellular membranes. Membrane partitioning will affect the physicochemical properties of the membrane domain(s) into which the drug partitions. This latter effect could significantly impact the function of adjacent or nearby proteins that are dependent upon the properties of their lipid environment for function (Lenaz et al., 1978). Such proteins could include growth factor receptors, membrane ER (Nelson et al., 1987; Watson et al., 1999), and other membrane-associated signaling molecules, such as G-proteins, phosphoinositides, and members of the PKC family. For example, TAM induces a selective membrane association of PKCε (Cabot et al., 1997).

TAM alters the physical attributes of breast cancer cells by decreasing membrane fluidity (Clarke et al., 1990). Fluidity was estimated by determining the

steady-state polarization of fluorescence of the probe 1,6-diphenyl-1,3,5-hexatriene, which reflects the rotational ability of the probe resulting from the molecular packing of the lipids comprising the membrane domains into which the probe is inserted. The reduced fluidity occurs regardless of ER status, as would be expected for an effect independent of ER. Similar effects have subsequently been reported in artificial membranes (Custodio et al., 1993b) and liposomes (Custodio et al., 1993a; Kayyali et al., 1994).

In breast cancer cells, these changes in membrane structure are associated with increasing cytotoxicity (Clarke et al., 1990). TAM has been reported to affect other membrane-associated events, including calcium ion influx (Morley and Whitfield, 1995), P-glycoprotein-mediated drug efflux (Leonessa et al., 1994), and membrane phospholipid metabolism (Cabot et al., 1995). Although potentially nonspecific, in terms of ER expression, there may be some degree of specificity conferred by the physicochemical characteristics of the domains into which TAM is inserted. If these domains are functionally linked to the activity of key membrane proteins, resistance could arise by cells switching to other pathways that do not require these membrane-dependent events, or by altering local membrane structure to reduce the stabilizing effects of TAM. The possibility that TAM-induced changes in membrane function are necessary, but not sufficient for its antiestrogenicity or antiproliferative effects, cannot be excluded. For example, these events might interact with specific ER-mediated signaling events that do not occur in ER-negative cells.

C. Protein Kinase C

PKC is a membrane protein that has been implicated as an important signal transduction molecule in several cellular systems. There are at least 10 isoforms that fall into one of three families. The classical family contains PKC isoforms α, β, and γ; the novel family comprises isoforms δ, ϵ, η, θ, and μ; and the atypical family contains isoforms ζ and λ (Datta et al., 1997). PKC is activated by the diacylglycerol produced following the hydrolysis of membrane inositol phospholipids by phospholipase C (Nishizuka, 1992; Olson et al., 1993). The hydrolytic activities of phospholipases D and A_2 may enhance this activation (Nishizuka, 1992).

Like many membrane-associated proteins, the function of PKC is probably dependent upon its lipid environment. The ability of TAM to alter the structural properties of membranes could indirectly alter PKC function. It also is apparent that TAM can bind directly to PKC (O'Brian et al., 1986, 1988). However, there is some controversy relating to whether TAM inhibits or activates PKC. TAM inhibits PKC activity with an IC_{50} = 25 μM in studies performed on partially purified PKC (O'Brian et al., 1986). In intact cells, TAM does not inhibit PKC activity (Issandou et al., 1990), whereas others have reported PKC activation by triphenylethylenes (Bignon et al., 1991). More recent studies have shown that TAM causes both a membrane translocation and a down-regulation of the enzyme. This translocation is generally associated with PKC activation and appears to require release of arachidonic acid (Gundimeda et al., 1996). TAM can activate phospholipases C and D and translocate PKCϵ, but not the α, β, γ, δ, and ζ PKC isoforms, to the membrane (Lavie et al., 1998). These effects occur at concentrations similar to those affecting membrane fluidity (Clarke et al., 1990). Thus, the membrane signaling effects of TAM on PKC activation may be related to its ability to alter membrane structure/function.

Signaling from PKC is often complex and the end result can be cell specific. For example, overexpression of PKCα in MCF-7 cells has produced conflicting results. Manni et al. (1996) observed a less aggressive phenotype, whereas Ways et al. (1995) reported a more aggressive phenotype. The latter observation is more reflective of the abilities of PKC to influence attachment, motility, and invasiveness (Palmantier et al., 1996; Platet et al., 1998). The difference between these studies might be explained by the concurrent changes in expression of other PKC isoforms. Ways et al. observed increased expression of the δ- and η-isoforms, whereas their expression was not changed in the Manni et al. study.

There are several potential signaling pathways following PKC activation that could produce the responses seen in normal and neoplastic breast tissues. PKC has been implicated in mediating the mitogenic activity of the *ras* proto-oncogene (Lacal et al., 1987). PKC activation causes the formation of *ras/raf-1* complexes, but activates *ras* in a manner that differs from its activation by receptor tyrosine kinases (Marais et al., 1998). Expression of $p21^{waf1/cip1}$, which is associated with cell cycle arrest, is induced by PKC independently of p53 through a posttranscriptional mechanism (Akashi et al., 1999). In contrast, cleavage of PKCθ by caspase 3 induces apoptosis (Datta et al., 1997).

PKC activity is greater in neoplastic breast tissues when compared with normal breast (O'Brian et al., 1989). Most appear to be the Ca^{2+}-dependent PKC isoforms (Gordge et al., 1995), which are more highly expressed in ER-negative tumors (Borner et al., 1987). Induction of PKC activity can inhibit ER function (Martin et al., 1995), whereas the ability of growth factors to alter ER function occurs independently of PKC (Ignar-Trowbridge et al., 1996). PKC affects ER signaling in osteoblasts (Migliaccio et al., 1993, 1998), similar to its effects in breast cancer cells (Martin et al., 1995). The consequences of PKC activation in breast cancer cells include cell cycle arrest (Seynaeve et al., 1993) and induction of prostaglandin E_2 synthesis (Boorne et al., 1998).

TAM can inhibit PKC activity following a transient activation (Gundimeda et al., 1996). If PKC activity

were rate-limiting for proliferation, any significant inhibition of its activity may be sufficient to induce a reduction in cellular proliferation. The importance of PKC in the regulation of mitogenic signals implies that, if TAM does regulate its function in vivo, this inhibition likely contributes to the overall effect on cellular proliferation. Perturbations in either the level of expression of PKC, or its sensitivity to inhibition by TAM, could contribute to acquired TAM resistance in some cells. The implications of altered PKC activation on ER function also require clarification, and these may differ among cells.

Any events related to TAM/PKC interactions could be most important in a subset of ER-positive cells. Since the effects of overexpression of PKCα appear cell-specific, additional studies are required to determine whether some isoforms are more important than others. Nevertheless, it seems likely that TAM's ability to influence PKC activity is important in mediating the effects of antiestrogens in some breast cancer cells. Some of these effects may be mediated through the ability of PKC to activate AP-1 and/or influence ER activity at AP-1 sites.

D. Calmodulin

Estrogen can depolarize plasma membranes and initiate internal calcium signaling (Nadal et al., 1998). Calmodulin is an intracellular Ca^{2+} binding protein and an important signal transduction molecule that participates in the signaling to several endpoints in different cells (Means, 2000). A major intermediary in this signaling is the calmodulin-dependent kinase II. For example, calmodulin kinase II activates the protooncogene *c-fos* (Wang and Simonson, 1996), is implicated in signaling to *fas*-mediated apoptosis (Pan et al., 1996; Wright et al., 1997), and can affect ER-mediated signaling. Calmodulin can phosphorylate the ER protein on tyrosine (Migliaccio et al., 1984), an event that effects ligand binding (Migliaccio et al., 1989). More recently, Biswas et al. (1998) have shown that calmodulin binds directly to ER, is an integral component of an active ERE-ER complex, and is required for the formation of a productive transcription complex. Calmodulin also is involved in cyclic nucleotide metabolism. Some aspects of ER-mediated gene transcription can be regulated by cAMP (Aronica and Katzenellenbogen, 1993). Calmodulin antagonists can inhibit breast cancer cell proliferation, arresting cells in the same cell cycle phase as TAM (Musgrove et al., 1989).

TAM could indirectly influence ER function through its ability to inhibit calmodulin's activities. A high-affinity interaction between TAM and calmodulin has been reported, with a K_d value of approximately 6 nM (Lopes et al., 1990). A second, lower affinity, interaction occurs with an apparent IC_{50} of 6 to 9 μM (Rowlands et al., 1995; Greenberg et al., 1987). 4-Iodination and elongation of the basic side chain length increase both the calmodulin and PKC antagonist activities of TAM (Rowlands et al., 1995).

An inhibition of calmodulin and/or calmodulin kinase II could contribute to the antiproliferative effects of antiestrogens. The extent of inhibition will be determined by the intratumor availability of TAM and its appropriate metabolites. The high-affinity TAM-calmodulin interaction occurs at concentrations well below those associated with an estrogen-reversible growth inhibition by the triphenylethylenes in vitro. These high-affinity sites should be occupied in the majority of TAM-treated tumors. A proportion of the low- affinity sites also may be occupied, since intratumor TAM concentrations in the range of their K_i can be detected in human tumors. These observations raise the possibility that inhibition of calmodulin is necessary, but not sufficient for TAM's activities. If calmodulin levels are dose-limiting for ER activation, a modest level of inhibition may be sufficient to influence ER function. It is tempting to speculate that one reason why TAM is a weak partial agonist is because it concurrently limits calmodulin's ability to produce a fully productive ER-ERE transcription complex.

E. Comments on the Possible Role of Nongenomic Effects

Cellular context may substantially affect how a cell perceives and responds to an occupied ER protein. Thus, a major contribution of nongenomic effects may be to influence the cellular context, such that other key regulators of the antiestrogen-induced signaling network are appropriately expressed/repressed. It can readily be appreciated that this could be facilitated by perturbations in the activities of key intracellular signaling proteins such as calmodulin, PKC, or the various factors associated with the induction of an oxidative stress response. For example, cellular stress is often accompanied by changes in the expression of apoptosis modulating factors such as NFκB or AP-1. Preliminary data from our laboratory indicate that NFκB activity is significantly elevated in the antiestrogen-resistant MCF7/LCC9 cells, as are several other genes regulated by oxidative stressors.

Some of these events are likely to be regulated independently of the ER. Thus, there may be a necessary interaction between ER-mediated and nongenomic events for the full induction of an antiestrogenic response in cells expressing ER. It might be predicted that the expression of some of the nongenomic targets will be different in ER-positive cells because they are more responsive. The levels of calmodulin in breast tumors appear higher than in normal tissue (O'Brian et al., 1989), and ER-negative tumors tend to express higher levels than ER-positive cells (Borner et al., 1987). Ultimately, it should be clearly demonstrated that the concentrations at which nongenomic effects occur represent achievable intracellular TAM concentrations in tumors. Many of the nongenomic effects are observed at micro-

molar concentrations of TAM in vitro. The cell culture conditions used contain only low concentrations of serum, generally ≤10%, which may not reduce availability to the same degree as occurs in blood/tissues.

IX. Immunologic Mechanisms of Tamoxifen Resistance

The immunosuppressive activities of estrogens have been known for many years, and antiestrogenic effects on these endpoints might be expected to affect host immunity and tumorigenicity. Not surprisingly, there is considerable evidence demonstrating the ability of antiestrogens to influence many aspects of immunity. Some of these effects are likely to be ER-mediated, since expression of steroid hormone receptors is widely reported among some lymphoreticular cells. For example, peripheral blood mononuclear cells, thymus and splenic cells, and CD8+ T cells express ER (reviewed in Schguurs and Verheul, 1990). Other immunologic effects of antiestrogens may well reflect perturbations in the activities of the ER-independent targets described elsewhere in this review.

Tumors proliferating successfully in the presence of cytotoxic host cells clearly indicate that the cells have evaded cytolytic effectors. The precise mechanisms involved remain unknown, but modification or masking of surface antigens, the secretion of factors that inhibit effector function, and an altered sensitivity to the direct cytolytic effects of effector cells are probably involved (Key et al., 1982). Where antiestrogens can influence these events, they also may impact the immune status of the host and alter its response to the tumor. Thus, the immunomodulatory activities of antiestrogens have considerable potential to contribute to their mechanism(s) of action and resistance.

A. Cell-Mediated Immunity

Cell-mediated or adaptive immunity (CMI) is primarily conferred by the interactions between T lymphocytes and cells expressing the antigens they recognize. There are several key lymphoid cell populations implicated in the control of cancer, including NK and lymphokine-activated killer (LAK) cells. Both NK and LAK cells are distinct from cytotoxic T lymphocytes, lysing cells lacking significant expression of the MHC genes. NK and LAK cells can infiltrate solid tumors and malignant effusions (Blanchard et al., 1988). Macrophages, which are of myeloid lineage, also exhibit antitumor activity (Wheelock and Robinson, 1983). Changes in CMI and the infiltration of its effectors are evident in many breast tumors. A common component of the desmoplastic response to breast cancers is the infiltration of reticuloendothelial cells (Clarke et al., 1992b). The skin window procedure, which provides an estimate of the extent of CMI, correlates inversely with metastatic disease (Humphrey et al., 1980; Black et al., 1988). The functional competence of T lymphocytes is impaired in 58% of breast cancer patients, with a high proportion observed in those with lymph node involvement (Head et al., 1993).

B. Natural Killer Cells

NK cells make up approximately 1 to 2.5% of peripheral lymphocytes and have been widely demonstrated to possess antitumor activity (Wheelock and Robinson, 1983). Low levels of NK cell activity are associated with familial breast cancer (Strayer et al., 1986), with these levels also seen in patients with stage III/IV disease (Akimoto et al., 1986; An et al., 1987; Contreras and Stoliar, 1988). Some tumors can suppress NK activity (Mantovani et al., 1980), perhaps explaining why this activity is generally low or absent in the axillary lymph nodes of patients with demonstrable metastatic disease (Horst and Horny, 1987; Bonilla et al., 1988). Other tumors may become resistant to NK cell-mediated cytolysis (Arteaga et al., 1999). Since NK cell activity may contribute to the control of metastasis, the poor metastatic potential of many human xenografts growing in nude mice may reflect their elevated NK cells activities (Clarke, 1996).

Estrogens and endocrine therapies clearly affect NK cell activity. Aminoglutethimide, which reduces serum estrogen concentrations, increases NK activity in breast cancer patients (Berry et al., 1987b). In mice, estrogens induce a biphasic response on NK cell activity. An initial increase in activity is generally followed by a subsequent reduction of activity to below pretreatment/untreated levels (Seaman et al., 1978; Seaman and Talal, 1980; Hanna and Schneider, 1983; Screpanti et al., 1987). TGF-α transgenic mice have lower NK cell activity, consistent with increases in their serum estrogens (Hilakivi-Clarke et al., 1992).

TAM stimulates NK activity both in vitro (Mandeville et al., 1984) and in vivo in rodents (Gottardis et al., 1989; Baral et al., 1995). In humans, TAM can produce estrogenic effects on lymphocyte function (Myers and Peterson, 1985). Short-term TAM treatment (1 month) increases NK activity (Berry et al., 1987a), whereas longer term treatment (1.5 to 2 years) reduces NK activity (Rotstein et al., 1988). TAM can also sensitize the target cells to lysis (Baral et al., 1995), an effect that does not appear to require ER expression (Baral et al., 1995). Long-term TAM-induced reduction in immunity, and/or changes in the susceptibility of the tumor cells to lysis, could contribute to the emergence of a TAM-stimulated phenotype by eliminating the cytolytic or inhibitory effects of tumor infiltrates.

A loss of responsiveness to TAM-induced NK cell activation could contribute to the appearance of resistance. Using the MCF7/LCC2 TAM resistance model (Brünner et al., 1993b), the potential importance of inhibiting NK cell activity as a mechanism of TAM resistance has been demonstrated. The MCF7/LCC2 cells secrete significant

amounts of the cytokine TGF-β_2, which can inhibit NK cell activity (Arteaga et al., 1999). TAM inhibits the growth of MCF7/LCC2 xenografts in nude mice, which have high NK cell activity (Clarke, 1996), when concurrently treated with antibodies that block TGF-β_2 activity (Arteaga et al., 1999). These data suggest that the antitumor effects of TAM are partly conferred by increased NK cell activity and that one form of resistance is for cells to secrete growth factors or cytokines that can block this activity (Arteaga et al., 1999).

C. Macrophages

Macrophages are widely observed to infiltrate solid tumors and can kill tumor cells, perhaps recognizing some tumors on the basis of their abnormal growth (Hibbs et al., 1972) or by surface modifications (Key et al., 1982). Macrophages can produce both antigen-specific and nonspecific cytolysis. These tumoricidal properties are acquired following activation by contact with either the target cell and/or its secreted products (Fidler, 1988). Cell kill is produced both by phagocytic and nonphagocytic processes (Key et al., 1982), the latter cytolysis probably involving the release of lysosomal enzymes by exocytosis.

In some cases, macrophage infiltration is associated with tumor progression rather than inhibition, implying that macrophages may secrete factors mitogenic for tumor cells (Acero et al., 1984). One possibility is their apparent ability to produce estradiol (Mor et al., 1998), which might limit their mitogenic effects to ER-positive breast cancer cells. However, macrophages secrete many cytokines and growth factors, and focal macrophage infiltration in breast tumors is associated with increased angiogenesis and poor prognosis (Leek et al., 1999).

The effects of endocrine treatments on macrophage activity have not been widely studied. However, estrogens can significantly alter the expression of several cytokines implicated in the activation of macrophages (Hunt et al., 1998; Rogers and Eastell, 1998). TAM blocks the estrogen-induced release of the interleukin-6 soluble receptor (Singh et al., 1995), tumor necrosis factor (Zuckerman et al., 1995), and induction of JE/MCP-1 mRNA (Frazier-Jessen and Kovacs, 1995). TAM also blocks the inhibitory effects of estradiol on macrophage function (Savita and Rai, 1998) and modulates the antiproliferative signal of interferon-α on premacrophage proliferation (Balint et al., 1992). These observations are consistent with a potential role for perturbations in macrophage function in both responsiveness and resistance to TAM therapy.

D. Lymphokine-Activated Killer Cells, Cytotoxic T Cells, and Other Cell-Mediated Immunity Effector Cells

LAK cells are clearly distinct from NK cells, a determination initially derived from studies of mice bearing different immune-deficiency mutations [i.e., *nu* and *bg* (Andriole et al., 1985)]. LAK cells are capable of killing neoplastic cells and can kill tumor cells resistant to NK cytolysis (Grimm et al., 1982). Some tumors produce material capable of blocking the development of LAK cells (Ebert et al., 1990). LAK cells are often present in the axillary lymph nodes of patients with demonstrable metastatic disease (Bonilla et al., 1988). Both TAM and estradiol can increase the sensitivity of target cells to lysis by LAK cells (Albertini et al., 1992; Baral et al., 1996a). TAM and Toremifene increase the immunotherapeutic effect of coadministered LAK cells both in vivo and in vitro (Baral et al., 1996b). Where such effects are lost, target cells could become resistant to cytolysis and appear TAM resistant.

Cytotoxic T cells are T lymphocytes that recognize surface antigens bound to MHC class I molecules. Binding to the T cell receptor causes the release of the effector molecules that induce lysis of the target cell. Infiltration of breast tumors (Kirii et al., 1998; Nguyen et al., 1999) and lymph nodes (Ito et al., 1997) by cytotoxic T cells has been clearly demonstrated. Whereas the full series of antigens recognized by these cells remains to be established, antigenic proteins with a mucin polypeptide core are clearly involved (Kirii et al., 1998). Cytotoxic T cells isolated from patients immunized with a synthetic MUC1 peptide exhibit class 1-restricted killing of MUC1-expressing cells (Reddish et al., 1998). Both TAM and estradiol increase the sensitivity of target cells to lysis by cytotoxic T cells (Baral et al., 1994). A combination of antiestrogens increased the cytotoxic effects of cytotoxic T cells against the H2712 mouse mammary tumor (Baral et al., 1997). The proliferation of some cytotoxic T cells is arrested in G1 following TAM treatment (Lyon and Watson, 1996).

Endocrine treatments also have been reported to affect less well defined mediators of CMI. For example, TAM increases TNF-α production by mononuclear cells (Teodorczyk-Injeyan et al., 1993). TAM, Toremifene, and ICI 164,384 exhibit immunosuppressive activities when their effects are measured on human mononuclear cells (Teodorczyk-Injeyan et al., 1993).

E. Humoral Immunity

Humoral immunity is conferred by the antibody-mediated response to antigens. There are cooperative interactions between humoral and CMI, since the interaction of tumor cells with CMI effectors likely alters the balance of cytokines such that the functional differentiation of CD4 T cells is affected (Janeway et al., 1997). Steroids are known to affect humoral immunity in several species (Leitner et al., 1996). For example, estrogens can increase IgM secretion (Myers and Peterson, 1985).

Generally, the ability of antiestrogens to affect specific aspects of humoral immunity are less well reported than their effects on CMI. TAM can block the effects of estrogens on an antigen-specific antibody response in vitro

(Clerici et al., 1991) and improve the persistent proteinuria and immune complex deposition in the kidneys of mice with experimental systemic lupus erythematosus (Sthoeger et al., 1994). The ability of pokeweed mitogen to induce IgG and IgM secretion is inhibited by ICI 164,384, TAM, and Toremifene (Teodorczyk-Injeyan et al., 1993). Long-term Toremifene therapy is associated with lower immunoglobulin levels, including IgA, IgM, and IgG, despite a short-term increase in the number of immunoglobulin-secreting cells (Paavonen et al., 1991a). Antiestrogens can also inhibit the rate of DNA synthesis in peripheral blood lymphocytes (Paavonen et al., 1991b). Estrogen enhances B cell maturation (Paavonen et al., 1981), whereas a short TAM incubation reduces C'3 complement receptor expression in B cells (Baral et al., 1985). A TAM-dependent platelet antibody response has been reported that may contribute to the thrombocytopenia that occurs in some patients (Candido et al., 1993).

Several proteins associated with estrogen independence and TAM resistance have recently been identified (Skaar et al., 1998). Autoantibodies to one of these proteins (nucleophosmin; NPM), which is induced by estrogens and inhibited by antiestrogens in estrogen-dependent cells, are produced in breast cancer patients. The levels of anti-NPM autoantibodies increase 6 months before recurrence (Brankin et al., 1998). The levels of other autoantibodies generally do not have substantial predictive and/or prognostic power in breast cancer (Lee et al., 1985; Ronai and Sulitzeanu, 1986). For example, autoantibodies to p53 are detected in a relatively small proportion of breast cancer patients (Schlichtholtz et al., 1992; Mudenda et al., 1994; Vojtesek et al., 1995; Regidor et al., 1996) and appear to be of little predictive/prognostic value (Regidor et al., 1996). Early studies suggesting an association between autoantibody levels and poor prognosis in breast cancer (Wasserman et al., 1975; Turnbull et al., 1978) have not subsequently been confirmed (Swissa et al., 1990).

The levels of anti-NPM autoantibodies are significantly reduced in patients that have received TAM, consistent with the antiestrogenic regulation of the antigen (Brankin et al., 1998). This suggests that monitoring anti-NPM levels could be a useful intermediate biomarker for assessing TAM responses and failures. It seems unlikely that TAM's effects on autoantibodies reflect its ability to influence immunity. TAM does not affect the production of 16/6 idiotype-induced autoantibodies in experimental systemic lupus erythematosus (Sthoeger et al., 1994).

X. Conclusions and Future Prospects

The precise mechanisms of resistance to antiestrogens remain to be established. Clearly, the most important mechanism driving de novo resistance is lack of ER expression, since >90% of ER-negative tumors will not respond to antiestrogens. For ER-positive tumors, it seems likely that no single mechanism predominates for either de novo or acquired resistance. Indeed, each tumor, or each subpopulation within a tumor, may utilize a different resistance mechanism (genomic and/or nongenomic). Nonetheless, some critical event(s) driving response and resistance to TAM are related to activities regulated, at least initially, through the ER signaling pathway(s). This may explain why so few ER-negative tumors respond to antiestrogens, and why a majority of initially responsive tumors acquiring resistance continue to express ER.

With the exception of pharmacokinetic or receptor mutational events, the precise contributions of which remain to be established, defects at, and/or downstream of, receptor-ligand interactions seem important. Modifications in the assembly/function of the ER-regulated transcription complex that drives different gene networks could be involved. The ability of cells to acquire an estrogen-independent phenotype without concurrently acquiring antiestrogen resistance, and the lack of a consistent cross-resistance between triphenylethylenes and steroidal antiestrogens, could reflect the differential regulation of interrelated and/or interdependent gene networks (Clarke and Brünner, 1995; Clarke and Lippman, 1996).

The biophysical events regulating these gene networks could be explained by the conformational changes induced in the ER protein when occupied by different ligands. The physical properties of the ER protein appear associated with its ability to recruit coregulator proteins and regulate reporter gene expression. These properties are dependent upon the occupying ligand and the composition of the transcription complex formed.

Resistance to one class of antiestrogens would not necessarily produce crossresistance to others if the regulated gene networks are interrelated but not interdependent. There may be several pathways that are concurrently influenced by the transcriptional activity of ER occupied by estrogen, but the end result of activation in terms of the choice to proliferate, differentiate, or die may be the same. Thus, cells could switch from one pathway to another as these are selectively blocked by the action of different receptor-ligand complexes (Clarke and Lippman, 1996).

The genes that make up the critical networks pathways involved in antiestrogen responsiveness and resistance may be identified in the next few years. The application of new molecular techniques like serial analysis of gene expression, gene microarray analyses, proteomics, and other state-of-the-art molecular techniques are proving powerful in the identification of molecular patterns associated with specific phenotypes. Already, some novel candidate genes have been identified.

One example is Bcar1/p130Cas. Identified as a putative resistance gene by insertion of a retrovirus into TAM-responsive cells, overexpression of this protein can

produce antiestrogen resistance in ZR-75–1 cells (Brinkman et al., 2000). The protein is clearly expressed in a significant proportion of breast cancers, and there is limited evidence that high levels of this expression are associated with poor response to TAM (van der Flier et al., 2000). Although more studies need to be done to further evaluate the possible contribution of Bcar1/p130Cas to clinical antiestrogen resistance, these studies provide an elegant example of one approach to identify potentially clinically useful molecular information.

The precise contribution of nongenomic effects to TAM's inhibitory effects will probably remain controversial for the moment. A necessary but not sufficient role seems plausible, given the importance of cellular context in determining response to ER activation/inhibition. As our understanding of how antiestrogens affect the function of the ER and its signaling network, this contribution may become more apparent.

Other areas of investigation include searches for endpoints that can predict TAM responders versus nonresponders. These should provide clinically important information because useful second line endocrine and cytotoxic therapies are available for tumors that begin to fail TAM. For example, investigators are looking for serum or other intermediate biomarkers of response/resistance to endocrine therapies. In this regard, changes in the levels of pS2 and apolipoprotein D in nipple aspirate fluids from patients on TAM may have predictive value (Harding et al., 2000). Autoantibodies to the nucleolar phosphoprotein NPM are significantly lower in patients who have received TAM (Brankin et al., 1998). Measuring changes in mammographic density, following initiation of TAM therapy, may also have predictive value (Atkinson et al., 1999).

Additional approaches are to find therapies that may modulate response to antiestrogens. For example, the addition of γ-linoleic acid to TAM may accelerate clinical response (Kenny et al., 2000). This may reflect the ability of polyunsaturated fatty acids to block TAM binding to AEBS (Hoh et al., 1990), which should increase intracellular availability to bind ER. Estrogens can activate telomerase expression through an imperfect ERE (Kyo et al., 1999). Thus, combinations of antiestrogens and telomerase inhibitors may have clinical value. Similarly, the association of increased angiogenesis with TAM resistance suggests that combinations of angiogenesis inhibitors with antiestrogens may be useful.

Our understanding of how the ER works, the complexity of its transcriptional regulatory apparatus, and the importance of cellular context are beginning to change how we think of antiestrogen action and the mechanisms of acquired and de novo resistance. The identification of new selective ER modulators, particularly those with reduced risk of increasing the incidence of endometrial carcinomas, also holds considerable promise for the development of new antiestrogen-based therapies. The pace of change in this field continues to increase, and has every prospect of providing exciting new developments in our ability to improve and refine antiestrogen-based therapeutic strategies for breast cancer.

Acknowledgments. This work was supported in part by Grants NIH R01-CA/AG58022, NIH P30-CA51008, and NIH P50-CA58185 (Public Health Service), and USAMRMC (Department of Defense) BC980629, BC980586, and BC990358.

REFERENCES

Aamdal S, Bormer O, Jorgensen O, Host H, Eilassen G, Kaalhus O and Pihl A (1984) Estrogen receptor and long term prognosis in breast cancer. *Cancer* **53:**2525–2529.

Acero R, Polentarutti N, Bottazzi B, Alberti S, Ricci MR, Bizzi A and Mantovani A (1984) Effect of hydrocortisone on the macrophage content, growth and metastasis of transplanted murine tumors. *Int J Cancer* **33:**95–105.

Adamo ML, Shao ZM, Lanau F, Chen JC, Clemmons DR, Roberts CT Jr, LeRoith D and Fontana JA (1992) Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs): IGFBP-2, -3 and -4 gene expression and protein secretion in a breast cancer cell line. *Endocrinology* **131:**1858–1866.

Akashi M, Osawa Y, Koeffler HP and Hachiya M (1999) $p21^{waf1}$ expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: Important role of RNA stabilization. *Biochem J* **337:**607–616.

Akimoto M, Ishii H, Nakajima Y and Iwasaki H (1986) Assessment of host immune response in breast cancer patients. *Cancer Detect Prev* **9:**311–317.

Albert JL, Boyle JP, Roberts JA, Challiss RA, Gubby SE and Boarder MR (1997) Regulation of brain capillary endothelial cells by P2 receptors coupled to Ca^{2+}, phospholipase C and mitogen-activated protein kinase. *Br J Pharmacol* **122:**935–941.

Albertini MR, Gibson DF, Robinson SP, Howard SP, Tans KJ, Robinson RR, Tormey DC, Jordan VC and Sondel PM (1992) Influence of estradiol and tamoxifen on susceptibility of human breast cancer cell lines to lysis by lymphokine-activated killer cells. *J Immunother* **11:**30–39.

Amundadottir LT and Leder P (1998) Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. *Oncogene* **16:**737–746.

An T, Sood U, Pietruk T and Cummings G (1987) In situ quantitation of inflammatory mononuclear cells in ductal infiltrating breast carcinoma. *Am J Pathol* **128:**52–60.

Anderson E, Vinson GP, Puddefoot JR, Raven PW and Gilmore OJA (1986) Steroid sequestration and tightly bound estrogen-protein complexes in human breast tumors and a breast cancer cell line. *J Steroid Biochem* **24:**489–495.

Andriole GL, Mule JJ, Hansen CT, Linehan WM and Rosenberg SA (1985) Evidence that lymphokine-activated killer cells and natural killer cells are distinct based on an analysis of congenitally immunodeficient mice. *J Immunol* **135:**2911–2913.

Angel P and Karin M (1991) The role of jun, fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* **1072:**129–157.

Antoniotti S, Maggiora P, Dati C and De Bortoli M (1992) Tamoxifen up-regulates c-*erb*B-2 expression in estrogen-responsive breast cancer cells *in vitro*. *Eur J Cancer* **28:**318–321.

Archer SG, Eliopoulous A, Spandidos D, Barnes D, Ellis IO, Blamey RW, Nicholson RI and Robertson JF (1995) Expression of ras, p21, p53 and c-erbB-2 in advanced breast cancer and response to first line hormone therapy. *Br J Cancer* **72:**1259–1266.

Arnold SF, Obourn JD, Yudt MR, Carter TH and Notides AC (1995) In vivo and in vitro phosphorylation of the human estrogen receptor. *J Steroid Biochem Mol Biol* **52:**159–171.

Aronica SM and Katzenellenbogen BS (1993) Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Mol Endocrinol* **7:**743–752.

Arteaga CL, Coronado E and Osborne CK (1988) Blockade of the epidermal growth factor receptor inhibits transforming growth factor α-induced but not estrogen-induced growth of hormone-dependent human breast cancer. *Mol Endocrinol* **2:**1064–1069.

Arteaga CI and Holt JT (1996) Tissue-targeted antisense c-*fos* retroviral vector inhibits established breast cancer xenografts in nude mice. *Cancer Res* **56:**1098–1103.

Arteaga CL, Kitten L, Coronado E, Jacobs S, Kull F and Osborne CK (1989) Blockade of the type-I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. *J Clin Invest* **84:**1418–1423.

Arteaga CL, Koli KM, Dugger TC and Clarke R (1999) Reversal of tamoxifen resistance of human breast carcinomas *in vivo* with neutralizing anti-transforming growth factor (TGF)-β antibodies involves paracrine mechanisms. *J Natl Cancer Inst* **91:**46–53.

Arteaga CL and Osborne CK (1989) Growth inhibition of human breast cancer cells in vitro with an antibody against the Type I somatomedin receptor. *Cancer Res* **49:**6237–6241.

Atkinson C, Warren R, Bingham SA and Day NE (1999) Mammographic patterns as a predictive biomarker of breast cancer risk: Effect of tamoxifen. *Cancer Epidemiol Biomarkers Prev* **8:**863–866.

Balint E, Grimley PM, Michalic R and Aszalos A (1992) Modulation of the antiproliferative signal of interferon α by tamoxifen in U937 cells. *Cancer Lett* **67:**13–19.

Baral E, Blomgren H, Rotstein S and Virving L (1985) Antiestrogen effects on human blood lymphocyte subpopulations in vitro. *J Clin Lab Immunol* **17:**33–35.

Baral E, Nagy E and Berczi I (1994) Target cells are sensitized for cytotoxic T-

lymphocyte-mediated destruction by estradiol and tamoxifen. *Int J Cancer* **58:**64–68.

Baral E, Nagy E and Berczi I (1995) Modulation of natural killer cell-mediated cytotoxicity by tamoxifen and estradiol. *Cancer* **75:**591–599.

Baral E, Nagy E and Berczi I (1996a) Modulation of lymphokine-activated killer cell-mediated cytotoxicity by estradiol and tamoxifen. *Int J Cancer* **66:**214–218.

Baral E, Nagy E, Kangas L and Berczi I (1996b) Antiestrogens enhance the therapeutic effects of lymphokine-activated killer cells on the P815 murine mastocytoma. *Int J Cancer* **67:**580–585.

Baral E, Nagy E and Berczi I (1997) Combination therapy of the H2712 murine mammary carcinoma with cytotoxic T lymphocytes and anti-estrogens. *Anticancer Res* **17:**3647–3651.

Bates SE, Davidson NHE, Valverius EM, Dickson RB, Freter CE, Tam JP, Kudlow JE, Lippman ME and Salomon S (1988) Expression of transforming growth factor-α and its mRNA in human breast cancer: Its regulation by estrogen and its possible functional significance. *Mol Endocrinol* **2:**543–545.

Batra S and Iosif CS (1996) Elevated concentrations of antiestrogen binding sites in membrane fractions of human ovarian tumors. *Gynecol Oncol* **60:**228–232.

Beatson GT (1896) On the treatment of inoperable cases of carcinoma of the mamma: Suggestions from a new method of treatment, with illustrative cases. *Lancet* **ii:**104–107.

Beex LVAM, Pieters GFFM, Smals AGH, Koenders AJM, Benraad TJ and Kloppenborg PWC (1981) Diethylstilbestrol versus tamoxifen in advanced breast cancer. *N Engl J Med* **304:**1041.

Bei M, Foegh M, Ramwell PR and Clarke R (1996) Specific high affinity binding sites for 17β-estradiol in rat heart smooth muscle cells. *J Steroid Biochem Mol Biol* **58:**83–88.

Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shephard HM and Osborne CK (1993) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* **24:**85–95.

Berrino F, Muti P, Micheli A, Krogh V, Sciajno R, Pisani P, Panico S and Secreto G (1996) Serum sex hormone levels after menopause and subsequent breast cancer. *J Natl Cancer Inst* **88:**291–296.

Berry J, Green BJ and Matheson DS (1987a) Modulation of natural killer cell activity in stage I postmenopausal breast cancer patients on low-dose aminogluthemide. *Cancer Immunol Immunother* **24:**72–75.

Berry J, Green BJ and Matheson DS (1987b) Modulation of natural killer cell activity by tamoxifen in stage I post-menopausal breast cancer. *Eur J Cancer Clin Oncol* **23:**517–520.

Bhimani RS, Troll W, Grunberger D and Frankel K (1993) Inhibition of oxidative stress in HeLa cells by chemopreventive agents. *Cancer Res* **53:**4528–4533.

Bignon E, Pons M, Dore JC, Gilbert J, Ojasoo T, Miquel JF, Raynaud JP and De Paulet AC (1991) Influence of di- and tri-phenylethylene estrogen/antiestrogen structure on the mechanisms of protein kinase C inhibition and activation as revealed by a multivariate analysis. *Biochem Pharmacol* **42:**1373–1383.

Biswas DK, Reddy PV, Pickard M, Makkad B, Pettit N and Pardee AB (1998) Calmodulin is essential for estrogen receptor interaction with its motif and activation of responsive promoter. *J Biol Chem* **273:**33817–33824.

Black MM, Zachrau RE, Hankey BF and Wesley M (1988) Skin window reactivity to autologous breast cancer. An index of prognostically significant cell-mediated immunity. *Cancer* **62:**72–83.

Blanchard DK, Kavanagh JJ, Sinoviks JG and Cavanagh D (1988) Infiltration of IL-2-inducible killer cells in ascitic fluid and pleural effusions of advanced cancer patients. *Cancer Res* **48:**6321–6327.

Bonilla F, Alvarez-Mon M, Merino F and de la Hera A (1988) IL-2 induces cytotoxic activity in lymphocytes from regional axillary nodes of breast cancer patients. *Cancer* **61:**629–634.

Bonney RC, Reed MJ, Davidson K, Beranek PA and James VH (1983) The relationship between 17β-hydroxysteroid dehydrogenase activity and estrogen concentrations in human breast tumors and in normal breast tissue. *Clin Endocrinol* **19:**727–739.

Boorne AJ, Donnelly N and Schrey MP (1998) Differential effects of protein kinase C agonists on prostaglandin production and growth in human breast cancer cells. *Breast Cancer Res Treat* **48:**117–124.

Borg A, Baldetrop B, Ferno M, Killander D, Olsson H, Ryden S and Sigurdsson H (1994) *ERBB2* amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Lett* **81:**137–144.

Borner C, Wyss R, Regazzi R, Eppenberger U and Fabbro D (1987) Immunological quantitation of phospholipid/Ca^{2+}-dependent protein kinase of human mammary carcinoma cells: Inverse relationship to estrogen receptors. *Int J Cancer* **40:**344–348.

Bornfeldt KE, Campbell JS, Koyama H, Argast GM, Leslie CC, Raines EW, Krebs EG and Ross R (1997) The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J Clin Invest* **100:**875–885.

Boyd S (1900) On oophorectomy in cancer of the breast. *Br Med J* **ii:**1161–1167.

Brandes JJ (1984) A diphenylmethane derivative selective for the anti-estrogen binding site may help to define its biological role. *Biochem Biophys Res Comm* **124:**244–249.

Brankin B, Skaar TC, Trock BJ, Berris M and Clarke R (1998) Autoantibodies to numatrin: An early predictor for relapse in breast cancer. *Cancer Epidemiol Biomarkers Prev* **7:**1109–1115.

Bratherton DG, Brown CH, Buchanan R, Hall V, Kingsley Pillers EM and Williams CJ (1984) A comparison of two doses of tamoxifen (Nolvadex) in postmenopausal women with advanced breast cancer: 10 mg b.d. versus 20 mg b.d. *Br J Cancer* **50:**199–205.

Brinkman A, van der Flier S, Kok EM and Dorssers LC (2000) Bcar1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. *J Natl Cancer Inst* **92:**112–120.

Bronzert DA, Greene GL and Lippman ME (1985) Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY 117018. *Endocrinology* **117:**1409–1417.

Browder TM, Dunbar CE and Nienhuis AW (1989) Private and public autocrine loops in neoplastic cells. *Cancer Cells* **1:**9–17.

Brünner N, Boulay V, Fojo A, Freter C, Lippman ME and Clarke R (1993a) Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res* **53:**283–290.

Brünner N, Frandsen TL, Holst-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME and Clarke R (1993b) MCF7/LCC2: A 4-hydroxytamoxifen resistant human breast cancer variant which retains sensitivity to the steroidal antiestrogen ICI 182,780. *Cancer Res* **53:**3229–3232.

Brünner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SAW and Clarke R (1997) MCF7/LCC9: An antiestrogen resistant MCF-7 variant where acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early crossresistance to the non-steroidal antiestrogen tamoxifen. *Cancer Res* **57:**3486–3493.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson J-A and Carlquist M (1997) Molecular basis of agonism and antagonism in the estrogen receptor. *Nature (Lond)* **389:**753–758.

Buckely MMT and Goa KL (1989) Tamoxifen: A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic use. *Drugs* **37:**451–490.

Bulun SE and Simpson ER (1994) Breast cancer and expression of aromatase in breast adipose tissue. *Trends Endocrinol Metab* **5:**113–120.

Bunone G, Briand PA, Miksicek RJ and Picard D (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J* **15:**2174–2183.

Burgering BMT, de Vries-Smits AMM, Medema RH, van Weeren P, Tertoolen LGJ and Bos JL (1993) Epidermal growth factor induces phosphorylation of extracellular signal-regulated kinase 2 via multiple pathways. *Mol Cell Biol* **13:**7248–7256.

Butler WB, Berlinski PJ, Hillman RM, Kelsey WH and Toenniges MM (1986) Relation of *in vitro* properties to tumorigenicity for a series of sublines of the human breast cancer cell line MCF-7. *Cancer Res* **46:**6339–6348.

Butler WB and Fontana JA (1992) Responses to retinoic acid of tamoxifen-sensitive and -resistant sublines of human breast cancer cell line MCF-7. *Cancer Res* **52:**6164–6167.

Cabot MC, Zhang Z-C, Cao HT, Lavie Y, Giuliano AE, Han TY and Jones RC (1997) Tamoxifen activates cellular phospholipase C and D and elicits protein kinase C translocation. *Int J Cancer* **70:**567–574.

Cabot MC, Zhang Z-C and Giuliano AE (1995) Tamoxifen elicits rapid transmembrane lipid signal responses in human breast cancer cells. *Breast Cancer Res Treat* **36:**299–306.

Cameron DA, Keen JC, Dixon JM, Bellamy C, Hanby A, Anderson TJ and Miller WR (2000) Effective tamoxifen therapy of breast cancer involves both antiproliferative and pro-apoptotic changes. *Eur J Cancer* **36:**845–851.

Candido A, Bussa S, Tartaglione R, Mancini R, Rumi C, Rossi P and Bizzi B (1993) Tamoxifen-induced immune-mediated platelet destruction. A case report. *Tumori* **79:**231–234.

Canney PA, Griffiths T, Latief TN and Priestman TJ (1989) Clinical significance of tamoxifen withdrawal response. *Lancet* **1:**36.

Carlomagno C, Perrone F, Gallo C, De Laurentiis M, Lauria R, Morabito A, Pettinato G, Panico L, D'Antonio A, Bianco R and De Placido S (1996) c-*erb*B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J Clin Oncol* **14:**2702–2708.

Castano E, Vorojeikina DP and Notides AC (1997) Phosphorylation of serine-167 on the human estrogen receptor is important for estrogen response element binding and transcriptional activation. *Biochem J* **326:**149–157.

Cattoretti G, Andreola S, Clemente C, D'Amato L and Rilke F (1988) Vimentin and P53 expression in epidermal growth factor receptor-positive estrogen receptor-negative breast carcinomas. *Br J Cancer* **57:**353–357.

Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL and Rogan EG (1997) Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc Natl Acad Sci USA* **94:**10937–10942.

Chan CM, Lykkesfeldt AE, Parker MG and Dowsett M (1999) Expression of nuclear receptor interacting proteins TIF-1, SUG-1, receptor interacting protein 140, and corepressor SMRT in tamoxifen-resistant breast cancer. *Clin Cancer Res* **5:**3460–3467.

Chang H, Riese DJ, Gilbert W, Stern DF and McMahan UJ (1997) Ligands for ErbB-family receptors encoded by a neuregulin-like gene. *Nature (Lond)* **387:**509–512.

Chen RH, Su YH and Chang TY (1998) Suppression of transforming growth factor-β-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. *Oncogene* **17:**1959–1968.

Chen TK, Smith LM, Gebhart DK, Birrer MJ and Brown PO (1996) Activation and inhibition of the AP-1 complex in human breast cancer cells. *Mol Carcinogenesis* **15:**215–226.

Chiappetta C, Kirkland JL, Loose-Mitchell DS, Murthy L and Stancel GM (1992) Estrogen regulates expression of the *jun* family of protooncogenes in the uterus. *J Steroid Biochem Mol Biol* **41:**113–123.

Clark GM and McGuire WL (1988) Steroid receptors and other prognostic factors in primary breast cancer. *Semin Oncol* **15:**20–25.

Clarke R (1996) Human breast cancer cell line xenografts as models of breast cancer: The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. *Breast Cancer Res Treat* **39:**69–86.

Clarke R and Brünner N (1995) Cross resistance and molecular mechanisms in antiestrogen resistance. *Endocr Related Cancer* **2:**59–72.

Clarke R and Brünner N (1996) Acquired estrogen independence and antiestrogen resistance in breast cancer: Estrogen receptor-driven phenotypes? *Trends Endocrinol Metab* **7:**25–35.

Clarke R, Brünner N, Thompson EW, Glanz P, Katz D, Dickson RB and Lippman ME (1989a) The inter-relationships between ovarian-independent growth, antiestrogen resistance and invasiveness in the malignant progression of human breast cancer. *J Endocrinol* **122:**331–340.

Clarke R, Brünner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME and Dickson RB (1989b) Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. *Proc Natl Acad Sci USA* **86:**3649–3653.

Clarke R, Brünner N, Katz D, Glanz P, Dickson RB, Lippman ME and Kern F (1989c) The effects of a constitutive production of TGF-α on the growth of MCF-7 human breast cancer cells in vitro and in vivo. *Mol Endocrinol* **3:**372–380.

Clarke R, Dickson RB and Lippman ME (1992a) Hormonal aspects of breast cancer: Growth factors, drugs and stromal interactions. *Crit Rev Oncol Hematol* **12:**1–23.

Clarke R, Currier S, Kaplan O, Lovelace E, Boulay V, Gottesman MM and Dickson RB (1992b) Effect of P-glycoprotein expression on sensitivity to hormones in MCF-7 human breast cancer cells. *J Natl Cancer Inst* **84:**1506–1512.

Clarke R, Leonessa F, Brunner N and Thompson EW (1996) *In vitro* models of human breast cancer, in *Diseases of the Breast* (Harris JR, Hellman S, Lippman ME and Morrow M eds) pp 245–261, J. B. Lippincott & Co., Philadelphia.

Clarke R and Lippman ME (1992) Antiestrogen resistance: Mechanisms and reversal, in *Drug Resistance in Oncology* (Teicher BA ed) pp 501–536, Marcel Dekker, Inc, New York.

Clarke R and Lippman ME (1996) Mechanism of resistance to antiestrogens and their implications for crossresistance, in *Tamoxifen: Beyond the Antiestrogen* (Kellen JA ed) pp 231–266, Birkhauser, Boston.

Clarke R, van den Berg HW and Murphy RF (1990) Tamoxifen and 17β-estradiol reduce the membrane fluidity of human breast cancer cells. *J Natl Cancer Inst* **82:**1702–1705.

Clemmons DR, Camacho-Hubner C, Coronado E and Osborne KJ (1990) Insulin-like growth factor binding protein secretion by breast carcinoma cell lines: Correlation with estrogen receptor status. *Endocrinology* **127:**2679–2686.

Clerici E, Bergamasco E, Ferrario E and Villa ML (1991) Influence of sex steroids on the antigen-specific primary antibody responses in vitro. *J Clin Lab Immunol* **34:**71–78.

Cobb MH and Goldsmith EJ (1995) How MAP kinases are regulated. *J Biol Chem* **270:**14843–14846.

Cobleigh MA, Norlock FE, Oleske DM and Starr A (1999) Hormone replacement therapy and high S-phase in breast cancer. *J Am Med Assoc* **281:**1528–1530.

Cole MP, Jones CTA and Todd IDH (1971) A new antioestrogenic agent in late breast cancer. An early clinical appraisal of ICI 46474. *Br J Cancer* **25:**270–275.

Collaborative Group on Hormonal Factors in Breast Cancer (1997) Collaborative Group on Hormonal Factors in Breast Cancer—Breast cancer and hormone replacement therapy: Collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. *Lancet* **350:**1047–1059.

Colotta F, Polentarutti N, Sironi M and Mantovani A (1992) Expression and involvement of c-*fos* and c-*jun* protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. *J Biol Chem* **267:**18278–18283.

Contreras OO and Stoliar A (1988) Immunologic changes in human breast cancer. *Eur J Gynecol Oncol* **9:**502–514.

Coopman P, Garcia M, Brünner N, Derocq D, Clarke R and Rochefort H (1994) Antiproliferative and antiestrogenic effects of ICI 164,384 in 4-OH-tamoxifen-resistant human breast cancer cells. *Int J Cancer* **56:**295–300.

Coutts AS and Murphy LC (1998) Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. *Cancer Res* **58:**4071–4074.

Crump M, Sawaka CA, DeBoer G, Buchanan RB, Ingle JN, Forbes J, Meakin JW, Shelley W and Pritchard KI (1997) An individual patient-based meta analysis of tamoxifen versus ovarian ablation as first line endocrine therapy for premenopausal women with metastatic breast cancer. *Breast Cancer Res Treat* **44:**2012–210.

Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnson NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A and Jordan VC (1999) 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* **281:**2189–2197.

Cunha GR (1999) Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. *Cancer* **74:**1030–1044.

Curtis SW, Washburn T, Sewall C, DiAugustine RP, Lindzey J, Couse JF and Korach KS (1996) Physiological coupling of growth factor and steroid receptor signaling pathways: Estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proc Natl Acad Sci USA* **93:**12626–12630.

Custodio JBA, Almeida LM and Madeira VMC (1993a) The anticancer drug tamoxifen induces changes in the physical properties of model and native membranes. *Biochim Biophys Acta Membranes* **123–129**.

Custodio JBA, Almeida LM and Madeira VMC (1993b) The active metabolite hydroxytamoxifen of the anticancer drug tamoxifen induces structural changes in membranes. *Biochem Biophys Acta* **1153:**308–314.

Custodio JBA, Dinis TCP, Almeida LM and Madeira VMC (1994) Tamoxifen and hydroxytamoxifen as intramembraneous inhibitors of lipid peroxidation. Evidence for peroxyl radical scavenging activity. *Biochem Pharmacol* **47:**1989–1998.

Daidone MG, Luisi A, Martelli G, Benini E, Veneroni S, Tomasic G, De Palo G and Silvestrini R (2000) Biomarkers and outcome after tamoxifen treatment in node-positive breast cancers from elderly women. *Br J Cancer* **82:**270–277.

D'Angelo G, Lee H and Weiner RI (1997) cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking raf activation. *J Cell Biochem* **67:**353–346.

Daniel P, Gaskell SJ, Bishop H, Campbell C and Nicholson I (1981) Determination of tamoxifen and biologically active metabolites in human breast tumors and plasma. *Eur J Cancer Clin Oncol* **17:**1183–1189.

Dati C, Antoniotti S, Taverna D, Perroteau I and De Bortoli M (1990) Inhibition of c-erbB-2 oncogene expression by estrogens in human breast cancer cells. *Oncogene* **5:**1001–1006.

Datta R, Kojima H, Yoshida K and Kufe DW (1997) Caspase-3-mediated cleavage of protein kinase C θ in induction of apoptosis. *J Biol Chem* **272:**20317–20320.

Dauvois S, Danielian PS, White R and Parker MG (1992) Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci USA* **89:**4037–4041.

Davidson NE, Gelmann EP, Lippman ME and Dickson RB (1987) Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol Endocrinol* **1:**216–223.

Davies GC, Huster WJ, Lu Y, Plouffe L and Lakshmanan M (1999) Adverse events reporter by postmenopausal women in controlled trials with raloxifene. *Obstet Gynecol* **93:**558–565.

Day BW, Tyurin VA, Tyurina YY, Liu M, Facey JA, Carta G, Kisin ER, Dubey RK and Kagan VE (1999b) Peroxidase-catalyzed pro- versus antioxidant effects of 4-hydroxytamoxifen: Enzyme specificity and biochemical sequelae. *Chem Res Toxicol* **12:**28–37.

Day RM, Cioce V, Breckenridge D, Castagnino P and Bottaro DP (1999a) Differential signaling by alternative HGF isoforms through c-met: Activation of both MAP kinase and PI 3-kinase pathways is insufficient for mitogenesis. *Oncogene* **18:** 1399–1406.

de Cremoux P, Martin EC, Vincent-Salomon A, Dieras V, Barbaroux C, Liva S, Pouillart P, Sastre-Garau X and Magdelenat H (1999) Quantitative PCR analysis of c-erb B-2 (HER2/neu) gene amplification and comparison with p185 (HER2/neu) protein expression in breast cancer drill biopsies. *Int J Cancer* **83:**157–161.

de Jong PC, van de Ven J, Nortier HW, Maitimu-Smeele I, Donker TH, Thijssen JH, Slee PH and Blankenstein RA (1997) Inhibition of breast cancer tissue aromatase activity and estrogen concentrations by the third-generation aromatase inhibitor vorozole. *Cancer Res* **57:**2109–2111.

De Larco JE and Todaro GJ (1978) Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci USA* **75:**4001–4005.

de Launoit Y, Dauvois S, Dufour M, Simard J and Labrie F (1991) Inhibition of cell cycle kinetics and proliferation by the androgen 5α-dihydrotestosterone and antiestrogen N, n-butyl-N-methyl-11-[16′α-chloro-3′,17β-dihydroxy-estra-1′,3′, 5′-(10′)triene-7′α-yl] undecamide in human breast cancer ZR-75–1 cells. *Cancer Res* **51:**2797–2802.

Decensi A, Robertson C, Rotmensz N, Severi G, Maisonneuve P, Sacchini V, Boyle P, Costa A and Veronesi U (1998) Effect of tamoxifen and transdermal hormone replacement therapy on cardiovascular risk factors in a prevention trial. Italian Chemoprevention Group. *Br J Cancer* **78:**572–578.

Delmas PD, Bjarnson NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, Draper M and Christiansen C (1997) Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N Engl J Med* **337:**1641–1647.

Dennis PA and Rifkin DB (1991) Cellular activation of latent transforming growth factor β requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proc Natl Acad Sci USA* **88:**580–584.

Dhodapkar MV, Ingle JN and Ahmann DL (1995) Estrogen replacement therapy withdrawal and regression of metastatic breast cancer. *Cancer* **75:**43–46.

Dickson RB and Lippman ME (1995) Growth factors in breast cancer. *Endocrine Rev* **16:**559–589.

Dickson RB, McManaway ME and Lippman ME (1987) Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. *Science (Wash DC)* **232:**1540–1543.

Diel P, Smolnikar K and Michna H (1999) The pure antiestrogen ICI 182780 is more effective in the induction of apoptosis and down regulation of BCL-2 than tamoxifen in MCF-7 cells. *Breast Cancer Res Treat* **58:**87–97.

DiGiovanni MP, Carter D, Flynn SD and Stern DF (1996) Functional assay for HER-2/*neu* demonstrates active signalling in a minority of HER-2/*neu*-overexpressing invasive human breast tumors. *Br J Cancer* **74:**802–806.

Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ and Stallcup MR (1998) Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): Multiple motifs with different binding specificities. *Mol Endocrinol* **12:**302–313.

Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Samudio I, Kladde MP, Vyhlidal C and Safe S (1999) Mechanisms of transcriptional activation of bcl-2 gene expression by 17β-estradiol in breast cancer cells. *J Biol Chem* **274:**32099–32107.

Dotzlaw H, Leygue E, Watson PH and Murphy LC (1999) Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: Relationship to steroid receptor status and regulation by progestins. *Cancer Res* **59:**529–532.

Doucas V, Spyrou G and Yaniv M (1991) Unregulated expression of c-jun or c-fos proteins but not jun D inhibits oestrogen receptor activity in human breast cancer derived cells. *EMBO J* **10:**2237–2245.

Dowsett M (1997) Future use of aromatase inhibitors in breast cancer. *J Steroid Biochem Mol Biol* **61:**261–266.

Dowsett M, Johnston SRD, Iveson TJ and Smith IE (1995) Response to specific anti-oestrogen (ICI182780) in tamoxifen-resistant breast cancer. *Lancet* **345:**525.

Drafta D, Priscu A, Neacsu E, Gangura M, Schindler AE, Stroe E, Anghel C and Panaitescu G (1983) Estradiol and progesterone receptor levels in human breast cancer in relation to cytosol and plasma estrogen level. *J Steroid Biochem* **18:**459–463.

Duh JL, Yu R, Jiao JJ, Matwyshyn GA, Li W, Tan TH and Kong AN (1997) Activation of signal transduction kinases by tamoxifen. *Pharm Res* **14:**186–189.

Dumont JA, Bitoni AJ, Wallace CD, Baumann RJ, Cashman EA and Cross-Doersen DE (1996) Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity. *Cell Growth Diff* **7:**351–359.

Duthie SJ, Melvin WT and Burke MD (1995) Drug toxicity mechanisms in human hepatoma HepG2 cells: Cyclosporin A and tamoxifen. *Xenobiotica* **25:**1151–1164.

Duval D, Durant S and Homo-Delarche F (1983) Non-genomic effects of steroids.

Interactions of steroid molecules with membrane structures and functions. *Biochim Biophys Acta* **737:**409–442.
EBCTCG (1992) Early Breast Cancer Trialists Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. *Lancet* **399:**1–15.
EBCTCG (1998) Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: An overview of the randomized trials. *Lancet* **351:**1451–1467.
Ebert EC, Roberts AI, Devereaux D and Nagase H (1990) Selective immunosuppressive action of a factor produced by colon cancer cells. *Cancer Res* **50:**6158–6161.
Edery M, Goussard J, Dehennin L, Scholler R, Reiffsteck J and Drosdowsky MA (1981) Endogenous estradiol-17β concentration in breast tumors determined by mass fragmentography and by radioimmunoassay: Relationship to receptor content. *Eur J Cancer* **17:**115–120.
El-Ashry D, Miller DL, Kharbanda S, Lippman ME and Kern FG (1997) Constitutive raf-1 kinase activity in breast cancer cells induces both estrogen-independent growth and apoptosis. *Oncogene* **15:**423–435.
El Tanani MK and Green CD (1996) Insulin/IGF-1 modulation of the expression of two estrogen-induced genes in MCF-7 cells. *Mol Cell Endocrinol* **121:**29–35.
El-Tanani MK and Green CD (1997) Interaction between estradiol and growth factors in the regulation of specific gene expression in MCF-7 human breast cancer cells. *J Steroid Biochem Mol Biol* **60:**269–276.
El-Tanani MK and Green CD (1998) Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Mol Endocrinol* **11:**928–937.
Elledge RM, Green S, Ciocca D, Pugh R, Allred DC, Clark GM, Hill J, Ravdin P, O'Sullivan J, Martino S and Osborne CK (1998) HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: A Southwest Oncology Group study. *Clin Cancer Res* **4:**7–12.
Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ and Joyce MJ (1978) Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. *Cancer Res* **38:**3352–3364.
Enmark E and Gustafsson J-A (1998) Estrogen receptor β—A novel receptor opens up new possibilities for cancer diagnosis and treatment. *Endocr Related Cancer* **5:**213–222.
Etienne MC, Milano G, Fischcel JL, Frenay M, Francois E, Formento JL, Gioanni J and Namer M (1989) Tamoxifen metabolism: Pharmacokinetic and in vitro study. *Br J Cancer* **60:**30–35.
Fargin A, Bayard F, Faye JC, Traore M, Poirot M, Klaebe A and Perie JJ (1988) Further evidence for a biological role of antiestrogen binding sites in mediating the growth inhibitory action of diphenylmethane derivatives. *Chem Biol Interactions* **66:**101–109.
Farhat MY, Abi-Younes S, Dingaan B, Vargas R and Ramwell PW (1996) Estradiol increases cAMP in rat pulmonary vascular smooth muscle cells by a nongenomic mechanism. *J Pharmacol Exp Ther* **276:**652–657.
Fawell SE, White R, Hoare S, Sydenham M, Page M and Parker MG (1990) Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. *Proc Natl Acad Sci USA* **87:**6883–6887.
Faye J-C, Jozan S, Redeuilh G, Beaulieu EE and Bayard F (1983) Physicochemical and genetic evidence for specific antiestrogen binding sites. *Proc Natl Acad Sci USA* **80:**3158–3162.
Fenig E, Wieder R, Paglin S, Wang H, Persaud R, Haimovitz-Friedman A, Fuks Z and Yahalom J (1997) Basic fibroblast growth factor confers growth inhibition and mitogen-activated protein kinase activation in human breast cancer cells. *Clin Cancer Res* **3:**135–142.
Ferguson AT, Lapidus AG, Baylin SB and Davidson NE (1995) Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. *Cancer Res* **55:**2279–2283.
Ferlini C, Scambia G, Marone M, Distefano M, Gaggini C, Ferrandina G, Fattorossi A, Isola G, Panici PB and Mancuso S (1999) Tamoxifen induces oxidative stress and apoptosis in oestrogen receptor- negative human cancer cell lines. *Br J Cancer* **79:**257–263.
Ferrazzi E, Cartei G and Matarazzo R (1977) Estrogen-like effect of tamoxifen on vaginal epithelium. *Lancet* **1:**1351–1352.
Fidler IJ (1988) Eradication of cancer metastasis by tumoricidal macrophages. *Adv Exp Med Biol* **233:**415–423.
Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov M, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L and Wolmark N (1998) Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* **90:**1371–1388.
Fishman J, Nisselbaum JS, Menendez-Botet C and Schwartz MK (1977) Estrone and estradiol content in human breast tumors: Relationship to estradiol receptors. *J Steroid Biochem* **8:**893–896.
Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH and Schultz GS (1984) Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progestin receptor levels. *Cancer Res* **44:**3448–3453.
Flury N, Eppenberger U and Mueller H (1997) Tumor-necrosis factor-α modulates mitogen-activated protein kinase activity of epidermal-growth-factor-stimulated MCF-7 breast cancer cells. *Eur J Biochem* **249:**421–426.
Fontana JA, Miksis G, Miranda DM and Durham JP (1987) Inhibition of human mammary carcinoma cell proliferation by retinoids and intracellular cAMP-elevating compounds. *J Natl Cancer Inst* **78:**1107–1112.
Frazier-Jessen MR and Kovacs EJ (1995) Estrogen modulation of JE/monocyte chemoattractant protein-1 mRNA expression in murine macrophages. *J Immunol* **154:**1838–1845.
Frey RS and Mulder KM (1997a) TGF-β regulation of mitogen-activated protein kinases in human breast cancer cells. *Cancer Lett* **117:**41–50.
Frey RS and Mulder KM (1997b) Involvement of extracellular signal-regulated kinase 2 and stress-activated protein kinase Jun N-terminal kinase activation by transforming growth factor beta in the negative growth control of breast cancer cells. *Cancer Res* **57:**628–633.
Fritz G, Just I and Kaina B (1999) Rho GTPases are over-expressed in human tumors. *Int J Cancer* **81:**682–687.
Fromson JM and Sharp DS (1974) The selective uptake of tamoxifen by human uterine tissue. *J Obstet Gynecol Br Commonwealth* **81:**321–323.
Furlanetto RW and DiCarlo JN (1984) Somatomedin C receptors and growth effects in human breast cancer cells maintained in long term tissue culture. *Cancer Res* **44:**2122–2128.
Gabelman BM and Emerman JT (1992) Effects of estrogen, epidermal growth factor, and transforming growth factor-α on the growth of human breast epithelial cells in primary culture. *Exp Cell Res* **201:**113–118.
Gagliardi A and Collins DC (1993) Inhibition of angiogenesis by antiestrogens. *Cancer Res* **53:**533–535.
Garcia JJ, Reiter RJ, Oritz GG, Oh CS, Tang L, Yu BP and Escames G (1998) Melatonin enhances tamoxifen's ability to prevent the reduction in microsomal membrane fluidity induced by lipid peroxidation. *J Membr Biol* **162:**59–65.
Garnett T, Studd JW, Henderson A, Watson N, Savvas M and Leather A (1990) Hormone implants and tachyphylaxis. *Br J Obstet Gynaecol* **97:**917–921.
Gasparini G, Barbareschi M, Doglioni C, Palma PD, Mauri FA, Boracchi P, Bevilacqua P, Caffo O, Morelli L and Verderio P (1995) Expression of bcl-2 protein predicts efficacy of adjuvant treatments in operable node-positive breast cancer. *Clin Cancer Res* **1:**189–198.
Gasparini G, Fox SB, Verderio P, Bonoldi E, Bevilacqua P, Boracchi P, Dante S, Marubini E and Harris AL (1996) Determination of angiogenesis adds information to estrogens receptor status in predicting the efficacy of adjuvant tamoxifen in node-negative breast cancer patient. *Clin Cancer Res* **2:**1191–1198.
Gee JM, Barroso AF, Ellis IO, Robertson JF and Nicholson RI (2000) Biological and clinical associations of c-jun activation in human breast cancer. *Int J Cancer* **89:**177–186.
Gee JMW, Ellis IO, Robertson JFR, Willsher PC, McClelland RA, Hewitt KN, Blamey RW and Nicholson RI (1995) Immunocytochemical localization of fos protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. *Int J Cancer* **64:**269–273.
Gee JMW, Willsher PC, Kenney FS, Robertson JF, Pinder SE, Ellis IO and Nicholson RI (1999) Endocrine response and resistance in breast cancer: A role for the transcription factor fos. *Int J Cancer* **84:**54–61.
Gopalakrishna R, Gundimeda U, Fontana JA and Clarke R (1999) Differential distribution of protein phosphatase 2A in human breast carcinoma cell lines and its relation to estrogen receptor status. *Cancer Lett* **136:**143–151.
Gordge PC, Clegg RA and Miller WR (1995) Ca^{2+}-dependent and Ca^{2+}-independent protein kinase C activity in human breast biopsies. *Biochem Soc Trans* **23:**449S.
Gottardis MM, Wagner RJ, Borden EC and Jordan VC (1989) Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Res* **49:**4765–4769.
Greaves P, Goonetilleke R, Nunn G, Topham J and Orton T (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res* **53:**3919–3924.
Greenberg DA, Carpenter CL and Messing RO (1987) Calcium channel antagonist properties of the antineoplastic antiestrogen tamoxifen in the PC12 neurosecretory cell line. *Cancer Res* **47:**70–74.
Grese TA, Sluka JP, Bryant HU, Cullinan GJ, Glasebrook AL, Jones CD, Matsumoto K, Palkowitz AD, Sato M, Termine JD, Winter MA, Yang NN and Dodge JA (1997) Molecular determinants of tissue selectivity in estrogen receptor modulators. *Proc Natl Acad Sci USA* **94:**14105–14110.
Grimm EA, Mazumder A, Zhang HZ and Rosenberg SA (1982) Lymphokine-activated killer cell phenomena. Lysis of natural killer resistant solid tumour cells by IL-2 activated autologous human peripheral blood lymphocytes. *J Exp Med* **155:**1823–1827.
Gu Q, Korach KS and Moss RL (1999) Rapid action of 17β-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. *Endocrinology* **140:**660–666.
Gundimeda U, Chen Z-H and Gopalakrishna R (1996) Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells. *J Biol Chem* **271:**13504–13514.
Guvakova MA and Surmacz E (1997) Tamoxifen interfers with the insulin-like growth factor-I receptor (IGF-IR) signaling pathway in breast cancer cells. *Cancer Res* **57:**2606–2610.
Hafner F, Holler E and von Angerer E (1996) Effect of growth factors on estrogen receptor mediated gene expression. *J Steroid Biochem Mol Biol* **58:**385–393.
Hanna N and Schneider M (1983) Enhancement of tumor metastases and suppression of natural killer cell activity by β-estradiol treatment. *J Immunol* **130:**974–980.
Haran EF, Maretzek AF, Goldberg I, Horowitz A and Degani H (1994) Tamoxifen enhances cell death in implanted MCF-7 breast cancer by inhibiting endothelium growth. *Cancer Res* **54:**5511–5514.
Harding C, Osundeko O, Tetlow L, Faragher EB, Howell A and Bundred NJ (2000) Hormonally-regulated proteins in breast secretions are markers of target organ sensitivity. *Br J Cancer* **82:**354–360.
Harper MJK and Walpole AL (1967) A new derivative of triphenylethylene: Effect on implantation and mode of action in rats. *J Reprod Fertil* **13:**101–119.
Head JF, Elliott RL and McCoy JL (1993) Evaluation of lymphocyte immunity in breast cancer patients. *Breast Cancer Res Treat* **26:**77–88.
Heinzel T, Lavinsky RM, Mullen T-M, Soderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK and Rosenfeld MG (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature (Lond)* **387:**43–48.
Helle SI, Holly JM, Tally M, Hall K, Vander Stappen J and Lonning PE (1996a) Influence of treatment with tamoxifen and change in tumor burden on the IGF-system in breast cancer patients. *Int J Cancer* **69:**335–339.
Helle SI, Anker GB, Tally M and Lonning PE (1996b) Influence of droloxifene on plasma levels of insulin-like growth factor (IGF)-I, pro-IGF-IIE, insulin-like

growth factor binding protein (IGFBP)-1 and IGFBP-3 in breast cancer patients. *J Steroid Biochem Mol Biol* **57:**167–171.

Herman ME and Katzenellenbogen BS (1996) Response-specific antiestrogen resistance in a newly characterized MCF-7 human breast cancer cell line resulting from long-term exposure to trans-hydroxytamoxifen. *J Steroid Biochem Mol Biol* **59:** 121–134.

Herrlinger C, Braungfels M, Fink E, Kinzig M, Metz R, Sorgel F and Vergin H (1992) Pharmacokinetics and bioavailability of tamoxifen in healthy volunteers. *Int J Clin Pharmacol Ther Toxicol* **30:**487–489.

Hibbs JB, Lambert LH and Remington JS (1972) Control of carcinogenesis: A possible role for the activated macrophage. *Science (Wash DC)* **177:**9980–1000.

Hilakivi-Clarke L, Cho E, Raygada M and Kenney N (1997) Alterations in mammary gland development following neonatal exposure to estradiol, transforming growth factorα, and estrogen receptor antagonist ICI 182,780. *J Cell Physiol* **170:**279–289.

Hilakivi-Clarke LA, Arora PK, Sabol MB, Clarke R, Dickson RB and Lippman ME (1992) Alterations in behavior, steroid hormones and natural killer cell activity in male transgenic TGF-α mice. *Brain Res* **588:**97–103.

Ho GH, Ji CY, Phang BH, Lee KO, Soo KC and Ng EH (1998) Tamoxifen alters levels of serum insulin-like growth factors and binding proteins in postmenopausal breast cancer patients: A prospective paired cohort study. *Ann Surg Oncol* **5:**361–367.

Hoh YK, Lim EH, Ooi SO and Kon OL (1990) Fatty acid modulation of antiestrogen action and antiestrogen-binding protein in cultured lymphoid cells. *Experientia* **46:**1032–1037.

Holli K, Isola J and Cuzick J (1997) Hormone replacement therapy and biological aggressiveness of breast cancer. *Lancet* **350:**1704–1705.

Honig SF (1996) Treatment of metastatic disease, in *Diseases of the Breast* (Harris JR, Lippman ME, Morrow M and Hellman S eds) pp 669–734, Lippincott-Raven, Philadelphia.

Horst HA and Horny HP (1987) Characterization and frequency distribution of lymphreticular infiltrates in axillary lymph node metastases of invasive ductal carcinoma of the breast. *Cancer* **60:**3001–3007.

Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS and Tung L (1996) Nuclear receptor coactivators and corepressors. *Mol Endocrinol* **10:**1167–1177.

Horwitz KB, McGuire WL and Pearson OH (1975) Predicting response to endocrine therapy in human breast cancer: A hypothesis. *Science (Wash DC)* **189:**726.

Howell A, DeFriend D, Robertson JFR, Blamey RW and Walton P (1995) Response to a specific antioestrogen (ICI 182,780) in tamoxifen-resistant breast cancer. *Lancet* **345:**29–30.

Howell A, Dodwell DJ, Anderson H and Redford J (1992) Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. *Ann Oncol* **3:**611–617.

Huff KK, Knabbe C, Lindsey R, Kaufman D, Bronzert DA, Lippman ME and Dickson RB (1988) Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. *Mol Endocrinol* **2:**200–208.

Hulka BS and Stark AT (1995) Breast cancer: Cause and prevention. *Lancet* **346:** 883–887.

Hulley S, Grady D, Bush T, Herrington D, Riggs B and Vittinghoff E (1998) Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/Progestin Replacement Study (HRES) Research Group. *J Am Med Assoc* **280:**605–613.

Humphrey LJ, Singla O and Volenec FJ (1980) Immunologic responsiveness of the breast cancer patient. *Cancer* **46:**893–898.

Hunt JS, Miller L and Platt JS (1998) Hormonal regulation of uterine macrophages. *Dev Immunol* **6:**105–110.

Hunyh H, Nickerson TP, Pollack M and Yang X (1996a) Regulation of insulin-like growth factor-I receptor expression by the pure antiestrogen ICI 182,780. *Clin Cancer Res* **2:**2037–2042.

Hunyh H, Yang XF and Pollack M (1996b) A role for insulin-like growth factor binding protein 5 in the antiproliferative action of the antiestrogen ICI 182,780. *Cell Growth Differ* **7:**1501–1506.

Huovinen R, Warri A and Collan Y (1993) Mitotic activity, apoptosis and TRPM-2 mRNA expression in DMBA-induced rat mammary carcinoma treated with antiestrogen toremifene. *Int J Cancer* **55:**685–691.

Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA and Korach KS (1992) Coupling of dual signaling pathways: Epidermal growth factor action involves the estrogen receptor. *Proc Natl Acad Sci USA* **89:**4658–4662.

Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA and Korach KS (1996) Peptide growth factor cross-talk with estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology* **137:**1735–1744.

Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T and McDonnell DP (1999) Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. *Proc Natl Acad Sci USA* **96:**4686–4691.

Ince BA, Montano MM and Katzenellenbogen BS (1994) Activation of transcriptionally inactive human estrogen receptors by cyclic adenosine 3,5′-monophosphate and ligands including antiestrogens. *Mol Endocrinol* **8:**1397–1406.

Ingram DM, Nottage EM, Willcox DL and Roberts A (1990) Estrogen binding and risk factors for breast cancer. *Br J Cancer* **61:**303–307.

Issandou M, Faucher C, Bayard F and Darbon JM (1990) Opposite effects of tamoxifen on in vitro protein kinase C activity and endogenous protein phosphorylation in intact MCF-7 cells. *Cancer Res* **50:**5845–5850.

Ito K, Fetten J, Khalili H, Hadju S, Busch E, Pergolizzi R, Vinciguerra V and Chang MD (1997) Oligoclonality of CD8+ T cells in breast cancer patients. *Mol Med* **3:**836–851.

Iwase H, Omoto Y, Iwata H, Toyama T, Hara Y, Ando Y, Ito Y, Fujii Y and Kobayashi S (1999) DNA methylation analysis at distal and proximal promoter regions of the oestrogen receptor gene in breast cancers. *Br J Cancer* **80:**1982–1986.

Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L and Horwitz KB (1997) The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. *Mol Endocrinol* **11:**693–705.

Jamil A, Croxtall JD and White JO (1991) The effect of anti-estrogens on cell growth and progesterone receptor concentration in human endometrial cancer cells (Ishikawa). *J Mol Endocrinol* **6:**215–221.

Janeway CA, Travers P, Hunt S and Walport M (1997) *Immunobiology: The Immune System in Health and Disease*. Garland Publishing, Inc., New York.

Jeng MH, Shupnik MA, Bender TP, Westin EH, Bandyopadhyay A, Kumar R, Masamura S and Santen RJ (1998) Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. *Endocrinology* **139:**4164–4174.

Jensen BL, Skouv J and Lykkesfeldt AE (1999) Differential regulation of specific genes in MCF-7 and the ICI 182,780-resistant cell line MCF-7182^{R}6. *Br J Cancer* **79:**386–392.

Jiang S-Y, Langan-Fahey SM, Stella AL, McCague R and Jordan VC (1992) Point mutation of estrogen receptor (ER) in the ligand-binding domain changes the pharmacology of antiestrogens in ER-negative human breast cancer cells stably expressing complementary DNAs for ER. *Mol Endocrinol* **6:**2167–2174.

Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J and Lannigan DA (1998a) pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of ser-167. *Mol Cell Biol* **18:**1978–1984.

Joel PB, Traish AM and Lannigan DA (1998b) Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. *J Biol Chem* **273:**13317–13323.

Johnson MR, Valentine C, Basilico C and Mansukhani A (1998) FGF signaling activates STAT1 and p21 and inhibits the estrogen response and proliferation of MCF-7 cells. *Oncogene* **16:**2647–2656.

Johnston SR, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M and Benz CC (1999) Increased activator protein-1 DNA binding and c-jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. *Clin Cancer Res* **5:**251–256.

Johnston SRD, Haynes BP, Smith IE, Jarman M, Sacks NPM, Ebbs SR and Dowsett M (1993) Acquired tamoxifen resistance in human breast cancer and reduced intra-tumoral drug concentration. *Lancet* **342:**1521–1522.

Johnston SRD, Saccanti-Jotti G, Smith IE, Newby J and Dowsett M (1995) Change in estrogen receptor expression and function in tamoxifen-resistant breast cancer. *Endocr Related Cancer* **2:**105–110.

Jonat W (1998) Luteinizing hormone-releasing hormone analogs: The rationale for adjuvant use in premenopausal women with breast cancer. *Br J Cancer* **78 (Suppl 4):**5–8.

Jones LA, Ota DM, Jackson GA, Jackson PM, Kemp K, Anderson DE, McCamant SK and Bauman DH (1987) Bioavailability of estradiol as a marker for breast cancer risk assessment. *Cancer Res* **47:**5224–5229.

Jordan VC, Bain RR, Brown RR, Gordon B and Santos MA (1983) Determination and pharmacology of a new hydroxylated metabolite of tamoxifen observed in patient sera during therapy for advanced breast cancer. *Cancer Res* **43:**1446–1450.

Jordan VC and Robinson SP (1987) Species-specific pharmacology of antiestrogens: Role of metabolism. *Fed Proc* **46:**1870–1874.

Kalkhoven E, Beraldi E, Panno ML, De Winter JP, Thijssen JH and van der Burg B (1996) Growth inhibition by anti-estrogens and progestins in TGF-β- resistant and -sensitive breast-tumor cells. *Int J Cancer* **65:**682–687.

Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270:**16483–16486.

Karnik PS, Kulkarni S, Liu XP, Budd GT and Bukowski RM (1994) Estrogen receptor mutations in tamoxifen-resistant breast cancer. *Cancer Res* **54:**349–353.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D and Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science (Wash DC)* **270:**1491–1494.

Katzenellenbogen BS, Kendra KL, Norman MJ and Berthois Y (1987) Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res* **47:**4355–4360.

Katzenellenbogen BS, Miller AM, Mullick A and Sheen YY (1985) Antiestrogen action in breast cancer cells: Modulation of proliferation and protein synthesis, and interaction with estrogen receptors and additional antiestrogen binding sites. *Breast Cancer Res Treat* **5:**231–243.

Katzenellenbogen BS, Norman MJ, Eckert RL, Pelyz SW and Mangel WF (1984) Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxytamoxifen isomers in MCF-7 human breast cancer cells. *Cancer Res* **44:**112–119.

Kayyali R, Marriott C and Wiseman H (1994) Tamoxifen decreases drug efflux from liposomes: Relevance to its ability to reverse multidrug resistance in cancer cells? *FEBS Lett* **344:**221–224.

Kazlauskas A and Cooper JA (1988) Protein kinase C mediates platelet-derived growth factor-induced tyrosine phosphorylation of p42. *J Cell Biol* **106:**1395–1402.

Kellen JA, Wong ACH, Szalai JP, Gardner HA and Wood EM (1986) Characteristics of a tamoxifen-tolerant breast cancer cell subpopulation. *J Nutr Growth Cancer* **3:**183–187.

Kelley MJ, Lagrange AH, Wagner EJ and Ronnekleiv OK (1999) Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids* **64:**64–75.

Kemp JV, Adam HK, Wakeling AE and Slater R (1983) Identification and biological activity of tamoxifen metabolites in human serum. *Biochem Pharmacol* **32:**2045–2052.

Kenney NJ, Saeki T, Gottardis M, Kim N, Garcia-Morales P, Martin MB, Normanno N, Ciardello F, Day A and Cutler ML (1993) Expression of transforming growth α antisense mRNA inhibits the estrogen-induced production of TGF α and human estrogen-induced proliferation of estrogen-responsive human breast cancer cells. *J Cell Physiol* **156:**497–514.

Kenny FS, Pinder SE, Ellis IO, Gee JM, Nicholson RI, Bryce RP and Robertson JF

(2000) Gamma linolenic acid with tamoxifen as primary therapy in breast cancer. *Int J Cancer* **85:**643–648.

Key ME, Hoyer L, Bucana C and Hanna MG (1982) Mechanisms of macrophage-mediated tumor cytolysis. *Adv Exp Med Biol* **146:**265–311.

Kirii Y, Magarian-Blander J, Alter MD, Kotera Y and Finn OJ (1998) Functional and molecular analysis of T cell receptors used by pancreatic- and breast tumor-(mucin-) specific cytotoxic T cells. *J Immunother* **21:**188–197.

Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Derynck R and Dickson RB (1987) Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* **48:**417–428.

Koga M and Sutherland RL (1987) Epidermal growth factor partially reverses the inhibitory effects of antiestrogens on T47D human breast cancer cell growth. *Biochem Biophys Res Comm* **146:**738–745.

Koli KM, Ramsey TT, Ko Y, Dugger TC, Brattain MC and Arteaga CL (1997) Blockade of transforming growth factor-β signaling does not abrogate antiestrogen-induced growth inhibition of human breast carcinoma cells. *J Biol Chem* **272:**8296–8302.

Kopp A, Jonat W, Schmahl M and Knabbe C (1995) Transforming growth factor beta 2 (TGF-β 2) levels in plasma of patients with metastatic breast cancer treated with tamoxifen. *Cancer Res* **55:**4512–4515.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson J-A (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* **93:**5925–5939.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S and Gustafsson J-A (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors *α and β. Endocrinology* **138:**863–870.

Kumar V, Green S, Stack G, Berry M, Jin JR and Chambon P (1987) Functional domains of the human estrogen receptor. *Cell* **51:**941–951.

Kumar R, Mandal M, Lipton A, Harvey H and Thompson CB (1996) Overexpression of HER2 modulates Bcl-2 and BCL-X_L, and tamoxifen-induced apoptosis human MCF-7 breast cancer cells. *Clin Cancer Res* **2:**1215–1219.

Kurebayashi J, McLeskey SW, Johnson MD, Lippman ME, Dickson RB and Kern FG (1993) Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and LacZ. *Cancer Res* **53:**2178–2187.

Kurebayashi J, Otsuki T, Yamamoto S, Kurosumi M, Nakata T, Akinaga S and Sonoo H (1998) A pure antiestrogen, ICI 182,780, stimulates the growth of tamoxifen-resistant KPL-1 human breast cancer cells in vivo but not in vitro. *Oncology* **55:**23–34.

Kyo S, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, Orimo A and Inoue M (1999) Estrogen activates telomerase. *Cancer Res* **59:**5917–5921.

Kyprianou N, English HF, Davidson NE and Isaacs JT (1991) Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res* **51:**162–166.

L'Allemain G, Sturgill TW and Weber MJ (1991) Defective regulation of mitogen-activated protein kinase activity in a 3T3 cell variant mitogenically nonresponsive to tetradeconyl phorbol acetate. *Mol Cell Biol* **11:**1002–1008.

Lacal JC, Fleming TP, Warren BS, Blumberg PM and Aaronson SA (1987) Involvement of functional protein kinase C in the mitogenic response to the H-*ras* oncogene product. *Mol Cell Biol* **7:**4146–4169.

Lacassagne A (1932) Apparition de cancers de la mammelle chez la souris male a des injections de folliculine. *C R Acad Sci (Paris)* **195:**639–632.

Lavie Y, Zhang Z-C, Cao HT, Han TY, Jones RC, Liu YY, Jarman M, Hardcastle IR, Giuliano AE and Cabot MC (1998) Tamoxifen induces selective membrane association of protein kinase C epsilon in MCF-7 human breast cancer cells. *Int J Cancer* **77:**928–932.

Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen T-M, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG and Rose DW (1998) Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* **95:**2920–2925.

Leake RE, George WD, Godfrey D and Rinaldi F (1988) Regulation of epidermal growth factor receptor synthesis in breast cancer cells. *Br J Cancer* **58:**521.

Lee AV, Jackson JGG, Hilsenbeck SGC, Osborne CK and Yee D (1999) Enhancement of insulin-like growth factor signaling in human breast cancer: Estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo. *Mol Endocrinol* **13:**787–796.

Lee AV, Weng CN, Jackson JG and Yee D (1997) Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J Endocrinol* **152:**39–47.

Lee Y-TM, Sheikh KMA, Quismorio FP and Friou GJ (1985) Circulating anti-tumor and autoantibodies in breast carcinoma: Relationship to stage and prognosis. *Breast Cancer Res Treat* **6:**57–65.

Leek RD, Landers RJ, Harris AL and Lewis CE (1999) Necrosis correlates with high vascular density and focal macrophage infiltraion in invasive carcinoma of the breast. *Br J Cancer* **79:**991–995.

Leitner G, Landsman T, Blum O, Zaltsmann N and Heller ED (1996) Effects of gonadal steroids and their antagonists on the humoral immune response of immune-selected broiler chickens. *Poult Sci* **75:**1373–1382.

LeJeune S, Leek R, Horak E, Plowman G, Greenall M and Harris AL (1993) Amphiregulin, epidermal growth factor receptor, and estrogen receptor expression in human primary breast cancer. *Cancer Res* **53:**3597–3602.

Lenaz G, Curatola G, Mazzanti L and Parenti-Castelli G (1978) Biophysical studies on agents affecting the state of membrane lipids: Biochemical and pharmacological implications. *Mol Cell Biochem* **22:**3–32.

Leonessa F, Jacobson M, Boyle B, Lippman J, McGarvey M and Clarke R (1994) The effect of tamoxifen on the multidrug resistant phenotype in human breast cancer cells: Isobologram, drug accumulation and gp-170 binding studies. *Cancer Res* **54:**441–447.

Leonessa F, Kemp D, Green G and Clarke R (1998) Effect of gp170 expression on human breast cancer cell growth inhibition by the antiestrogen ICI 182,780. *Proc Am Assoc Cancer Res* **39:**218.

Levinson AS and Jordan VC (1997) MCF-7 the first hormone-responsive breast cancer cell line. *Cancer Res* **57:**3071–3078.

Leygue E, Dotzlaw H, Watson PH and Murphy LC (1998) Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. *Cancer Res* **58:**3197–3201.

Liehr JG and Ricci MR (1996) 4-Hydroxylation of estrogens as a marker of human mammary tumors. *Proc Natl Acad Sci USA* **93:**3294–3296.

Lien EA and Lonning PE (2000) Selective estrogen receptor modifiers (SERMs) and breast cancer therapy. *Cancer Treat Rev* **26:**205–227.

Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S and Ueland PM (1989) Distribution of 4-hydroxy-N-desmethyltamoxifen and their tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res* **49:**2175–2183.

Lien EA, Wester K, Lonning PE, Solheim E and Ueland PM (1991) Distribution of tamoxifen and metabolites into brain tissue and brain metastases in breast cancer patients. *Br J Cancer* **63:**641–645.

Lim JS, Frenkel K and Troll W (1992) Tamoxifen suppresses tumor promoter-induced hydrogen peroxide formation by human neutrophils. *Cancer Res* **52:**4969–4972.

Lin L and Hwang PL (1991) Antiproliferative effects of oxygenated sterols: Positive correlation with binding affinities for the antiestrogen-binding sites. *Biochim Biophys Acta* **1082:**177–184.

Lindner DJ and Borden EC (1997) Effects of tamoxifen and interferon-β or the combination on tumor-induced angiogenesis. *Int J Cancer* **71:**456–461.

Lingham RB, Stancel GM and Loose-Mitchel DS (1988) Estrogen regulation of epidermal growth factor messenger ribonucleic acid. *Mol Endocrinol* **2:**230–235.

Lippman ME, Dickson RB, Kasid A, Gelmann E, Davidson N, McManaway M, Huff K, Bronzert D, Bates S, Swain S and Knabbe C (1986) Autocrine and paracrine growth regulation of human breast cancer. *J Steroid Biochem* **24:**147–154.

Liu JF, Issad T, Chevet E, Ledoux D, Courty J, Caroulee JP, Barritault D, Crepin M and Bertin B (1998) Fibroblast growth factor-2 has opposite effects on human breast cancer MCF-7 cell growth depending on the activation level of the mitogen-activated protein kinase pathway. *Eur J Biochem* **258:**271–276.

Liu Y, El-Ashry D, Chen D, Ding IYF and Kern FG (1995) MCF-7 breast cancer cells overexpressing transfected *c-erb*B-2 have an *in vitro* growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity *in vivo. Breast Cancer Res Treat* **34:**97–117.

Lonning PE, Dowsett M and Powles TJ (1989a) Treatment of breast cancer with aromatase inhibitors—Current status and future prospects. *Br J Cancer* **60:**5–8.

Lonning PE, Johannesson DC and Thoresen T (1989b) Alterations in the production and clearance rate of estrone and estrone sulfate in breast cancer patients treated with aminoglutethimide. *Br J Cancer* **60:**107–111.

Lonning PE, Dowsett M and Powles TJ (1990) Postmenopausal estrogen biosynthesis and metabolism: Alterations caused by aromatase inhibitors used for the treatment of breast cancer. *J Steroid Biochem* **35:**355–366.

Lonning PE, Lien EA, Lundgren S and Kvinnsland S (1992a) Clinical pharmacokinetics of endocrine agents used in advanced breast cancer. *Clin Pharmacokinet* **22:**327–358.

Lonning PE, Hall K, Aakvaag A and Lien EA (1992b) Influence of tamoxifen on plasma levels of insulin-like growth factor I and insulin-like growth factor binding protein I in breast cancer patients. *Cancer Res* **52:**4719–4723.

Lonning PE, Helle SI, Johannessen DC, Adlercreutz H, Lien EA, Tally M, Ekse D, Fotsis T, Anker GB and Hall K (1995) Relations between sex hormones, sex hormone binding globulin, insulin-like growth factor-I and insulin-like growth binding protein-1 in postmenopausal breast cancer patients. *Clin Endocrinol* **42:**23–30.

Lopes MCF, Vale MGP and Carvalho AP (1990) Ca^{2+}-dependent binding of tamoxifen to calmodulin isolated from bovine brain. *Cancer Res* **50:**2753–2758.

Lopez-Ilasaca M (1998) Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem Pharmacol* **56:**269–277.

Lower EE, Blau R, Gazder P and Stahl DL (1999) The effect of estrogen usage on the subsequent hormone receptor status of primary breast cancer. *Breast Cancer Res Treat* **58:**205–211.

Lu Q, Liu Y, Long BJ, Grigoryev D, Gimbel M and Brodie A (1999) The effect of combining aromatase inhibitors with antiestrogens on tumor growth in a nude mouse model for breast cancer. *Breast Cancer Res Treat* **57:**183–192.

Luconi M, Muratori M, Forti G and Baldi E (1999) Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interfers with progesterone effects. *J Clin Endocrinol Metab* **84:**1670–1678.

Luo W, Sharif TR, Houghton PJ and Sharif M (1997) CGP41251 and tamoxifen selectively inhibit mitogen-activated protein kinase activation and c-fos phosphoprotein induction by substance P in human astrocytoma cells. *Cell Growth Diff* **8:**1225–1240.

Lykkesfeldt AE, Madsen MW and Briand P (1994) Altered expression of estrogen-regulated genes in a tamoxifen-resistant and ICI 164,384 and ICI 182,790 sensitive human breast cancer cell line, MCF-7/TAM^R-1. *Cancer Res* **54:**1587–1595.

Lyon JJ and Watson RJ (1996) Intereference of Myb transactivation activity by a conditional dominant negative protein: Functional interference in a cytotoxic T-cell line results in G1 arrest. *Gene* **182:**123–128.

Ma L, Gauville C, Berthois Y, Degeorges A, Millot G, Martin PM and Calvo F (1998) Role of epidermal growth factor receptor in tumor progression in transformed human mammary epithelial cells. *Int J Cancer* **78:**112–119.

MacCallum J, Cummings J, Dixon JM and Miller WR (2000) Concentrations of tamoxifen and its major metabolites in hormone responsive and resistant breast tumours. *Br J Cancer* **82:**1629–1635.

MacCallum J, Keen JC, Bartlett JM, Thompson AM, Dixon JM and Miller WR (1996) Changes in the expression of transforming growth factor beta mRNA isoforms in patients undergoing tamoxifen therapy. *Br J Cancer* **74:**474–478.

Madigan MP, Troisi R, Potischman N, Dorgan JF, Brinton LA and Hoover RN (1998) Serum hormone levels in relation to reproductive and lifestyle factors in postmenopausal women (United States). *Cancer Causes Control* **9:**199–207.

Maenpaa J, Wiebe V, Wurz G, Koester S, Emshoff V, Seymour R and DeGregorio M

(1994) Reduced tamoxifen accumulation is not associated with stimulated growth in tamoxifen resistance. *Cancer Chemother Pharmacol* **35:**149–152.
Magnusson C, Baron JA, Correia N, Bergstrom R, Adami HO and Persson I (1999) Breast-cancer risk following long-term estrogen- and estrogen-progestin-replacement therapy. *Int J Cancer* **81:**339–344.
Mandeville R, Ghali SS and Chausseau JP (1984) In vitro stimulation of NK activity by an estrogen antagonist (tamoxifen). *Eur J Cancer Clin Oncol* **20:**983–985.
Manni A, Buckwalter E, Etindi R, Kunselma S, Rossini A, Mauger D, Dabbs D and Demers L (1996) Induction of a less aggressive breast cancer phenotype by protein kinase C-α. and -β overexpression. *Cell Growth Diff* **7:**1187–1198.
Mantovani A, Allavena P, Sessa C, Bolis G and Mangioni C (1980) Natural killer activity of lymphoid cells isolated from human ascitic ovarian tumors. *Int J Cancer* **25:**573–582.
Marais R, Light Y, Mason C, Paterson H, Olson MF and Marshall CJ (1998) Requirement of ras-GTP-raf complexes for activation of raf-1 by protein kinase C. *Science (Wash DC)* **280:**109–112.
Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80:**179–185.
Martin MB, Garcia-Morales P, Stoica A, Solomon HB, Pierce M, Katz D, Zhang S, Danielsen M and Saceda M (1995) Effects of 12-*O*-tetradecanolyphorbol-13-acetate on estrogen receptor activity in MCF-7 cells. *J Biol Chem* **270:**25244–25251.
Martin MB, Saceda M and Lindsey RK (1991) Estrogen and progesterone receptors, in *Regulatory Mechanisms in Breast Cancer* (Lippman ME and Dickson RB eds) pp 273–288, Kluwer Academic Publishers, Boston.
Masamura S, Santner SJ, Gimotty P, Georger J and Santen RJ (1997) Mechanism for maintenance of high breast tumor estradiol concentrations in the absence of ovarian function: Role of very high affinity tissue uptake. *Breast Cancer Res Treat* **42:**215–226.
Masamura S, Santner SJ, Heitjan DF and Santen RJ (1995) Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *J Clin Endocrinol Metab* **80:**2918–2925.
Matsui Y, Halter SA, Holt JT, Hogan BL and Coffey RJ (1990) Development of mamary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. *Cell* **61:** 1147–1155.
Maxwell P and van den Berg HW (1999) Changes in the secretion of insulin-like growth factor binding proteins -2 and -4 associated with the development of tamoxifen resistance and estrogen independence in human breast cancer cell lines. *Cancer Lett* **139:**121–127.
Mayal Y, Shiu RPC, Bhaumick B and Bala M (1984) Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T-47D) in culture. *Cancer Res* **44:**5486–5490.
Maynard PV, Brownsey BG and Griffiths K (1978) Estradiol levels in fractions of human breast tumors. *J Endocrinol* **77:**62P–63P.
McDonald CC, Alexander FE, Whyte BW, Forrest AP and Stewart HJ (1995) Cardiac and vascular morbidity in women receiving adjuvant tamoxifen for breast cancer in a randomised trial. The Scottish Cancer Trials Breast Group. *Br Med J* **311:** 977–980.
McKenna NJ, Lanz RB and O'Malley BW (1999) Nuclear receptor coregulators: Cellular and molecular biology. *Endocr Rev* **20:**321–344.
McLeskey SW, Zhang L, El-Ashry D, Trock BJ, Lopez CA, Kharbanda S, Tobias CA, Lorant LA, Hannum RS, Dickson RB and Kern FG (1998) Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are crossresistant *in vivo* to the antiestrogen ICI 182,780 and two aromatase inhibitors. *Clin Cancer Res* **4:**697–711.
Means AR (2000) Regulatory cascades involving calmodulin-dependent protein kinases. *Mol Endocrinol* **14:**4–13.
Mehta RR, Valcourt L, Graves J, Green R and Das Gupta TK (1987) Subcellular concentrations of estrone, estradiol, androstenedione and 17β-hydroxysteroid dehydrogenase (17-β-OH-SDH) activity in malignant and non-malignant human breast tissues. *Int J Cancer* **40:**305–308.
Mermelstein PG, Becker JB and Surmeier DJ (1996) Estradiol reduces calcium current in rat neostriatal neurons via a membrane receptor. *J Neurosci* **16:**595–604.
Mésange F, Sebbar M, Kedjouar B, Capdevielle J, Guillemot J-C, Ferrara P, Bayard F, Delarue F, Faye J-C and Poirot M (1998) Microsomal epoxide hydrolase of rat liver is a subunit of the anti-estrogen-binding site. *Biochem J* **334:**107–112.
Migliaccio S, Bernardini S, Wetsel WC, Korach KS, Faraggiana T and Teti A (1998) Protein kinase C modulates estrogen receptors in differentiated osteoblastic cells in vitro. *Steroids* **63:**352–354.
Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E and Auricchio F (1996) Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J* **15:**1292–1300.
Migliaccio A, Di Domenico M, Green S, de Falco A, Kajtaniak EL, Chambon P and Auricchio F (1989) Phosphorylation on tyrosine of *in vitro* synthesized human estrogen receptor activates its hormone binding. *Mol Endocrinol* **3:**1061–1069.
Migliaccio A, Rotondi A and Auricchio F (1984) Calmodulin-stimulated phosphorylation of 17β-estradiol receptor on tyrosine. *Proc Natl Acad Sci USA* **81:**5921–5925.
Migliaccio S, Wetsel WC, Fox WM, Washburn TF and Korach KS (1993) Endogenous protein kinase-C activation in osteoblast-like cells modulates responsiveness to estrogen and estrogen receptor levels. *Mol Endocrinol* **7:**1133–1143.
Miller WR (1997) Uptake and synthesis of steroid hormones by the breast. *Endocr Related Cancer* **4:**307–311.
Millington DS (1975) Determination of hormonal steroid concentrations in biological extracts by high resolution mass fragmentography. *J Steroid Biochem* **6:**239–245.
Millward TA, Zolnierowicz S and Hemmings BA (1999) Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci* **24:**186–191.
Miner JN, Diamond MI and Yamamoto KR (1991) Joints in the regulatory lattice: Composite regulation by steroid receptor-AP1 complexes. *Cell Growth Diff* **2:**525–530.
Mistry P, Griffiths K and Maynard PV (1986) Endogenous C19-steroids and estradiol levels in human primary breast tumor tissues and their correlation with androgen and estrogen receptors. *J Steroid Biochem* **24:**1117–1125.
Molino A, Micciolo R, Bonetti F, Piubello Q, Corgnati A, Sperotto L, Recaldin E, Spagnolli P, Manfrin E, Bonetti A, Nortilli R, Tomezzoli A, Pollini GP, Modena S and Cetto GL (1997) Prognostic significance of estrogen receptors in 405 primary breast cancers: A comparison of immunohistochemical and biochemical methods. *Breast Cancer Res Treat* **45:**241–249.
Montano MM and Katzenellenbogen BS (1997) The quinone reductase gene: A unique estrogen receptor-regulated gene that is activated by antiestrogens. *Proc Natl Acad Sci USA* **94:**2581–2586.
Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su J-L, Kliewer SA, Lehmann JM and Willson TM (1998) Cloning and characterization of human estrogen receptor β isoforms. *Biochem Biophys Res Comm* **247:**75–78.
Moore JW, Clark GM, Hoare SA, Millis RR, Hayward JL, Quinlan MK, Wang DY and Bulbrook RD (1986) Binding of estradiol to blood proteins and aetiology of breast cancer. *Int J Cancer* **38:**625–630.
Mor G, Yue W, Santen RJ, Gutierrez L, Eliza M, Berstein LM, Harada N, Wang J, Diano S and Naftolin F (1998) Macrophages, estrogen and the microenvironment of breast cancer. *J Steroid Biochem Mol Biol* **67:**403–411.
Morel Y and Barouki R (1999) Repression of gene expression by oxidative stress. *Biochem J* **342:**481–496.
Morley P and Whitfield JF (1995) Effect of tamoxifen on carbachol-triggered intracellular calcium responses in chicken granulosa cells. *Cancer Res* **54:**69–74.
Mosselman S, Polman J and Dijkema R (1996) ERβ: Identification and characterization of a novel human estrogen receptor. *FEBS Lett* **392:**49–53.
Mudenda B, Green JA, Green B, Jenkins JR, Robertson L, Tarunina M and Leinster SJ (1994) The relationship between serum p53 autoantibodies and characteristics of human breast cancer. *Br J Cancer* **69:**1115–1119.
Muhlemann K, Cook LS and Weiss NS (1994) The incidence of hepatocellular carcinoma in US white women with breast cancer after the introduction of tamoxifen in 1977. *Breast Cancer Res Treat* **30:**201–204.
Muller V, Jensen EV and Knabbe C (1998) Partial antagonism between steroidal and nonsteroidal antiestrogens in human breast cancer cell lines. *Cancer Res* **58:**263–267.
Murphy LC, Dotzlaw H, Leygue E, Coutts A and Watson P (1998) The pathophysiological role of estrogen receptor variants in human breast cancer. *J Steroid Biochem Mol Biol* **65:**175–180.
Murphy LC and Sutherland RL (1985) Differential effects of tamoxifen and analogs with nonbasic side chains on cell proliferation in vitro. *Endocrinology* **116:**1071–1078.
Musgrove EA, Wakeling AE and Sutherland RL (1989) Points of action of estrogen antagonists and a calmodulin antagonist within the MCF-7 human breast cancer cell cycle. *Cancer Res* **49:**2398–2404.
Myers MJ and Peterson BH (1985) Estradiol indiced alterations in the immune system. I. Enhancement of IgM production. *Int J Immunopharmacol* **7:**207–213.
Nadal A, Rovira JM, Laribi O, Leon-quinto T, Andreu E, Ripoll C and Soria B (1998) Rapid insulinotropic effect of 17β-estradiol via a plasma membrane receptor. *FASEB J* **12:**1341–1348.
Nawata H, Bronzert D and Lippman ME (1981) Isolation and characterization of a tamoxifen resistant cell line derived from MCF-7 human breast cancer cells. *J Biol Chem* **256:**5016–5021.
Nelson J, Clarke R and Murphy RF (1987) The unoccupied estrogen receptor: Some comments on localization. *Steroids* **48:**121–124.
Newby JC, Johnston SRD, Smith IE and Dowsett M (1997) Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer. *Clin Cancer Res* **3:**1643–1651.
Nguyen T, Naziruddin B, Dintzis S, Doherty GM and Mohanakumar T (1999) Recognition of breast cancer-associated peptides by tumor-reactive, HLA-class I restricted allogeneic cytotoxic T lymphocytes. *Int J Cancer* **81:**607–615.
Nicholson RI, McClelland RA, Gee JM, Manning DL, Cannon P, Robertson JF, Ellis IO and Blamey RW (1994) Epidermal growth factor receptor expression in breast cancer: Association with response to endocrine therapy. *Breast Cancer Res Treat* **29:**117–125.
Nickerson TP, Hunyh H and Pollack M (1997) Insulin-like growth factor binding protein-3 induces apoptosis in MCF-7 breast cancer cells. *Biochem Biophys Res Comm* **240:**690–693.
Niemeyer CC, Persico MG and Adamson ED (1998) Cripto: Roles in mammary cell growth, survival, differentiation and transformation. *Cell Death Differ* **5:**440–449.
Nishizuka Y (1992) Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science (Wash DC)* **258:**607–614.
Nomura Y, Tashiro H and Hisamatsu K (1990) Differential effects of estrogen and antiestrogen on in vitro clonogenic growth of human breast cancers in soft agar. *J Natl Cancer Inst* **82:**1146–1149.
Notides AC, Vorojeikina DP and Notides AC (1997) Phosphorylation of serine-167 on the human estrogen receptor is important for estrogen response element binding and transcriptional activation. *Biochem J* **326:**149–157.
Novak-Hofer I, Kung W, Fabbro D and Eppenberger U (1987) Estrogen stimulates growth mammary tumor cells ZR-75 without activation of the S6 kinase and S6 phosphorylation. Difference from epidermal growth factor and transforming growth factor-α-induced proliferation. *Eur J Biochem* **164:**445–451.
Nuedling S, Kahlert S, Loebbert K, Meyer R, Vetter H and Grohe C (1999) Differential effects of 17β-estradiol on mitogen-activated protein kinase pathways in rat cardiomyocytes. *FEBS Lett* **454:**271–276.
Nuwaysir EF, Dragan YP, Jefcoate CR, Jordan VC and Pitot HC (1995) Effects of tamoxifen administration on the expression of xenobiotic metabolizing enzymes in rat liver. *Cancer Res* **55:**1780–1786.
O'Brian CA, Housey GM and Weinstein IB (1988) Specific and direct binding of protein kinase C to an immobilized tamoxifen analog. *Cancer Res* **48:**3626–3629.
O'Brian CA, Liskamp RM, Solomon DH and Weinstein IB (1986) Triphenylethylenes: A new class of protein kinase C inhibitors. *J Natl Cancer Inst* **76:**1243–1246.
O'Brian CA, Vogel VG, Singletary SE and Ward NE (1989) Elevated protein kinase

C expression in human breast tumor biopsies relative to normal breast tissue. *Cancer Res* **49:**3215–3217.
Okubo T, Nagai F, Ushiyama K, Yokoyama Y, Ozawa S, Kano K, Tomita S, Kubo H and Kano I (1998) DNA cleavage and 8-hydroxydeoxyguanosine formation caused by tamoxifen derivatives in vitro. *Cancer Lett* **122:**9–15.
Olson EN, Burgess R and Staudinger J (1993) Protein kinase C as a transducer of nuclear signals. *Cell Growth Diff* **4:**699–705.
Osborne CK, Coronado E, Allred DC, Wiebe V and DeGregorio M (1991) Acquired tamoxifen resistance: Correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* **83:**1477–1482.
Osborne CK, Coronado EB and Robinson JP (1987) Human breast cancer in athymic nude mice: Cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* **23:**1189–1196.
Osborne CK, Coronado-Heinsohn EB, Hilsenbeck S, McCue BL, Wakeling A, McClelland RA, Manning DL and Nicholson RI (1995) 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.
Osborne CK, Jarman M, McCague R, Coronado E, Hilsenbeck S and Wakeling A (1994) The importance of tamoxifen metabolism in tamoxifen-stimulated breast tumor growth. *Cancer Chemother Pharmacol* **34:**89–95.
Osborne CK, Wiebe VJ, McGuire WL, Ciocca DR and DeGregorio M (1992) Tamoxifen and the isomers of 4-hydroxytamoxifen in tamoxifen resistant tumors from breast cancer patients. *J Clin Oncol* **10:**304–310.
Paavonen T, Andersson LC and Adlercreutz H (1981) Sex hormone regulation of in vitro immune response. Estradiol enhances human B cell maturation via inhibition of suppressor T cells in pokeweed mitogen stimulated cultures. *J Exp Med* **154:**1935–1945.
Paavonen T, Aronen H, Pyrhoenen S, Hajba A and Andersson LC (1991a) The effect of toremifene therapy on serum immunoglobulin levels in breast cancer. *APMIS* **99:**849–853.
Paavonen T, Aronen H, Pyrhoenen S, Hajba A and Andersson LC (1991b) The effects of anti-estrogen therapy on lymphocyte functions in breast cancer patients. *APMIS* **99:**163–170.
Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J-A, Kushner PJ and Scanlan TS (1997) Differential ligand activation of estrogen receptors ER-α and ER-β at AP1 sites. *Science (Wash DC)* **277:**1508–1510.
Palmantier R, Roberts JD, Glasgow WC, Eling TE and Olden K (1996) Regulation of the adhesion of a human breast carcinoma cell line to type IV collagen and vitronectin: Roles for lipoxygenase and protein kinase C. *Cancer Res* **56:**2206–2212.
Pan Z, Radding W, Zhou T, Hunter E, Mountz J and McDonald JM (1996) Role of calmodulin in HIV-potentiated fas-mediated apoptosis. *Am J Pathol* **149:**903–910.
Papa V, Biancamaria G, Clark GM, McGuire WL, Moore D, Fujita-Yamagouchi Y, Riccardo V, Goldfine ID and Pezzino V (1993) Insulin-like growth factor receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Res* **53:**2448–2454.
Parisot JP, Hu XF, DeLuise M and Zalcberg JR (1999) Altered expression of the IGF-1 receptor in a tamoxifen-resistant human breast cancer cell line. *Br J Cancer* **79:**693–700.
Parker MG (1998) Transcriptional activation by estrogen receptors. *Biochem Soc Symp* **63:**45–50.
Pasqualini JR, Chetrite G, Blacker C, Feinstein MC, Delalonde L, Talbi M and Maloche C (1996) Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *J Clin Endocrinol Metab* **81:**1460–1464.
Pasqualini JR, Cortes-Prieto J, Chetrite G, Talbi M and Ruiz A (1997) Concentrations of estrone, estradiol and their sulfates, and evaluation of sulfatase and aromatase activities in patients with breast fibroadenoma. *Int J Cancer* **70:**639–643.
Pasqualini JR, Sumida C and Giambiagi N (1988) Pharmacodynamic and biological effects of anti-estrogens in different models. *J Steroid Biochem* **31:**613–643.
Patrone C, Gianazza E, Santagati S, Agrati P and Maggi A (1998) Divergent pathways regulate ligand-independent activation of ER α in SK-N-BE neuroblastoma and COS-1 renal carcinoma cells. *Mol Endocrinol* **12:**835–841.
Pavlik EJ, Nelson K, Srinivasan S, Powell DE, Kenady DE, DePreist PD, Gallion HH and van Nagell JR (1992) Resistance to tamoxifen with persisting sensitivity to estrogen: Possible mediation by excessive antiestrogen binding site activity. *Cancer Res* **52:**4106–4112.
Pazin MJ and Kadonaga JT (1997) What's up and down with histone deacetylation and transcription. *Cell* **89:**325–328.
Pelech SL and Charest DL (1995) MAP kinase-dependent pathways in cell cycle control. *Prog Cell Cycle Res* **1:**33–52.
Peres R, Betsholtz C, Westermark B and Heldin C-H (1987) Frequent expression of growth factors for mesenchymal cells in human mammary carcinoma cell lines. *Cancer Res* **47:**3425–3429.
Perroteau I, Salomon D, DeBertoli M, Kidwell W, Hazarika P, Pardue R, Dedman J and Tam J (1986) Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. *Breast Cancer Res Treat* **7:**201–210.
Pettersson K, Grandien K, Kuiper GGJM and Gustafsson J-A (1997) Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Mol Endocrinol* **11:**1486–1496.
Pfahl M (1993) Nuclear receptor/AP-1 interaction. *Endocr Rev* **14:**651–658.
Picotto G, Massheimer V and Boland R (1996) Acute stimulation of intestinal calcium influx induced by 17β-estradiol via the cAMP messenger system. *Mol Cell Endocrinol* **119:**129–134.
Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX and Slamon DJ (1995) HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* **10:**2435–2446.
Platet N, Prevostel C, Derocq D, Joubert D, Rochefort H and Garcia M (1998) Breast cancer cell invasiveness: Correlation with protein kinase C activity and differential regulation by phorbol ester in estrogen receptor-positive and -negative cells. *Int J Cancer* **75:**750–756.
Plotkin D, Lechner JJ, Jung WE and Rosen PJ (1978) Tamoxifen flare in advanced breast cancer. *J Am Med Assoc* **240:**2644–2646.
Poirot M, Garnier M, Bayard F, Riviere I, Traore M, Wilson M, Fargin A and Faye J-C (1990) The anti-proliferative propoerties of 4-benzylphenoy ethanamine derivatives are mediated by the anti-estrogen binding site (ABS), whereas the anti-estrogenic effects of trifluopromazine are not. *Biochem Pharmacol* **40:**425–429.
Pollack M (1998) IGF-I physiology and breast cancer. *Recent Res Cancer Res* **152:**63–70.
Pommier RF, Woltering EA, Keenan EJ and Fletcher WS (1999) The mechanism of hormone-sensitive breast cancer progression on antiestrogen therapy. Implications for treatment and protocol planning. *Arch Surg* **122:**1311–1316.
Ponta H, Cato AC and Herrlich P (1992) Interference of pathway specific transcription factors. *Biochim Biophys Acta* **1129:**255–261.
Powles T, Eeles R, Ashley S, Easton D, Chang J and Dowsett M (1998) Interim analysis of the incidence of breast cancer in the Royal Marsden Hospital tamoxifen randomized chemoprevention trial. *Lancet* **352:**98–101.
Prevot V, Croix D, Rialas CM, Poulian P, Fricchione GL, Stefano GB and Beauvillain JC (1999) Estradiol coupling to endothelial nitric oxide stimulates gonadotropin-releasing hormone release from rat median eminence via a membrane receptor. *Endocrinology* **140:**652–659.
Pritchard KI (2000) Current controversies in cancer. *Eur J Cancer* **36:**148–150.
Pyrhonen S, Ellmen J, Vuorinen J, Gershanovich M, Tominaga T, Kaufmann M and Hayes DF (1999) Meta-analysis of trials comparing toremifene with tamoxifen and factors predicting outcome of antiestrogen therapy in postmenopausal women with breast cancer. *Breast Cancer Res Treat* **56:**133–143.
Qian NX, Russell M and Johnson GL (1995) Acetylcholine muscarinic receptor regulation of the ras/raf/Map kinase pathway. *Life Sci* **56:**945–949.
Ram PT, Kiefer T, Silverman M, Song Y, Brown GM and Hill SM (1998) Estrogen receptor transactivation in MCF-7 breast cancer cells by melatonin and growth factors. *Mol Cell Endocrinol* **141:**53–64.
Ramu A, Glaubiger D and Fuks Z (1984) Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by tamoxifen and other triparanol analogs. *Cancer Res* **44:**4392–4395.
Ravdin PM, Fritz NF, Tormey DC and Jordan VC (1988) Endocrine status of premenopausal node-positive breast cancer pateints following adjuvant chemotherapy and long-term tamoxifen. *Cancer Res* **48:**1026–1029.
Razandi M, Pedram A, Greene GL and Levin ER (1999) Cell membrane and nucelar estrogen receptors (ERs) originate from a single transcript: Studies of ER-α and ER-β expressed in Chinese hamster ovary cells. *Mol Endocrinol* **13:**307–319.
Recchione C, Venturelli E, Manzari A, Cavalleri A, Martinetti A and Secreto G (1995) Testosterone, dihydrotestosterone and estradiol levels in postmenopausal breast cancer tissues. *J Steroid Biochem Mol Biol* **52:**541–546.
Reddish M, MacLean GD, Koganty RR, Kan-Mitchell J, Jones V, Mitchell MS and Longnecker BM (1998) Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. *Int J Cancer* **76:**817–823.
Reddy KB, Krueger JS, Kondapaka SB and Diglio CA (1999) Mitogen-activated protein kinase (MAPK) regulates the expression of progelatinase B (MMP-9) in breast epithelial cells. *Int J Cancer* **82:**268–273.
Reddy KB, Yee D, Hilsenbeck SG, Coffey RJ and Osborne CK (1994) Inhibition of estrogen-induced breast cancer cell proliferation by reduction in autocrine transforming growth factor α expression. *Cell Growth Differ* **5:**1275–1282.
Ree AH, Froysa A, Eskild W, Jahnsen T and Hansson V (1990) Biphasic regulation of the messenger ribonucleic acid coding for the estrogen receptor by cyclic adenosine 3′-5′-monophosphate in tumor Leydig cells. *Cancer Res* **50:**1528–1531.
Regidor PA, Regidor M, Callies R and Schindler AE (1996) Detection of p53 autoantibodies in the sera of breast cancer patients with a new recurrence using an ELISA assay. Does a correlation with the recurrence free interval exist? *Eur J Gynaecol Oncol* **17:**192–199.
Revillion F, Bonneterre J and Peyrat JP (1998) *ERBB2* oncogenes ni human breast cancer and its clinical significance. *Eur J Cancer* **34:**791–808.
Rogers A and Eastell R (1998) Effects of estrogen therapy of postmenopausal women on cytokines measured in peripheral blood. *J Bone Miner Res* **13:**1577–1586.
Rohlik QT, Adams D, Kull FC and Jacobs S (1987) An antibody to the receptor for insulin-like growth factor-I inhibits the growth of MCF-7 cells in tissue culture. *Biochem Biophys Res Comm* **149:**276–281.
Ronai Z and Sulitzeanu D (1986) (Auto)antibodies in human breast cancer sera against antigens associated with breast cancer cells, detected by immunoblotting. *J Natl Cancer Inst* **77:**1203–1209.
Ronnov-Jessen L, Petersen OW and Bissell MJ (1996) Cellular changes involved in conversion of normal to malignant breast: Importance of the stromal reaction. *Physiol Rev* **76:**69–125.
Roos W, Oeze L, Loser R and Eppenberger U (1983) Antiestrogenic action of 3-hydroxytamoxifen in the human breast cancer cell line. *J Natl Cancer Inst* **71:**55–59.
Ross EM (1996) Pharmacodynamics: Mechanisms of drug action and the relationship between drug concentration and effect, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Hardman JG, Limbird LE, Molinoff PB, Ruddon RW and Gilman AG eds) pp 29–41, McGraw-Hill, New York.
Rotstein S, Blomgren H, Petrini B, Wasserman J and von Stedingk L-V (1988) Influence of adjuvant tamoxifen on blood lymphocytes. *Breast Cancer Res Treat* **12:**75–79.
Rowlands MG, Budworth J, Jarman M, Hardcastle IR, McCague R and Gescher A (1995) Comparison between inhibition of protein kinase C and antagonism of calmodulin by tamoxifen analogues. *Biochem Pharmacol* **50:**723–726.
Rudolph R (1986) Response of metastatic breast cancer to tamoxifen (TAM) withdrawal. *Proc Am Soc Clin Oncol* **5:**69.
Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR and van Zwieten MJ

Toyokuni S, Okamoto K, Yodoi J and Hiai H (1995) Persistent oxidative stress in cancer. *FEBS Lett* **358:**1–3.

Travers MT, Barrett-Lee PJ, Berger U, Luqmani YA, Gazet J-C, Powles TJ and Coombes RC (1988) Growth factor expression in normal, benign and malignant breast tissue. *Br Med J* **296:**1621.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F and Giguere V (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* **11:**353–365.

Tremblay GB, Tremblay A, Labrie F and Giguere V (1998) Ligand-independent activation of the estrogen receptors α and β by mutations of a conserved tyrosine can be abolished by antiestrogens. *Cancer Res* **58:**877–881.

Tremblay GB, Tremblay A, Labrie F and Giguere V (1999) Dominant activity of activation function 1 (AF-1) and differential stoichiometric requirements for AF-1 and -2 in the estrogen receptor alpha-beta heterodimeric complex. *Mol Cell Biol* **19:**1919–1927.

Trock BJ, Leonessa F and Clarke R (1997) Multidrug resistance in breast cancer: A meta analysis of MDR1/gp170 expression and its possible functional significance. *J Natl Cancer Inst* **89:**917–931.

Trump DL, Smith DC, Ellis PG, Rogers MP, Schold SC, Winer EP, Panella TJ, Jordan VC and Fine RL (1992) High-dose oral tamoxifen, a potential multidrug-resistant- reversal agent: Phase I trial in combination with vinblastine. *J Natl Cancer Inst* **84:**1811–1816.

Tsunoda S, Ikezaki Y and Nagasawa H (1997) Transforming growth factor-α mRNA and epidermal growth factor receptor mRNA expression in normal and neoplastic mammary glands of four strains of mice with different mammary tumor potentials. *Zoolog Sci* **14:**803–807.

Turnbull AR, Turner DTL and Fraser JD (1978) Autoantibodies in early breast cancer: A stage related phenomenon? *Br J Cancer* **38:**461–467.

Tzukerman M, Zhang X-K, Hermann T, Wills KN, Graupner G and Pfahl M (1990) The human estrogen receptor has transcriptional activitor and repressor functions in the absence of ligand. *New Biologist* **2:**613–620.

van den Berg HW, Claffie D, Boylan M, McKillen J, Lynch M and McKibben B (1996) Expression of receptors for epidermal growth factor and insulin-like growth factor-I by ZR-75–1 human breast cancer cell variants is inversely related: The effect of steroid hormones on insulin-like growth factor I receptor expression. *Br J Cancer* **73:**477–481.

van den Berg HW, Leahey WJ, Lynch M, Clarke R and Nelson J (1987) Recombinant human interferon α increases estrogen receptor expression in human breast cancer cells (ZR-75–1) and sensitizes them to the anti-proliferative effects of tamoxifen. *Br J Cancer* **55:**255–257.

van den Berg HW, Lynch M, Martin J, Nelson J, Dickson GR and Crockard AD (1989) Characterization of a tamoxifen-resistant variant of the ZR-75–1 human breast cancer cell line (ZR-75–9a1) and stability of the resistant phenotype. *Br J Cancer* **59:**522–526.

van der Burg B, Isbrucker L, vasn Selm-Miltenburb AJP, de Laat SW and van Zoelen EJJ (1990) Role of estrogen-induced insulin-like growth factors in the proliferation of human breast cancer cells. *Cancer Res* **50:**7770–7774.

van der Burg B, Slager-Davidov R, van der Leede BM, de Laat SW and ven der Saag PT (1995) Differential regulation of AP1 activity by retinoic acid in hormone-dependent and -independent breast cancer cells. *Mol Cell Endocrinol* **112:**143–152.

van der Flier S, Brinkman A, Look MP, Kok EM, Meijer-van Gelder ME, Klijn JG, Dorssers LC and Foekens JA (2000) Bcar1/p130Cas protein and primary breast cancer: Prognosis and response to tamoxifen treatment. *J Natl Cancer Inst* **92:** 120–127.

van Landeghem AA, Poortman J, Nabuurs M and Thijssen JH (1985) Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Res* **45:**2900–2906.

Vermeulen A, Deslypere JP, Paridaens R, LeClercq G, Roy F and Heuson JC (1986) Aromatase, 17β-hydroxysteroid dehydrogenase and intratissular sex hormone concentrations in cancerous and normal glandular breast tissue in postmenopausal women. *Eur J Cancer* **22:**515–525.

Veronesi U, Maisonneuve P, Costa A, Sacchini V, Maltoni C and Robertson C (1998) Prevention of breast cancer with tamoxifen: Preliminary findings from the Italian randomized trial among hysterectomized women. *Lancet* **352:**93–97.

Vignon F, Bouton MM and Rochefort H (1987) Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochem Biophys Res Comm* **146:**1502–1508.

Visser JA and Themmen AP (1998) Downstream factors in transforming growth factor-β family signaling. *Mol Cell Endocrinol* **146:**7–17.

Vladusic EA, Hornby AE, Guerra-Vladusic FK and Lupu R (1999) Expression of estrogen receptor β messenger RNA variant in breast cancer. *Cancer Res* **58:**210–214.

Vogel CL, Shemano I, Schoenfelder J, Gams RA and Green MR (1993) Multicenter phase II efficacy trial of toremifene in tamoxifen-refractory patients with advanced breast cancer. *J Clin Oncol* **11:**345–350.

Vojtek AB and Cooper JA (1999) Rho family members: Activators of MAP kinase cascades. *Cell* **82:**527–529.

Vojtesek B, Kovarik J, Dolezalova H, Nenutil R, Havlis P, Brentani RR and Lane DP (1995) Absence of p53 autoantibodies in a significant proportion of breast cancer patients. *Br J Cancer* **71:**1253–1256.

Wakeling AE and Bowler J (1988) Novel antioestrogens without partial agonist activity. *J Steroid Biochem* **31:**645–653.

Walker RA and Cowl J (1991) The expression of c-*fos* protein in human breast. *J Pathol* **163:**323–327.

Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, Shah AS and Anderson PW (1998) Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. *J Am Med Assoc* **279:**1445–1451.

Wang H, Rubin M, Fenig E, DeBlasio A, Mendelsohn J and Yahalom J (1997) Basic fibroblast growth factor causes growth arrest in MVF-7 human breast cnacer cells while inducing both mitogenic and inhibitory G_1 events. *Cancer Res* **57:**1750–1757.

Wang Q, Maloof P, Wang H, Fenig E, Stein D, Nichols G, Denney TN, Yahalom J and Wieder R (1998) Basic fibroblast growth factor downregulates BCL-2 and promotes apoptosis in MCF-7 human breast cancer cells. *Exp Cell Res* **238:**177–187.

Wang Y and Simonson MS (1996) Voltage-insensitive Ca^{2+} channels and Ca^{2+}/calmodulin-dependent protein kinases propagate signals from endothelin-1 receptors to the c-fos promoter. *Mol Cell Biol* **16:**5915–5923.

Wasserman J, Glas U and Blomgren H (1975) Autoantibodies in patients with carcinoma of the breast. *Clin Exp Immunol* **19:**417–422.

Watanabe T, Inoue S, Ogawa S, Ishii Y, Hiroi H, Ikeda K, Orimo A and Muramatsu M (1997) Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context, and estrogen receptor subtype: Functional difference between estrogen receptors α and β. *Biochem Biophys Res Commun* **236:**140–145.

Watson CS, Norfleet AM, Pappas TC and Gametchu B (1999) Rapid actions of estrogens in GH3/B6 pituitary cells via a plasma membrane version of estrogen receptor-α. *Steroids* **64:**5–13.

Watters JJ, Campbell JS, Cunningham MJ, Krebs EG and Dorsa DM (1997) Rapid membrane effects of steroids in neuroblastoma cells: Effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. *Endocrinology* **138:**4030–4033.

Ways DK, Kukoly CA, de Vente JE, Hooker JL, Bryant WO, Posekany KJ, Fletcher DJ, Cook PP and Parker PJ (1995) MCF-7 breast cancer cells transfected with protein kinase C-α exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. *J Clin Invest* **95:**1906–1915.

Webb P, Lopez GN, Uht RM and Kushner PJ (1995) Tamoxifen activation of the estrogen receptor/AP-1 pathway: Potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* **9:**443–456.

Wei H, Cai Q, Tian L and Lebwohl M (1998) Tamoxifen reduces endogenous and UV light-induced oxidative damage to DNA lipid and protein in vitro and in vivo. *Carcinogenesis* **19:**1013–1918.

Welshons WV, Lieberman ME and Gorski J (1984) Nuclear localization of unoccupied estrogen receptors. *Nature (Lond)* **307:**747–749.

Wenger CR, Beardslee S, Owens MA, Pounds G, Oldaker T, Vendely P, Pandian MR, Harrington D, Clark GM and McGuire WL (1993) DNA ploidy, S-phase, and steroid receptors in more than 127,000 breast cancer patients. *Breast Cancer Res Treat* **28:**9–20.

Wheelock EF and Robinson MK (1983) Endogenous control of the neoplastic process. *Lab Invest* **48:**120–139.

Wiseman H (1994) The antioxidant action of a pure antiestrogen: Ability to inhibit lipid peroxidation compared to tamoxifen and 17β-estradiol and relevance to its anticancer potential. *Biochem Pharmacol* **47:**493–498.

Wiseman H, Paganga G and Halliwell B (1993b) Protective actions of tamoxifen and 4-hydroxytamoxifen against oxidative damage to low-density lipoproteins: A mechanism accounting for the cardioprotective action of tamoxifen? *Biochem J* **292:**635–638.

Wiseman LR, Johnson MD, Wakeling AE, Lykkesfeldt AE, May FEB and Westley BR (1993a) Type I IGF receptor and acquired tamoxifen resistance in estrogen-responsive human breast cancer cells. *Eur J Cancer* **29A:**2256–2264.

Wiseman LR, Wakeling AE, May FEB and Westley BR (1989) Effects of the antiestrogen, ICI 164,384, on estrogen induced RNAs in MCF-7 cells. *J Steroid Biochem* **33:**1–6.

Witters LM, Kumar R, Chinchilli VM and Lipton A (1997) Enhanced anti-proliferative activity of the combination of tamoxifen plus HER-2-neu antibody. *Breast Cancer Res Treat* **42:**1–5.

Wolf DM, Langan-Fahey SM, Parker CP, McCague R and Jordan VC (1993) Investigation of the mechanism of tamoxifen stimulated breast tumor growth; non-isomerizable analogues of tamoxifen and its metabolites. *J Natl Cancer Inst* **85:**806–812.

Woodgett JR, Avruch J and Kyriakis J (1996) The stress activated protein kinase pathway. *Cancer Surv* **27:**127–138.

Wright C, Nicholson S, Angus B, Sainsbury C Jr, Farndon JR, Cairns J, Harris AL and Horne CH (1992) Relationship between c-erbB2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br J Cancer* **65:** 118–121.

Wright S, Schellenberger U, Ji L, Wang H and Larrick JW (1997) Calmodulin-dependent protein kinase II mediates signal transduction to apoptosis. *FASEB J* **11:**843–849.

Xanthoudakis S and Curran T (1996) Redox regulation of AP-1. A link between transcription factor signaling and DNA repair, in *Biological Reactive Intermediates V* (Snyder R ed) pp 69–75, Plenum Press, New York.

Xing C and Imagawa W (1999) Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: Elevated basal activity and sustained response to EGF. *Carcinogenesis* **20:**1201–1208.

Xu J, Qiu Y, DeMayo FJ, Tsai SY and O'Malley BW (1998) Partial hormone resistance in mice with disruption of the steroid receptor co-activator-1 (SRC-1) gene. *Science (Wash DC)* **279:**1922–1925.

Yamauchi H, O'Neill A, Gelman R, Carney W, Tenney DY, Hosch S and Hayes DF (1997) Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-*neu* protein. *J Clin Oncol* **15:**2518–2532.

Yang NN, Venugopalan M, Hardikar S and Glasebrook A (1996) Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. *Science (Wash DC)* **273:**1222–1225.

Yarden RI, Lauber AH, El-Ashry D and Chrysogelos SA (1996) Bimodal regulation of epidermal growth factor receptor by estrogen in breast cancer cells. *Endocrinology* **137:**2739–2747.

Ye Q and Bodell WJ (1996) Production of 8-hydroxy-2′-deoxyguanosine in DNA by microsomal activation of tamoxifen and 4-hydroxytamoxifen. *Carcinogenesis* **17:** 1747–1750.

Yee D, Paik S, Lebovic GS, Marcus R, Favoni RE, Cullen KJ, Lippman ME and Rosen N (1989a) Analysis of insulin-like growth factor-I gene expression in malignancy: Evidence for a paracrine role in human breast cancer. *Mol Endocrinol* **3:**509–517.

(1990) Biology of disease: Comparative study of human and rat mammary tumorigenesis. *Lab Invest* **62:**244–278.
Saceda M, Lippman ME, Chambon P, Lindsey RK, Puente M and Martin MB (1988) Regulation of the estrogen receptor in MCF-7 cells by estradiol. *Mol Endocrinol* **2:**1157–1162.
Sahl B, Marotta A, Matthewson C, Ahluwalia M, Flint J, Owen D and Pelech S (1999) Investigation of the Mek-MAP kinase-Rsk pathway in human breast cancer. *Anticancer Res* **19:**731–740.
Saji S, Jensen EV, Nilsson S, Rylander T, Warner M and Gustafsson JA (2000) Estrogen receptors alpha and beta in the rodent mammary gland. *Proc Natl Acad Sci U S A* **97:**337–342.
Salerno M, Sisci D, Mauro L, Guvakova MA, Ando S and Surmacz E (1999) Insulin receptor substrate 1 is a target for the pure antiestrogen ICI 182,780 in breast cancer cells. *Int J Cancer* **81:**299–304.
Santen RJ, Yue W, Naftolin F, Mor G and Berstein L (1999) The potential of aromatase inhibitors in breast cancer prevention. *Endocr Relat Cancer* **6:**235–243.
Savita S and Rai U (1998) Sex steroid hormones modulate the activation of murine peritoneal macrophages: Receptor mediated modulation. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **119:**199–204.
Schaefer EJ, Lamon-Fava S, Spiegelman D, Dwyer JT, Lichtenstein AH, McNamara JR, Goldin BR, Woods MN, Morrill-LaBrode A and Hertzmark E (1995) Changes in plasma lipoprotein concentrations and composition in response to a low-fat, high-fiber diet are associated with changes in serum estrogen concentrations in premenopausal women. *Metabolism* **44:**749–756.
Schaffer HJ and Weber MJ (1999) Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. *Mol Cell Biol* **19:**2435–2444.
Schairer C, Gail M, Byrne C, Rosenberg PS, Sturgeon SR, Brinton LA and Hoover RN (1999) Estrogen replacement therapy and breast cancer survival in a large screening study. *J Natl Cancer Inst* **91:**264–270.
Schairer C, Lubin J, Troisi R, Sturgeon S, Brinton L and Hoover R (2000) Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk. *J Am Med Assoc* **283:**485–491.
Schguurs AHWM and Verheul HAM (1990) Effects of gender and sex steroids on the immune response. *J Steroid Biochem* **35:**157–172.
Schlichtholtz B, Legros Y, Gillet D, Gaillard C, Marty M, Lane D, Calvo F and Soussi T (1992) The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. *Cancer Res* **52:** 6380–6384.
Schor NF, Tyurina YY, Tyurin VA and Kagan VE (1999) Differential membrane antioxidant effects of immediate and long-term estradiol treatment of MCF-7 breast cancer cells. *Biochem Biophys Res Commun* **260:**410–415.
Schultze-Osthoff K, Los M and Baeuerle PA (1995) Redox cycling by transcription factors NF-kB and AP-1 in lymphocytes. *Biochem Pharmacol* **50:**735–741.
Screpanti I, Santoni A, Gulino A, Herberman RB and Frati L (1987) Estrogen and antiestrogen modulation of mouse natural killer activity and large granular lymphocytes. *Cell Immunol* **106:**191–202.
Seaman WE, Blackman MA, Gindhart TD, Roubinian JR, Loeb JM and Talal N (1978) β-Estradiol reduces natural killer cells in mice. *J Immunol* **121:**2193–2198.
Seaman WE and Talal N (1980) The effect of 17β-estradiol on natural killing in the mouse, in *Natural Cell-Mediated Immunity Against Tumors* (Herberman RB ed) pp 765–777, Academic Press, New York.
Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, Derynck R, Hom YK, Cunha GR and DiAugustine RP (1998) Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Diff* **9:**777–785.
Seibert K, Shafie SM, Triche TJ, Whang-Peng JJ, O'Brien SJ, Toney JH, Huff KK and Lippman ME (1983) Clonal variation of MCF-7 breast cancer cells in vitro and in athymic nude mice. *Cancer Res* **43:**2223–2239.
Seynaeve CM, Stetler-Stevenson M, Sebers S, Kaur G, Sausville EA and Worland PJ (1993) Cell cycle arrest and growth inhibition by the protein kinase antagonist UCN-01 in human breast carcinoma cells. *Cancer Res* **53:**2081–2086.
Shemshedini L, Knauthe R, Sassone-Corsi P and Gronemeyer H (1991) Cell-specific inhibitory and stimulatory effects of fos and jun on transcription activation by nuclear receptors. *EMBO J* **10:**3839–3849.
Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA and Greene GL (1999) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95:**927–937.
Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Kmar R and Santen RJ (2000) Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. *Endocrinology* **141:**396–405.
Silvestrini R, Benini E, Veneroni S, Daidone MG, Tomasic G, Squicciarini P and Salvadori B (1996) p53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. *J Clin Oncol* **14:**1604–1610.
Singer CA, Figueroa-Masot XA, Batchelor RH and Dorsa DM (1999) The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J Neurosci* **19:**2455–2463.
Singh A, Purohit A, Wang DY, Duncan LJ, Ghilchik MW and Reed MJ (1995) IL-6sR: Release from MCF-7 breast cancer cells and a role in regulating peripheral estrogen synthesis. *J Endocrinol* **147:**9–12.
Singh M, Setalo G, Guan X, Warren M and Toran-Allerand CD (1999) Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: Convergence of estrogen and neurotrophin signaling pathways. *J Neurosci* **19:**1179–1188.
Singh RK, Ruh MF, Butler WB and Ruh TS (1986) Acceptor sites on chromatin for receptor bound by estrogen versus antiestrogen in antiestrogen-sensitive and -resistant cells. *Endocrinology* **118:**1087–1097.
Skaar TC, Prasad SC, Sharaeh S, Lippman ME, Brunner N and Clarke R (1998) Two-dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. *J Steroid Biochem Mol Biol* **67:**391–402.
Smeyne RJ, Vendrell M, Hayward M, Baker SJ, Miao G, Schilling K, Robertson LM, Curran T and Morgan JI (1993) Continuous c-*fos* expression precedes programmed cell death *in vivo*. *Nature (Lond)* **363:**166–169.
Smith CL, Nawaz Z and O'Malley BW (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol Endocrinol* **11:**657–666.
Smith CL, Onate SA, Tsai M-J and O'Malley BW (1996) CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proc Natl Acad Sci USA* **93:**8884–8888.
Smith LM, Wise SC, Hendricks DT, Sabichi AL, Bos T, Reddy P, Brown PH and Birrer MJ (1999) cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. *Oncogene* **18:**6063–6070.
Soderstrom M, Vo A, Heinzel T, Lavinsky RM, Yang W-M, Seto E, Peterson DA, Rosenfeld MG and Glass CK (1997) Differential effects of nuclear receptor corepressor (N-CoR) expression levels on retinoic acid receptor-mediated repression support the existence of dynamically regulated corepressor complexes. *Mol Endocrinol* **11:**682–692.
Soule HD, Vasquez J, Long A, Albert S and Brennan M (1973) A human cell line from a pleural effusion derived from a human breast carcinoma. *J Natl Cancer Inst* **51:**1409–1416.
Speirs V, Parkes AT, Kerin MJ, Walton DS, Carelton PJ, Fox JN and Atkin SL (1999a) Coexpression of estrogen receptor α and β: Poor prognostic factors in human breast cancer. *Cancer Res* **59:**525–528.
Speirs V, Malone C, Walton DS, Kerin MJ and Atkin SL (1999b) Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res* **59:**5421–5424.
Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou JX, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai M-J and O'Malley BW (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature (Lond)* **389:**194–198.
Sthoeger ZM, Bentwich Z, Zinger H and Mozes E (1994) The beneficial effects of the estrogen antagonist, tamoxifen, on experimental systemic lupus erythematosus. *J Rheumatol* **21:**2231–2238.
Stoica A, Saceda M, Fakhro A, Joyner M and Martin MB (2000) Role of insulin-like growth factor-I in regulating estrogen receptor-α gene expression. *J Cell Biochem* **76:**605–614.
Strayer DR, Carter WA and Brodsky I (1986) Familial occurrence of breast cancer is associated with reduced natural killer cytotoxicity. *Breast Cancer Res Treat* **7:**187–192.
Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA and Katzenellenbogen BS (1999) Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-α or estrogen receptor-β. *Endocrinology* **140:**800–804.
Swissa M, Amital-Teplizki H, Haim N, Cohen Y and Shoenfeld Y (1990) Autoantibodies in neoplasia. *Cancer* **65:**2554–2558.
Szamel I, Hindy I, Vincze B, Eckhardt S, Kangas L and Hajba A (1994) Influence of toremifene on the endocrine regulation of breast cancer patients. *Eur J Cancer* **30A:**154–158.
Szapary D, Huang Y and Simons SS Jr (1999) Opposing effects of corepressor and coactivators in determining the dose-response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated gene expression. *Mol Endocrinol* **13:**2108–2121.
Szymczak J, Milewicz A, Thijssen JH, Blankenstein MA and Daroszewski J (1998) Concentration of sex steroids in adipose tissue after menopause. *Steroids* **63:**319–321.
Tai H, Kubota N and Kato S (2000) Involvement of nuclear receptor coactivator SRC-1 in estrogen-dependent cell growth of MCF-7 cells. *Biochem Biophys Res Commun* **267:**311–316.
Takahashi N and Breitman TR (1989) Covalent binding of 17β-estradiol and retinoic acid to proteins in the human breast cancer cell line MCF-7. *In Vitro Cell Dev Biol* **25:**1199–1200.
Tang BL, Teo CC, Sim KY, Ng ML and Kon OL (1989) Cytostatic effects of antiestrogens in lymphoid cells: Relationship to high affinity antiestrogen-binding sites and cholesterol. *Biochim Biophys Acta* **1014:**162–172.
Tang Z, Treilleux I and Brown M (1997) A transcriptional enhancer required for the differential expression of the human estrogen receptor in breast cancers. *Mol Cell Biol* **17:**1274–1280.
Tari AM, Hung MC, Li K and Lopez-Berenstein G (1999) Growth inhibition of breast cancer cells by Grb2 downregulation is correlated with inactivation of mitogen-activated protein kinase in EGFR, but not in ErbB2, cells. *Oncogene* **18:**1325–1332.
Taylor SG, Gelman RS, Falkson G and Cummings FJ (1986) Combination chemotherapy compared to tamoxifen as initial therapy for stage IV breast cancer in elderly women. *Ann Intern Med* **104:**455–461.
Teo CC, Kon OL, Sim KY and Ng SC (1992) Synthesis of 2-(*p*-chlorobenzyl)-3-aryl-6-methoxybenzofurans as selective ligands for antiestrogen binding sites. Effects on cell proliferation and cholesterol synthesis. *J Med Chem* **35:**1330–1339.
Teodorczyk-Injeyan J, Cembryzynska M, Lalani S and Kellen JA (1993) Modulation of biological responses of normal human mononuclear cells by antiestrogens. *Anticancer Res* **13:**279–284.
Thomas HV, Reeves GK and Key TJ (1997) Endogenous estrogen and postmenopausal breast cancer: A quantitative review. *Cancer Causes Control* **8:**922–928.
Thompson EW, Brünner N, Torri J, Johnson MD, Boulay V, Wright A, Lippman ME, Steeg PS and Clarke R (1993) The invasive and metastatic properties of hormone-independent and hormone-responsive variants of MCF-7 human breast cancer cells. *Clin Exp Metastasis* **11:**15–26.
Thompson EW, Katz D, Shima TB, Wakeling AE, Lippman ME and Dickson RB (1989) ICI 164,384: A pure antagonist of estrogen-stimulated MCF-7 cell proliferation and invasiveness. *Cancer Res* **49:**6929–6934.
Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, Strax P and Pasternack BS (1995) A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J Natl Cancer Inst* **87:**190–197.

Yee D, Favoni R, Lupu R, Cullen KJ, Lebovic GS, Huff KK, Lee PDK, Powell DR, Dickson RB, Rosen N and Lippman ME (1989b) The insulin-like growth factor binding protein BP-25 is expressed by human breast cancer cells. *Biochem Biophys Res Comm* **158**:38–44.

Zeleniuch-Jacquotte A, Toniolo P, Levitz M, Shore RE, Koenig KL, Banerjee S, Strax P and Pasternack BS (1995) Endogenous estrogens and risk of breast cancer by estrogen receptor status: A prospective study in postmenopausal women. *Cancer Epidemiol Biomarkers Prev* **4**:857–860.

Zhang GJ, Kimijima I, Onda M, Kanno M, Sato H, Watanabe T, Tsuchiya A, Abe R and Takenoshita S (1999) Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X(L), without alteration of p53 protein levels. *Clin Cancer Res* **5**:2971–2977.

Zhang L, Kharbanda S, Chen D, Bullocks J, Miller DL, Ding IY, Hanfelt J, McLeskey SW and Kern FG (1997) MCF-7 breast carcinoma cells overexpressing FGF-1 form vascularized, metastatic tumors in ovariectomized or tamoxifen-treated nude mice. *Oncogene* **15**:2093–2108.

Zhang L, Kharbanda S, Hanfelt J and Kern FG (1998) Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen-resistant growth of MCF-7 breast cancer cells. *Cancer Res* **58**:352–361.

Zhoa Y, Escot C, Maudelonde T, Peuch C, Rouanet P and Rochefort H (1993) Correlation between mannose-6-phosphate/IGF-II receptor and cathepsin D RNA levels by *in situ* hybridization in benign and malignant mammary tumors. *Cancer Res* **53**:2901–2905.

Zhu BT and Conney AH (1998) Functional role of estrogen metabolism in target cells: Review and perspectives. *Carcinogenesis* **19**:1–27.

Zou A, Marschke KB, Arnold KE, Berger EM, Fitzgerald P, Mais DE and Allegretto EA (1999) Estrogen receptor β activates the human retinoic acid receptor α-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to estrogen. *Mol Endocrinol* **13**:418–430.

Zuckerman SH, Bryan-Poole N, Evans GF, Short L and Glasebrook AL (1995) In vivo modulation of murine serum tumor necrosis factor and interleukin-6 levels during endotoxemia by oestrogen agonists and antagonists. *Immunology* **86**:18–24.

0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 4
Printed in U.S.A.

Constitutive Expression of the Steroid Sulfatase Gene Supports the Growth of MCF-7 Human Breast Cancer Cells *in Vitro* and *in Vivo**

MATTIE R. JAMES, TODD C. SKAAR, RICHARD Y. LEE, ALEXANDER MACPHERSON, JAMES A. ZWIEBEL, BALWANT S. AHLUWALIA, FRANKLIN AMPY, AND ROBERT CLARKE

Vincent T. Lombardi Cancer Center, Georgetown University School of Medicine (M.R.J., T.C.S., R.Y.L., A.M.), Washington, D.C. 20007; National Cancer Institute, National Institutes of Health (J.A.Z.), Bethesda, Maryland 20892; and Department of Genetics and Human Genetics, Howard University (B.S.A., F.A., M.R.J.), Washington, D.C. 20059

ABSTRACT

Many human breast tumors are driven by high intratumor concentrations of 17β-estradiol that appear to be locally synthesized. The role of aromatase is well established, but the possible contribution of the steroid sulfatase (STS), which liberates estrogens from their biologically inactive sulfates, has been inadequately assessed and remains unclear. To evaluate the role of STS further, we transduced estrogen-dependent MCF-7 human breast cancer cells with a retroviral vector directing the constitutive expression of the human STS gene. Gene integration was confirmed by Southern hybridization, production of the appropriately sized messenger RNA by Northern hybridization, and expression of functional protein by metabolism of [^{3}H]estrone sulfate to [^{3}H]estrone. Maximum velocity estimates of estrone formation are 64.2 pmol estrone/mg protein·h in STS-transduced cells (STS Clone 20), levels comparable to those seen in some human breast tumors. Lower levels of endogenous activity are seen in MCF-7 cells (13.0 pmol estrone/mg protein·h) and in cells transduced with vector lacking the STS gene (Vector 3 cells; 12.0 pmol estrone/mg protein·h).

17β-Estradiol sulfate induces expression of the progesterone receptor messenger RNA only in STS Clone 20 cells, whereas estrone sulfate produces the greatest stimulation of anchorage-independent growth in these cells. STS Clone 20 cells retain responsiveness to antiestrogens, which block the ability of estrogen sulfate to increase the proportion of cells in both the S and G_2/M phases of the cell cycle. Consistent with these *in vitro* observations, only STS Clone 20 cells exhibit a significant increase in the proportion of proliferating tumors in nude ovariectomized mice supplemented with 17β-estradiol sulfate. The primary activity *in vivo* appears to be from intratumor STS, rather than hepatic STS. Surprisingly, 17β-estradiol sulfate appears more effective than 17β-estradiol when both are administered at comparable concentrations. This effect, which is seen only in STS Clone 20 cells, may reflect differences in the cellular pharmacology of exogenous estrogens compared with those released by the activity of intracellular STS. These studies directly demonstrate that intratumor STS activity can support estrogen-dependent tumorigenicity in an experimental model and may contribute to the promotion of human breast tumors. (*Endocrinology* **142:** 1497–1505, 2001)

ESTROGENS ARE THE most important etiological factors in the growth and development of many breast carcinomas in both pre- and postmenopausal women. Breast tumors from postmenopausal women contain high levels of 17β-estradiol despite the presence of low plasma 17β-estradiol concentrations (1–3). Although breast tumors can clearly accumulate serum 17β-estradiol to concentrations higher than those seen in serum (3–5), 17β-estradiol is a relatively minor serum estrogen in postmenopausal women. It is now widely accepted that breast tumors can synthesize 17β-estradiol from adrenal androgen precursors. This occurs through the aromatization of androstenedione to estrone by aromatase, followed by the conversion of estrone to 17β-estradiol by 17β-hydroxysteroid dehydrogenase type 1 (6). However, breast cancer cells also express both steroid sulfotransferase (7–9) and steroid sulfatase (STS) activities (10, 11). The latter potentially obviate the need to synthesize significant amounts of estrone within some tumors. STS can release estrone from estrone sulfate, which is peripherally synthesized in adipose tissues.

Inhibition of aromatase provides significant benefit to many breast cancer patients (12, 13), establishing its importance in the production of estrogens. Nonetheless, as with other endocrine therapies, a significant proportion of tumors that express estrogen receptors fail aromatase therapy. Inhibition of aromatase activity does not readily discriminate between inhibition of peripheral and intratumoral aromatase and sulfatase activities, because it also should reduce both circulating and intratumor concentrations of estrone.

Although estrone sulfate is the predominant serum estrogen in postmenopausal women, the primary intratumor estrogen is 17β-estradiol (14, 15). Estrogen sulfates have been considered biologically inactive compounds, and the contri-

Received October 3, 2000.

Address all correspondence and requests for reprints to: Robert Clarke, Ph.D., D.Sc., Room W405A, Research Building, Vincent T. Lombardi Cancer Center, Georgetown University School of Medicine, 3970 Reservoir Road NW, Washington, D.C. 20007. E-mail: clarker@gunet.georgetown.edu.

* This work was supported by awards from the American Institute for Cancer Research (90BW65) and the USPHS (F31-CA-63923 to M.R.J., R01-CA/AG-58022, and USAMRMC BC980629 to R.C.). Several aspects of this work were supported by Shared Resources funded through the Lombardi Cancer Center's Cancer Center Support Grant (USPHS P30-CA-51008): Animal Research Resource (to R.C.), Macromolecular Synthesis and Sequencing (to T.C.S.), Tissue Culture (to Dr. Michael Johnson), and Flow Activated Cell Sorting (to Dr. Owen Blair).

bution of serum estrone sulfate to intratumor estrogens remains controversial. Sulfated steroids are not believed to penetrate cell membranes easily because of their polarity (8); the sulfate moiety at the C3 position essentially eliminates their ability to recognize estrogen receptors (16). Nonetheless, estrogenic effects in response to sulfated estrogens have been demonstrated, and desulfation probably occurs rapidly as the estrogen sulfates penetrate the cell membrane (8).

Data from clinical studies indirectly support the importance of serum estrogen sulfates. Two first generation aromatase inhibitors, aminoglutethimide and testololactone (17), reduce aromatase activity to comparable levels, but the clinical response rate to testololactone is much lower (18). However, in addition to its inhibition of aromatase activity, aminoglutethimide significantly increases estrone sulfate clearance (19, 20), an effect not seen with testololactone (17, 18). These data suggest that estrogen sulfates may contribute to intratumor 17β-estradiol concentrations in some human breast tumors.

Evidence that either aromatase or sulfatase expression is useful as a predictive/prognostic marker in breast cancer remains contradictory. Studies have failed to demonstrate that aromatase has any significant power as an independent prognostic indicator for breast cancer outcome (21, 22). STS messenger RNA (mRNA) expression was an independent predictor of recurrence in one study (21), but not in another (23). However, STS is more commonly detected than aromatase expression, being found in up to 90% of breast tumors (23, 23) compared with 60–70% for aromatase (21, 22). Pasqualini *et al.* (3) estimate that STS activity is 50–200 times greater than aromatase activity in both premenopausal and postmenopausal breast tumors. However, activity reflects the combination of maximum velocity (V_{max}), K_m, and substrate availability. The much greater V_{max} of sulfatase in breast tumors may be partly offset by the higher affinity interactions between aromatase and its substrate compared with the affinity of estrone sulfate for STS.

Although supportive, these observations only provide an indirect assessment of a possible role for intratumor STS. For example, it is not known whether the levels of STS activity are sufficient to support the growth of estrogen-dependent cells *in vivo*. We now describe an *in vivo* model, using ovariectomized nude mice supplemented with an estrogen sulfate and bearing STS-transduced human breast cancer cells, to test this hypothesis directly. Our data demonstrate that stable expression of high levels of STS activity can be obtained, and that this is sufficient to support the growth of estrogen-dependent tumors in mice supplemented with 17β-estradiol sulfate. Hepatic metabolism is not a major contributor of estrogen sulfate metabolism in these animals. Thus, our data directly support the hypothesis that STS activity can significantly contribute to the high intratumor 17β-estradiol concentrations seen in the tumors of some premenopausal and postmenopausal breast cancer patients.

Materials and Methods

Cell lines

The estrogen-responsive MCF-7/2 human breast cancer cell line was obtained from the Lombardi Cancer Center's Tissue Culture Shared Resource (provided by Dr. Michael Johnson, Lombardi Cancer Center, Washington, DC). MCF-7/2 is a subline of the parental MCF-7 cells derived by single cell cloning of the parental MCF-7 cells. MCF-7/2 cells are reproducibly estrogen responsive. The production of a high incidence of rapidly growing MCF-7/2 tumors in ovariectomized nude mice and proliferation *in vitro* are estrogen dependent. Thus, MCF-7/2 cells were routinely grown in Improved MEM containing phenol red (Biofluids, Rockville, MD) and supplemented with 5% FBS.

Estrogens are retained for prolonged periods in breast cancer cells *in vitro* (24). Thus, where experiments required either that cells be grown in the absence of estrogens and/or estrogenic supplementation, we first applied a rigorous stripping regimen to remove endogenous steroids (25). Briefly, cells were extensively washed and then maintained in Improved MEM without phenol red (Biofluids) and supplemented with 5% calf serum stripped of endogenous estrogens by treatment with dextran-coated charcoal and STS (26). Cell culture prepared in this manner contains less than 10 fM 17β-estradiol (27). Monolayers were washed with the stripped medium three times on the first day, twice on the second day, and once on the third day. All further treatments, *e.g.* with estrogens or sulfated estrogens, began on the fourth day (25).

Transduction of complementary DNAs (cDNAs) into MCF-7/2 cells

The full-length sulfatase cDNA was obtained from American Type Culture Collection (Manassas, VA). The LXSN retroviral expression vector contains appropriate cloning sites placing the cDNA of interest downstream of the 5′-long terminal repeat, with a constitutively expressed neomycin resistance gene as the selectable marker. A 2.4-kb STS cDNA was ligated into the *Eco*RI site of the LXSN vector (28) using T4 DNA ligase (Promega Corp.). The ligated product was stably transfected into GP+E86 packaging cell line (29) by the calcium phosphate coprecipitation method (30). Cells were grown to confluence, and the supernatant was collected. A transinfection with the viral supernatant was performed using the PA317β packaging cell line (31). Cells were selected, and individual colonies were collected and expanded, in Improved MEM containing phenol red and 600 μg/ml G418 and supplemented with 10% FBS (selection medium). Titration of the recombinant retrovirus stock showed that the highest titer was 8×10^5 colony-forming units/ml. This supernatant was tested for the helper virus using a standard helper virus detection assay protocol and was free of the helper virus. MCF-7/2 cells were infected by exposure to the virus, and G418-resistant colonies were isolated. Single cell clones, derived from these resistant colonies, were expanded for further study in Improved MEM containing phenol red and supplemented with 5% FBS. This estrogenic environment precluded the selection for either estrogen-independent or estrogen-supersensitive cells, which require prolonged estrogen deprivation both *in vivo* (32) and *in vitro* (32, 33). Clones transduced with retroviral vectors containing the STS genes were designated STS Clone #, *e.g.* STS Clone 20; those transduced with the vector but lacking the STS gene were designated Vector#, *e.g.* Vector 3.

Nucleic acid probes

The STS probes for Southern and Northern hybridizations were prepared using 25 ng of a 2.4-kb STS cDNA labeled with [^{32}P]deoxy-ATP (Amersham Pharmacia Biotech, Arlington Heights, IL) by random priming (34). Radiolabeled probes were purified by chromatography on a Quick Spin Column, Sephadex G-50 (Roche Molecular Biochemicals, Indianapolis, IN). To control for RNA loading on Northern hybridization analyses, the blots were probed with a radiolabeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe. For ribonuclease (RNase) protection analyses, a progesterone receptor (PgR) riboprobe was used that produces a 240-bp protected fragment (35). The pS2 riboprobe protects a 300-bp fragment, whereas the 36B4 riboprobe (loading control) (36) produces a 220-bp protected fragment (35).

Southern and Northern hybridizations and RNase protection studies

Genomic DNA was isolated from cultured cells by the DNAzol method (Life Technologies, Inc., Gaithersburg, MD). Total RNA was obtained using the TRIzol reagent method (Life Technologies, Inc.). Southern hybridizations were performed using standard techniques

(34). Northern hybridizations and RNase protection studies were performed as previously described (30, 37). RNA loading controls were GAPDH (Northern hybridizations) and 36B4 (RNase protection analyses). Where appropriate, phosphorimage analyses were performed on a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Isolation of subcellular fractions

Cellular homogenates of transduced clones, vector, and wild-type MCF-7/2 cells were prepared by the method of MacIndoe *et al.* (38). Cells were harvested by scraping and centrifuged at 2000 × *g* for 5 min, the cell pellets were allowed to swell on ice for 10 min in ice-cold 1.5 mM $MgCl_2$, and the cells were disrupted with a Dounce homogenizer. An equal volume of 0.04 M Tris-HCl buffer, pH 6.5, was added, and the homogenate was centrifuged at 200,000 × *g* for 30 min. Microsomal fractions (pellet) were washed once in 1 ml 0.02 M Tris-HCl buffer, pH 6.5, and further spun at 2000 × *g* for 5 min. These pellets were resuspended in Tris-HCl buffer, sonicated, and stored at −20 C until used.

STS biochemical assay

We used a modified method of MacIndoe *et al.* (38) to measure activity in cells growing either *in vitro* or *in vivo*. For *in vivo* tissues, tumors were obtained at necropsy, immediately frozen, and stored at −80 C until used. Samples were diluted to the appropriate protein concentration, *i.e.* each protein sample contained 0.05–0.15 mg protein/100 μl, and 100 μl were added to reaction buffer (200 μl 0.02 M Tris-HCl, pH 6.5). Next, 100 μl Tris-HCl buffer, pH 6.5, containing 0.4 nM [^{3}H]estrone sulfate (SA, 49 Ci/mmol; NEN Life Science Products, Boston, MA), diluted with a 100-fold excess of unlabeled estrone sulfate (Sigma, St. Louis, MO), were added to each tube to obtain the required final molarity. After incubation for 1 h, the reaction was terminated by placing the samples on ice for 10 min. To measure unconjugated radiolabeled metabolites, 100 μl of each sample were mixed with 5 ml of a highly nonpolar scintillation fluid (666 ml dioxane, 330 ml xylene, 80 g naphthalene, and 5 g 2,5-diphenyloxazole), the samples were vortexed, and radioactivity was measured by scintillation spectrometry. Subsequently, 5 ml dH_2O were added, each sample was vortexed, and scintillation spectrometry was repeated to assess radioactivity incorporated into the polar metabolites.

Cell cycle analyses

Cell monolayers were grown at 3 × 10^6 cells/T-75 cm^2 flasks and stripped of endogenous steroids for 3 days with extensive washing, as described above. After the 3-day stripping procedure, cells were treated with estrogen with or without antiestrogen (17β-estradiol 3-sulfate, 1 nM; ICI 182,780, 100 nM; tamoxifen 1 μM) in Improved MEM without phenol red-free medium and supplemented with 5% stripped calf serum. Cell cycle distribution was measured 48 h later. Briefly, 10^6 cells were suspended in a citrate buffer (250 mM sucrose, 40 mM trisodium-citrate, and 5% dimethylsulfoxide), and cell cycle analysis was performed using standard techniques (39) in the Lombardi Cancer Center Flow Cytometry Resource with a FACStar flow cytometer (Becton Dickinson and Co., Palo Alto, CA).

Anchorage-independent growth

Anchorage-independent colony formation was performed as previously described (26). Briefly, cells were stripped of endogenous steroids (25), 4 × 10^6 cells were suspended in 0.5 ml Improved MEM without phenol red-free medium and supplemented with 5% stripped calf serum, then added to a mixture containing 1.5 ml 1.2% agar (Difco, Detroit, MI) solution and 0.5 ml of treatment/vehicle solution. The suspension was poured onto a layer of solidified agar and incubated for 14 days at 37 C in a humidified 5% CO_2/95% air atmosphere. Colonies of 50 cells or more (≥60 μm in diameter) were counted using an Omicron 3600 image analysis system (Artek, Farmingdale, NY). Five replicates were made for each sample.

In vivo tumor growth

Ovariectomized nude mice were used as a model because these animals have serum estrogen levels similar to those observed in postmenopausal women (40, 41). Although many breast tumors can convert estrone to 17β-estradiol, we were concerned that the level of 17β-hydroxysteroid dehydrogenase type 1 activity in the STS Clone 20 and Vector 3 cells might be low. Consequently, we used 17β-estradiol sulfate rather than estrone sulfate for the *in vivo* studies. This approach should ensure that any failure to support tumorigenicity could be essentially attributed to an inability of STS activity to generate biologically relevant intratumor 17β-estradiol concentrations. To control for the possible contribution of hepatic STS activities, mice received STS Clone 20 cells on one flank and control Vector 3 cells on the opposite flank.

Cells growing *in vitro* were used for the xenograft inocula. Briefly, subconfluent monolayers (80%) were removed by gentle scraping, the cell suspensions were spun for 5 min at 1000 × *g*, and the pellets were resuspended in growth medium. Cell viability was estimated by trypan blue dye exclusion, and 2 × 10^6 viable cells were sc inoculated into the right and left flanks of 6- to 8-week-old, specific pathogen-free, ovariectomized, athymic, NCr-*nu/nu* nude mice (Taconic Farms, Germantown, NY). Estrogens were administered as sc implants of 60-day release pellets (Innovative Research of America, Sarasota, FL). The 17β-estradiol sulfate pellets were custom made by Innovative Research of America using 17β-estradiol 3-sulfate (Sigma). Mice received STS Clone 20 cells in one flank and Vector 3 cells in the opposite flank. Body weights were obtained on each group of animals twice weekly. The response to 17β-estradiol sulfate was determined by measuring tumor incidence, a standard end point for many *in vivo* studies (42, 43). Tumor incidence was defined as the proportion of proliferating tumors, *i.e.* those tumors that consistently increased in size throughout the study. To facilitate this determination, tumor size was recorded weekly.

Statistical analyses

Lineweaver-Burke transformations were fitted by simple least square linear regression, and the 99% confidence interval for each fit was estimated. These analyses were performed using the algorithms in SigmaPlot version 5.0 (Jandel Scientific, Carlsbad, CA). Statistical tests were performed using SigmaStat version 2.0 (Jandel Scientific). χ^2 analyses were performed to compare tumor incidence (proportions) among treated and control groups. ANOVA was used to compare multiple groups. Where only two groups were compared, Student's *t* test was applied. Values are represented as the mean ± SE unless otherwise indicated.

Results

Southern and Northern analyses of STS in transduced and control cells

Effective incorporation of the STS cDNA into the estrogen-responsive MCF-7/2 cells was demonstrated by Southern analysis. Expression of the appropriate size band from the *XbaI/BbsI* restriction digest of 5.2 kb was detected only in the STS-transduced cells (Fig. 1A). Placenta produces bands of 9.6, 3.0, 2.6, and 1.6 kb. MCF-7/2, Vector 3, and STS-transduced cells produced bands of 3.0, 2.6, and 1.6 kb, representing the endogenous STS gene.

Data from Northern hybridizations confirmed the expression of increased levels of the appropriately sized RNA transcript. Clones 1, 3, 12, and 20 synthesized an RNA transcript of 4.8 kb. No expression was seen in Clone 2 (Fig. 1B). A 5.2-kb band representing the placental RNA was observed (lane 2), consistent with the report by Yen *et al.* (44). However, these transcripts were not detected in placenta by others using similar Northern hybridization analyses (45, 46). STS mRNA expression in STS Clone 3 was approximately equivalent to that in placenta, with expression in Clone 12 and Clone 20 being approximately 2-fold higher than that in placenta. It is difficult to provide precise estimates relative to other controls because the MCF7/2 and Vector 3 values are consistently undetectable. As described by others (45, 46),

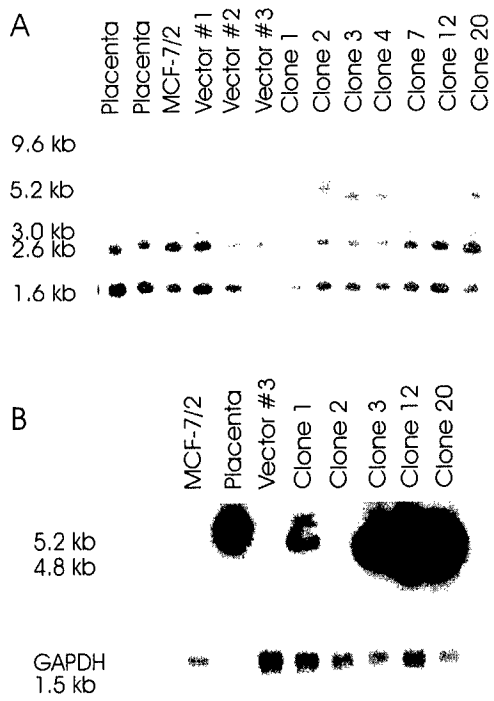


FIG. 1. STS nucleic acid analyses. A, Southern blot analysis of genomic DNA isolated from transduced STS cells and wild-type MCF-7/2 cells. B, Northern blot analysis of total RNA isolated from STS clones, MCF-7/2 cells, and human placenta. RNA loading was assessed by comparing expression of the GAPDH mRNA.

Northern hybridizations do not detect the endogenous STS transcripts in MCF-7 cells that should be similar to our MCF-7/2 and Vector 3 cells. These transcripts are readily detected by RT-PCR (45).

STS enzyme activity in control and transduced cells

STS enzyme activity was measured in cellular homogenates, cytosol, and microsomal fractions. As expected, activity was detected in both the homogenate and cytosol (not shown); the highest activity was found in the microsomal fractions. Microsomal fractions were used in all subsequent studies. STS activity displayed linear enzyme kinetics up to 0.50 mg protein/ml at 37 C for 1 h. Nonlinear kinetics were observed above this concentration (not shown). A level of 0.50 mg protein/ml was used in all other reactions.

Sulfatase activity was assessed from 30 min to 4 h. At 1 h, placenta produces 26.2 ± 0.27 pmol estrone /mg protein·h, MCF-7/2 produces 13.0 ± 0.42 pmol estrone/mg protein·h, Vector 3 produces 12.0 ± 0.26 pmol estrone/mg protein·h, and STS Clone 20 produces 64.2 ± 0.14 pmol estrone/mg protein·h. After 1 h, STS Clone 20 cells hydrolyze 87% of the [^{3}H]estrone sulfate, and STS Clone 12 cells hydrolyze 70% of the estrone sulfate. STS Clone 3 cells had the lowest percentage of enzyme activity, and this was comparable to that seen in MCF-7/2 (36%) and Vector 3 (42%) cells. The higher enzyme activity in the transfectants was less than might be predicted by the Northern hybridizations. However, it is not unusual to detect levels of mRNA expression among transfected cells that do not fully reflect the levels of an active protein.

As sufficient sulfatase activity could be detected with an incubation of 1 h in each of the cell lines, all sulfatase assays were performed using a 1-h incubation unless otherwise indicated. The optimum pH was estimated by incubating the microsomal fraction of STS clones, placental, vector, and MCF-7/2 cells for 1 h with [^{3}H]estrone sulfate in Tris-HCl buffer over a pH range of 5.0–8.0. The optimum pH for all the samples in all experiments was 6.5–7.5 in Tris-HCl buffer. Subsequent samples were assayed at a pH of 6.5. These data are consistent with the most frequently reported optimum pH of 6.5 for the sulfatase assay of MCF-7 cells (38, 47).

Consistent with the data from the Southern and Northern analyses, STS Clone 20 and STS Clone 12 cells express significantly elevated levels of STS activity, relative to MCF-7/2 and Vector 3 cells, as evidenced by their higher V_{max} estimates (Table 1). The estimated STS K_m values in the transduced cells (Table 1) are variable, but broadly comparable to both controls (MCF-7/2, Vector 3, and placenta) and previously published data (38, 48). Detection of endogenous STS activity in MCF-7/2 and Vector 3 cells is consistent with previous reports of this activity in other MCF-7 cells (49).

These data clearly demonstrate that the STS mRNA expressed is translated into functional protein in at least two of the clones studied (STS Clone 12 and STS Clone 20). This is supported by the comparability of the estimated K_m values with previously published studies (38, 48) and the increased V_{max} for estrone sulfate metabolism in the transduced cells. The STS activities detected in STS Clone 12 (0.61 nmol/min·mg) and STS Clone 20 (0.72 nmol/min·mg) cells are broadly comparable with the levels seen in breast tumors expressing high levels of STS activity (8, 23, 50). For example, a range of 0–0.40 nmol/min·mg^1 was reported in 93 of 104 human breast cancers (23).

Effects of sulfated estrogens on cell cycle distribution

Cell cycle analysis of STS Clone 20, MCF-7/2, and Vector 3 cells treated with 17β-estradiol sulfate in the presence or absence of either ICI 182,780 (100 nM) or tamoxifen (1 μM) was determined by flow cytometry (Table 2). Consistent with the reported effects of estrogens on cell cycle distribution (51), 1 nM 17β-estradiol sulfate increased the proportion of cells in the proliferative fraction ($S+G_2/M$), with a consequent reduction in G_0/G_1. The greatest change was evident in the STS Clone 20 cells, reflecting their higher levels of STS activity. To confirm that these are estrogenic effects, we determined the ability of antiestrogens to block the changes in cell cycle distribution induced by 17β-estradiol sulfate. Treatment of STS Clone 20 cells with either ICI 182,780 or tamox-

TABLE 1. Catalytic properties of steroid sulfatase transduced cells

Cells/tissue	K_m (μM)	V_{max} (nmol/mg protein·h)
Placenta	0.50 ± 0.07	22.5 ± 1.26
MCF-7/2	0.10 ± 0.63	8.4 ± 1.25
Vector 3	0.33 ± 0.10	9.0 ± 1.4
STS clone 3	0.50 ± 0.08	18.0 ± 1.08
STS clone 12	1.01 ± 0.90	36.0 ± 6.09
STS clone 20	1.25 ± 0.19	39.0 ± 1.46

Data represent the mean ± SE of each of three replicates in three or more experiments.

TABLE 2. Effect of 17β-estradiol sulfate on cell cycle distribution *in vitro*

Cell line	Treatment	%G_0/G_1	%S	%G_2/M
MCF-7/2	Vehicle	71	19	10
	E_2S	51	30	19
	E_2S + ICI 182,780	62	24	14
	E_2S + tamoxifen	78	13	9
Vector 3	Vehicle	69	23	8
	E_2S	38	42	20
	E_2S + ICI 182,780	76	17	7
	E_2S + tamoxifen	83	8	9
STS clone 20	Vehicle	65	23	12
	E_2S	24	48	26
	E_2S + ICI 182,780	76	13	11
	E_2S + tamoxifen	74	19	7

E_2S, 17β-Estradiol sulfate. The concentrations used are: E_2S, 1 nM; ICI 182,780, 100 nM; tamoxifen, 1 μM.

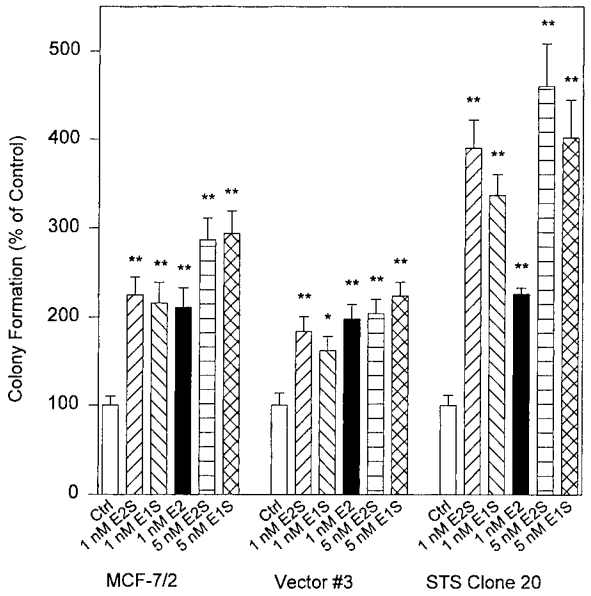


FIG. 2. Induction of anchorage-independent colony formation by estrogens and estrogen sulfates. Data represent the mean ± SE of each of five replicates in two or more experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with control).

ifen increased the proportion of cells in G_o/G_1 while reducing the proportion in S+G_2/M. These data clearly indicate that STS-transduced cells retain responsiveness to antiestrogens.

Estrogenic effects of estrogen sulfates in vitro

The effects of sulfated estrogens and 17β-estradiol on anchorage-independent colony-forming ability are shown in Fig. 2. At the physiologically relevant concentration of 1 nM, the sulfated estrogens act as potent mitogens for colony formation in both STS Clone 20 and MCF-7/2 cells. As expected, these data are broadly comparable to the changes in cell cycle profiles seen in Table 2. These mitogenic effects appear dose dependent, as 5-nM treatments are generally more effective than 1-nM treatments and are consistently higher in STS Clone 20 cells compared with either the MCF-7/2 or Vector 3 cells. These data reflect the higher level of STS expression in STS Clone 20 relative to endogenous STS activity. 17β-Estradiol is equally effective in all three cell lines.

Expression of the estrogen-regulated genes PgR (Fig. 3A) and pS2 (Fig. 3B) were evaluated in the presence of 1 nM estrone sulfate, 17β-estradiol sulfate, and 17β-estradiol. Estrone sulfate does not stimulate the expression of PgR in any of the cell lines (not shown). Both 17β-estradiol sulfate and 17β-estradiol increase PgR mRNA expression in STS Clone 20, but only 17β-estradiol is effective in MCF-7/2 and Vector 3 cells (Fig. 3A). In contrast, pS2 expression is induced by estrone sulfate in all three cells (Fig. 3B). The expression pattern seen for the apparently less estrogen-responsive PgR gene is consistent with higher STS activity in STS Clone 20 cells. Santner *et al.* (49) suggest that both PgR and pS2 are relatively insensitive to stimulation, requiring at least 1 μM

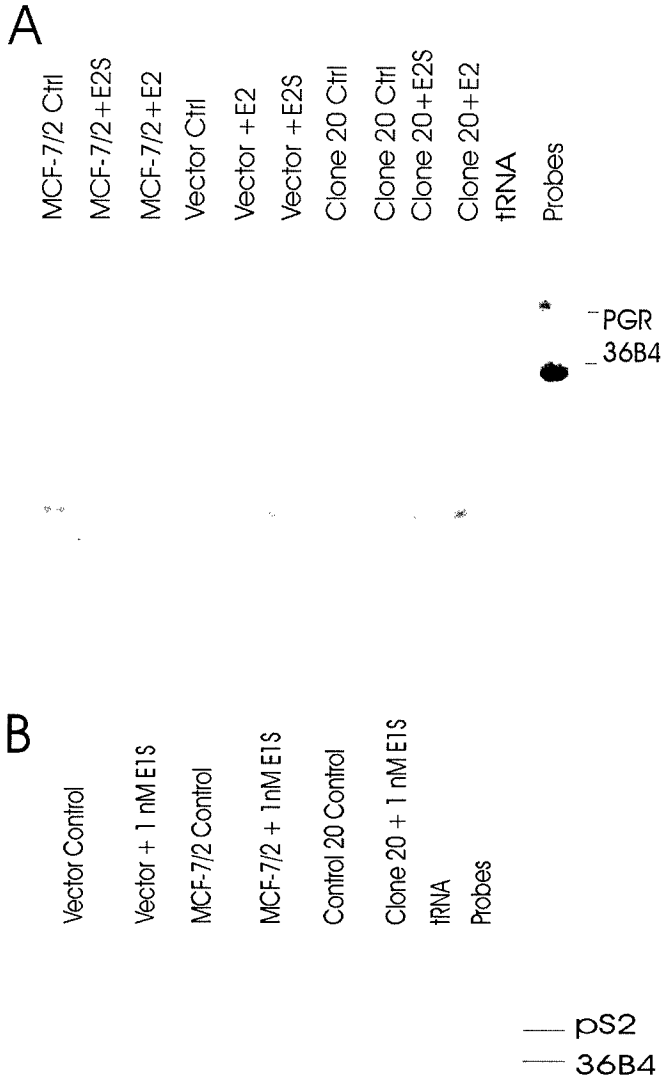


FIG. 3. Effects of estrogens and estrogen sulfates on transcription of the PgR (A) and pS2 mRNAs (B). In the RNase protection assays, the PgR probe produces a protected fragment of 250 bp, the pS2 probe protects a 300-bp fragment, and the 36B4 probe a generates a protected fragment of 220 bp.

estrone sulfate for induction of expression. Although we used only a single concentration (1 nM), this was clearly sufficient to induce pS2 in all cells (estrone sulfate) but to induce PgR expression only in STS clones (17β-estradiol sulfate). Cells that acquire an estrogen-independent phenotype also up-regulate pS2 mRNA, but not PgR expression, suggesting that pS2 induction is more sensitive to changes in estrogenicity than PgR (37).

Effect of intratumor STS activity on tumorigenicity

To address the possible *in vivo* relevance of estrogens released by intratumor estrogen sulfatase activity, we evaluated the ability of 17β-estradiol sulfate to support the establishment of estrogen-dependent tumors in ovariectomized athymic nude mice (Table 3). When evaluating endocrine therapies in these models, it is common to first establish tumors with 17β-estradiol, and then administer the endocrine manipulation, *e.g.* removal of the estrogenic stimulus by administration of an estrogen antagonist. Our experimental design is more rigorous and appropriate for the assessment of the effects of estrogen sulfates because it requires the hormone to promote the survival and establishment of a relatively small number of cells. In this regard, our approach is similar to that used to determine the ability of aromatase overexpression to support tumor growth *in vivo*. However, we did not implant cells embedded in Matrigel, an artificial basement membrane, which is required for the maintenance of MCF-7 cells transfected with the aromatase gene (52).

The growth rate of nonproliferating tumors could not be measured. We observed marked variation in tumor growth within each group, which would limit our ability to statistically detect modest differences in tumor growth rate. However, we were more interested in whether proliferating tumors could be established than in the rate at which any tumors might grow once established. From a clinical perspective, the presence of a proliferating, estrogen-dependent tumor is more relevant than the rate at which an established tumor proliferates. Thus, we compared the incidence of proliferating tumors in mice bearing vector control *vs.* transfectant (STS Clone 20) xenografts. The rate of growth is a useful prognostic indicator is some cases, but is not particularly informative in predicting response to endocrine therapies. Although toxicity was not anticipated, body weight measurements were obtained on each group of animals during the study. No significant difference in body weights was seen among the groups.

Mice receiving both STS Clone 20 cells and 17β-estradiol sulfate exhibited the highest incidence of proliferating tumors, more than 3-fold higher than that in their nonsupplemented controls (Table 3; $P < 0.001$). Although 17β-estradiol sulfate also supported the growth of some Vector 3 cells, tumor incidence was not significantly increased compared with the incidence in nonsupplemented mice (Table 3; $P = 0.37$). These data are consistent with the increased STS activity (~4-fold higher compared with Vector 3 tumors; $P < 0.001$) in the STS Clone 20 cells and indicate continued expression of high levels of the active enzyme *in vivo*. Tumorigenicity in the absence of 17β-estradiol sulfate supplementation was equivalent in both STS Clone 20 and Vector 3 cells ($P = 0.84$). Similarly, the tumorigenicity of STS Clone 20 and Vector 3 cells was equivalent in the presence of 17β-estradiol ($P = 1.00$). Thus, the differences in tumor incidence are not due to altered basal tumorigenicity between STS cells and controls.

TABLE 3. Effect of 17β-estradiol sulfate on tumor incidence in ovariectomized athymic nude mice

Group	Tumor incidence/tumorigenicity [no. (%)]	P value[a]
Vector 3 (untreated)	9/40 (22)	
Vector 3 + E_2S	17/51 (33)	0.37
STS clone 20 (untreated)	9/41 (22)	
STS clone 20 + E_2S	36/51 (71)	<0.001

E_2S, 17β-Estradiol sulfate.

[a] P values (χ^2 test) describe the effects of 17β-estradiol sulfate on tumorigenicity.

Discussion

To address the possible functional relevance of the STS gene further, we have overexpressed the STS cDNA in estrogen-dependent human breast cancer cells. Previous studies were limited to those characterizing endogenous levels from breast cancer tissues or various breast cancer cell lines. The STS-transduced clones we have generated now permit studies to address the potential importance of this activity in the production of biologically relevant concentrations of intratumor estrogens. These transfectants clearly exhibit differential responses to estrogen sulfates *in vitro* and *in vivo*, consistent with their elevated STS expression. For example, equimolar concentrations of estrogen sulfates are more effective in STS-transduced cells relative to controls *in vitro*. This is evident for estrogenic effects on PgR mRNA expression, cell cycle distribution, and anchorage-independent growth. Modest activity in controls is consistent with the low level of endogenous STS in the MCF-7/2 and Vector 3 cells and other MCF-7 populations (49).

Consistent with the estrogenic effects on cell cycle distribution and anchorage-independent growth, 17β-estradiol sulfate is a potent mitogen *in vivo*. In ovariectomized mice supplemented with 17β-estradiol sulfate, STS Clone 20 tumors arise with a higher incidence compared with that in untreated controls inoculated into the opposite flanks of the same mice. As control tumors are not supported by 17β-estradiol sulfate, any endogenous STS activity in these cells is not sufficient to support full tumorigenesis.

Hydrolysis of estrone sulfate can stimulate the growth of *N*-nitroso-*N*-methylurea-induced mammary adenocarcinomas in castrated rats. This observation provides only circumstantial evidence, because a direct requirement for sulfatase activity in the *in situ* synthesis of estrogens was not demonstrated (53). For example, hepatic sulfatases could release 17β-estradiol into the blood. Supplementation with 17β-estradiol sulfate is not sufficient to support an increase in the tumorigenicity of Vector 3 cells. If hepatic sulfatases released biologically relevant concentrations of 17β-estradiol, the incidence of Vector 3 tumors would have been increased. However, the small increase in Vector 3 tumor incidence, from 22% to 33%, is not statistically significant ($P = 0.37$). In marked contrast, the incidence of STS Clone 20

tumors, which arise in the opposite flanks of the same mice, is significantly increased ($P < 0.001$). These data, which provide a more direct assessment of the likely importance of intratumor STS in supporting tumor growth, clearly indicate that serum-derived estrogen sulfates can support estrogen-dependent tumorigenicity. Furthermore, hepatic STS activity seems biologically less important than intratumor STS.

Extrapolation of these data to human breast cancer requires a degree of caution. There may be differences in the pharmacokinetics of estrogen sulfates between mice and humans. For example, mice have different metabolic rates that require consideration in pharmacological/toxicological studies (54). MCF-7 cells probably reflect only one of several possible endocrine-responsive breast cancer phenotypes. Nonetheless, the consistent *in vitro* and *in vivo* responses exhibited by the STS-transfected cells, relative to their appropriate controls, strongly imply a likely role for this enzyme and serum estrogen sulfates in the biology of breast cancer. The *in vivo* data demonstrate that relevant levels of STS expression can support tumorigenesis and demonstrate the utility of this model to evaluate the role of this enzyme and estrogen sulfates further.

The *in vitro* and *in vivo* estrogenic effects of estrogen sulfates we observed are most likely a consequence of the liberation of free estrogens, as only free estrogens can bind and activate estrogen receptors (16). Treatment of STS Clone 20 cells with either ICI 182,780 or tamoxifen induces G_o/G_1 arrest, as is widely reported for MCF-7 cells (51). Antiestrogens primarily function by competing for estrogen activation of estrogen receptors (55). Thus, the mitogenic effects of the estrogen sulfates are primarily mediated though activation of estrogen receptors. The ultimate effector for estrone sulfate treatment is probably free intracellular 17β-estradiol, as MCF-7 cells have detectable 17β-hydroxysteroid dehydrogenase activity and can convert some estrone to 17β-estradiol (56).

Our data provide limited evidence that exogenous estrogens may have different biological activity than estrogens released from estrogen sulfates within cells. Indeed, the estrogen sulfates apparently exhibit greater biological activity than equimolar concentrations of 17β-estradiol, at least for some experimental end points. For example, 17β-estradiol sulfate is more mitogenic than 17β-estradiol *in vitro*, producing a greater increase in anchorage-independent colony formation in the STS Clone 20 cells. These differences are not consistently seen in the Vector 3 and MCF-7/2 cells. Although tumor volumes recorded at the end of a study contain only limited information (42), the volume of STS Clone 20 tumors in the 17β-estradiol sulfate-treated animals (mean tumor volume, 138 mm^3; n = 36) is greater than that of tumors arising in animals supplemented with 17β-estradiol (mean tumor volume, 51 mm^3; n = 6). The number of observations is limited, but the trend reflects our observations in anchorage-independent colony formation assays.

These *in vitro* and *in vivo* observations are surprising and clearly require further study. Why the sulfated estradiol may be more effective than the free hormone is unclear. If all estrogen sulfate was converted to 17β-estradiol, it might be expected that the estrogen sulfates and 17β-estradiol should be equieffective at equimolar concentrations. However, the increased activity is seen only in the STS cells. As the ability to increase free intracellular estrogen levels will reflect the relative activities of both the STS (increase unconjugated estrogen production) and steroid sulfotransferase enzymes (reduce unconjugated estrogen production), a high proportion of intracellular estrogen may be present as free 17β-estradiol in STS Clone 20 cells.

Biological activity will reflect the concentrations of available intracellular estrogens able to interact with their receptors. Availability will be determined by binding to other extracellular and intracellular proteins (5, 57, 58), sequestration in cellular membranes (59), and distribution within extranuclear compartments. Free estrogen molecules are highly lipophilic, with some partitioning into cellular membranes and affecting membrane function (59, 60) while others eventually reach their nuclear receptors and activate gene transcription (55). In marked contrast, sulfated estrogen molecules are less lipophilic and require removal of the sulfate moiety (8), perhaps within specific plasma membrane domains rich in the STS enzyme, before they can reach the nucleus in substantial numbers. Thus, the cellular pharmacologies of exogenous sulfated and free estrogens may produce significantly different subcellular distributions of those estrogen molecules capable of eventually activating their receptors. This also could contribute to the different activities of free and sulfated estrogens in the STS cells. Studies to further address this hypothesis are currently in progress.

The high levels of intratumor 17β-estradiol seen in breast cancers are probably multifocal in origin. These levels reflect a combination of uptake of 17β-estradiol, estrone, and their sulfated metabolites from blood (4, 5) and the metabolism of circulating adrenal androgen precursors in neoplastic epithelium (61) and adjacent adipocytes (62). The predominant pathway may vary among tumors. Nonetheless, our data clearly establish the feasibility of uptake of sulfated estrogens from blood and their conversion to biologically active estrogens within breast tumors. Furthermore, the intratumor release of estrogens from their sulfates appears more important than the production of free estrogens by hepatic activation or other peripheral metabolism.

Our data also suggest that tumors and normal tissues that concurrently express both the aromatase and STS enzymes may be the most efficient at maintaining a highly estrogenic environment. The utility of aromatase inhibitors is well established. Inhibitors of STS have been generated (63), but their clinical utility is unclear, as aromatase inhibitors reduce the available substrate concentrations for STS. Differences in tissue distributions of aromatase and STS could produce a tissue-specific advantage for some STS inhibitors. Perhaps a combination of aromatase and STS inhibitors would produce a greater inhibition of intratumor estrogen concentrations by blocking the activation of any remaining sulfated estrogens. STS Clone 20 cells provide a new model to begin to address several of these issues in more detail.

Acknowledgments

The authors thank Dr. Fabio Leonessa (Lombardi Cancer Center) for assistance with the growth assays, Mr. Robert Williams (Department of Pathology, Howard University) for the human placental tissue, and Ms. Kerrie Bouker for critical reading of the manuscript.

References

1. **van Landeghem AA, Poortman J, Nabuurs M, Thijssen JH** 1985 Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. Cancer Res 45:2900–2906
2. **McNeill JM, Reed MJ, Beranek PA, Bonney RC, Ghilchik MW, Robinson DJ, James VHT** 1986 A comparison of the *in vivo* uptake and metabolism of ^{3}H-oestrone and ^{3}H-oestradiol by normal breast and breast tumor tissues in post-menopausal women. Int J Cancer 38:193–196
3. **Pasqualini JR, Chetrite G, Blacker C, Feinstein MC, Delalonde L, Talbi M, Maloche C** 1996 Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J Clin Endocrinol Metab 81:1460–1464
4. **Miller WR** 1997 Uptake and synthesis of steroid hormones by the breast. Endocr Related Cancer 4:307–311
5. **Masamura S, Santner SJ, Gimotty P, Georger J, Santen RJ** 1997 Mechanism for maintenance of high breast tumor estradiol concentrations in the absence of ovarian function: role of very high affinity tissue uptake. Breast Cancer Res Treat 42:215–226
6. **Poutanen M, Miettinen M, Vihko R** 1993 Differential estrogen substrate specificities for transiently expressed human placental 17β-hydroxysteroid dehydrogenase and an endogenous enzyme expressed in cultured COS-m6 cells. Endocrinology 133:2639–2644
7. **Falany JL, Falany CN** 1996 Expression of cytosolic sulfotransferases in normal mammary epithelial cells and breast cancer cell lines. Cancer Res 56:1551–1555
8. **Pasqualini JR, Schatz B, Varin C, Nguyen BL** 1992 Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer. J Steroid Biochem Mol Biol 41:323–329
9. **Adams JB, Pewnim T, Chandra DP, Archibald L, Foo MS** 1979 A correlation between estrogen sulfotransferase levels and estrogen receptor status in human primary breast carcinoma. Cancer Res 39:5124–5126
10. **Pasqualini JR, Chetrite G, Nestour EL** 1996 Control and expression of oestrone sulphatase activities in human breast cancer. J Endocrinol 150:S99–S105
11. **Santner SJ, Feil PD, Santen RJ** 1984 In situ estrogen production via the estrone sulfate pathway in breast tumors: relative importance versus the aromatase pathway. J Clin Endocrinol Metab 59:29–33
12. **Dowsett M** 1997 Future use of aromatase inhibitors in breast cancer. J Steroid Biochem Mol Biol 61:261–266
13. **Miller WR, Hawkins RA, Forrest AP** 1982 Significance of aromatase activity in human breast cancer. Cancer Res 42:3365s–3368s
14. **Fishman J, Nisselbaum JS, Menendez-Botet C, Schwartz MK** 1977 Estrone and estradiol content in human breast tumors: relationship to estradiol receptors. J Steroid Biochem 8:893–896
15. **Vermeulen A, Deslypere JP, Paridaens R, LeClercq G, Roy F, Heuson JC** 1986 Aromatase, 17β-hydroxysteroid dehydrogenase and intratissular sex hormone concentrations in cancerous and normal glandular breast tissue in postmenopausal women. Eur J Cancer 22:515–525
16. **Clarke R, Hilakivi-Clarke LA, Cho E, James MR, Leonessa F** 1996 Estrogens, phytoestrogens and breast cancer. Adv Exp Biol Med 401:63–86
17. **Cocconi G** 1994 First generation aromatase inhibitors–aminoglutethimide and testololactone. Breast Cancer Res Treat 30:57–80
18. **Lonning PE, Dowsett M, Powles TJ** 1989 Treatment of breast cancer with aromatase inhibitors - current status and future prospects. Br J Cancer 60:5–8
19. **Lonning PE, Johannesson DC, Thoresen T** 1989 Alterations in the production and clearance rate of estrone and estrone sulfate in breast cancer patients treated with aminoglutethimide. Br J Cancer 60:107–111
20. **Lonning PE, Dowsett M, Powles TJ** 1990 Postmenopausal estrogen biosynthesis and metabolism: alterations caused by aromatase inhibitors used for the treatment of breast cancer. J Steroid Biochem 35:355–366
21. **Evans TR, Rowlands MG, Silva MC, Law M, Coombes RC** 1993 Prognostic significance of aromatase and estrone sulfatase enzymes in human breast cancer. J Steroid Biochem Mol Biol 44:583–587
22. **Lipton A, Santen RJ, Santner SJ, Harvey HA, Sanders SI, Matthews YL** 1992 Prognostic value of breast cancer aromatase. Cancer 70:1951–1955
23. **Evans TR, Rowlands MG, Law M, Coombes RC** 1994 Intratumoral oestrone sulphatase activity as a prognostic marker in human breast carcinoma. Br J Cancer 69:555–561
24. **Strobl JS, Lippman ME** 1979 Prolonged retention of estradiol by human breast cancer cells in tissue culture. Cancer Res 39:3319–3327
25. **Skaar TC, Prasad SC, Sharaeh S, Lippman ME, Brunner N, Clarke R** 1998 Two- dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. J Steroid Biochem Mol Biol 67:391–402
26. **Clarke R, Brünner N, Thompson EW, Glanz P, Katz D, Dickson RB, Lippman ME** 1989 The inter-relationships between ovarian-independent growth, antiestrogen resistance and invasiveness in the malignant progression of human breast cancer. J Endocrinol 122:331–340
27. **Darbre P, Yates J, Curtis S, King RJB** 1983 Effect of estradiol on human breast cancer cells in culture. Cancer Res 43:349–354
28. **Miller AD, Rosman GJ** 1989 Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980–982
29. **Markowitz DG, Goff SP, Bank A** 1988 Safe and efficient ecotropic and amphotropic packaging lines for use in gene transfer experiments. Trans Assoc Am Physicians 101:212–218
30. **Clarke R, Brünner N, Katz D, Glanz P, Dickson RB, Lippman ME, Kern F** 1989 The effects of a constitutive production of TGF-α on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. Mol Endocrinol 3:372–380
31. **Miller AD, Buttimore C** 1986 Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 6:2895–2902
32. **Clarke R, Brünner N, Katzenellenbogen BS, Thompson EW, Norman MJ, Koppi C, Paik S, Lippman ME, Dickson RB** 1989 Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. Proc Natl Acad Sci USA 86:3649–3653
33. **Masamura S, Santner SJ, Heitjan DF, Santen RJ** 1995 Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab 80:2918–2925
34. **Sambrook J, Fritsch EF, Maniatis T** 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
35. **Brunner N, Boysen B, Jirus S, Skaar T, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SAW, Clarke R** 1997 MCF7/LCC9: An antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182, 780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res 57:3486–3493
36. **LaBorda J** 1991 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal protein PO. Nucleic Acids Res 19:3998
37. **Brünner N, Boulay V, Fojo A, Freter C, Lippman ME, Clarke R** 1993 Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res 53:283–290
38. **MacIndoe JH, Woods G, Jeffries L, Hinkhouse M** 1988 The hydrolysis of estrone sulfate and dehydroepiandrostenedione sulfate by MCF-7 human breast cancer cells. Endocrinology 123:1281–1287
39. **Vindelov LL, Christensen IJ, Nissen NI** 1983 A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3:323–327
40. **Seibert K, Shafie SM, Triche TJ, Whang-Peng JJ, O'Brien SJ, Toney JH, Huff KK, Lippman ME** 1983 Clonal variation of MCF-7 breast cancer cells in vitro and in athymic nude mice. Cancer Res 43:2223–2239
41. **Brünner N, Svenstrup B, Spang-Thompsen M, Bennet P, Nielsen A, Nielsen JJ** 1986 Serum steroid levels in intact and endocrine ablated BALB nude mice and their intact litter mates. J Steroid Biochem 25:429–432
42. **Clarke R** 1997 Issues in experimental design and endpoint analysis in the study of experimental cytotoxic agents *in vivo* in breast cancer and other models. Breast Cancer Res Treat 46:255–278
43. **Clarke R** 1996 Human breast cancer cell line xenografts as models of breast cancer: the immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. Breast Cancer Res Treat 39:69–86
44. **Yen PH, Allen E, Marsh B, Mohandas T, Wang N, Taggart RT, Shapiro LT** 1987 Cloning and expression of steroid sulfatase cDNA and the frequent occurrence of deletions in STS deficiency: implications for X-Y interchange. Cell 49:443–454
45. **Evans TR, Rowlands MG, Luqmani YA, Chander SK, Coombes RC** 1993 Detection of breast cancer-associated estrone sulfatase in breast cancer biopsies and cell lines using polymerase chain reaction. J Steroid Biochem Mol Biol 46:195–201
46. **Pasqualini JR, Maloche C, Maroni M, Cherite G** 1994 Effect of the progestagen promegesterone (R-5020) on mRNA of the estrone sulfatase in the MCF-7 human mammary cancer cells. Anticancer Res 14:1589–1594
47. **Rabe T, Kiesel L, Radke G, Runnebaum B** 1984 New assay for steroid sulfatase (EC 3.1.6.2) and its application for studies of human placental and skin sulfatase. J Steroid Biochem 20:677–682
48. **Pasqualini JR, Gelly C, Lecerf F** 1986 Estrogen sulfates: biological and ultrastructural responses and metabolism in MCF-7 human breast cancer cells. Breast Cancer Res Treat 8:233–240
49. **Santner SJ, Ohlsson-Wilhelm B, Santen RJ** 1993 Estrone sulfate promotes human breast cancer cell replication and nuclear uptake of estradiol in MCF-7 cell cultures. Int J Cancer 54:119–124
50. **Pasqualini JR, Nguyen B-L** 1991 Estrone sulfatase activity and effect of antiestrogens on transformation of estrone sulfate in hormone-dependent vs independent human breast cancer cell lines. Breast Cancer Res Treat 18:93–98
51. **Taylor IW, Hodson PJ, Green MD, Sutherland RL** 1983 Effects of tamoxifen on cell cycle progression of synchronous MCF-7 human mammary carcinoma cells. Cancer Res 43:4007–4010
52. **Lu Q, Liu Y, Long BJ, Grigoryev D, Gimbel M, Brodie A** 1999 The effect of combining aromatase inhibitors with antiestrogens on tumor growth in a nude mouse model for breast cancer. Breast Cancer Res Treat 57:183–192
53. **Santner SJ, Levin MC, Santen RJ** 1990 Estrone sulfate stimulates growth of nitrosomethylurea-induced breast carcinoma in vivo in the rat. Int J Cancer 46:73–78
54. **Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE** 1966 Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 50:219–244
55. **Clarke R, Brünner N** 1996 Acquired estrogen independence and antiestrogen

resistance in breast cancer: estrogen receptor-driven phenotypes? Trends Endocrinol Metab 7:25–35
56. **Cherite G, Paris J, Botella J, Pasqualini JR** 1999 Effect of nomegestrol acetate on estrone-sulfatase and 17β-hydroxysteroid dehydrogenase activities in breast cancer cells. J Steroid Biochem Mol Biol 58:525–531
57. **Takahashi N, Breitman TR** 1989 Covalent binding of 17β-estradiol and retinoic acid to proteins in the human breast cancer cell line MCF-7. In Vitro Cell Dev Biol 25:1199–1200
58. **Anderson E, Vinson GP, Puddefoot JR, Raven PW, Gilmore OJA** 1986 Steroid sequestration and tightly bound oestrogen-protein complexes in human breast tumors and a breast cancer cell line. J Steroid Biochem 24:489–495
59. **Clarke R, van den Berg HW, Murphy RF** 1990 Tamoxifen and 17β-estradiol reduce the membrane fluidity of human breast cancer cells. J Natl Cancer Inst 82:1702–1705
60. **Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP** 1999 Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci USA 96:4686–4691
61. **Brodie AMH, Lu Q, Nakamura J** 1997 Aromatase in the normal breast and breast cancer. J Steroid Biochem Mol Biol 61:281–286
62. **Bulun SE, Simpson ER** 1994 Breast cancer and expression of aromatase in breast adipose tissue. Trends Endocrinol Metab 5:113–120
63. **Duncan L, Purohit A, Howarth NM, Potter BV, Reed MJ** 1993 Inhibition of estrone sulfatase activity by estrone-3-methylthiophosphonate: a potential therapeutic agent in breast cancer. Cancer Res 53:298–303

30th Session of the Advanced Course on Biology and Biochemistry of Normal and Cancer Cell Growth
Erice, Sicily, Italy
May 1–6, 2001
Classical and Non-Classical Issues from Prevention to Treatment of Hormone-Related Tumors

Venue: Centro Ettore Majorana for Scientific Culture

Scientific Committee: B. Agostara, H. L. Bradlow, L. Castagnetta, L. Massimo, G. Montalto

Secretariat: lucashbl@unipa.it. Registration Information: Professor L. Castagnetta, Fax 39 091 666435, e-mail lucashbl@unipa.it.

Publication: The proceedings will be published as a volume of the Annals of the New York Academy of Sciences

Topics:

1. Breast Cancer
2. Prostate Cancer
3. Colorectal Cancer
4. Liver Cancer
5. Nuclear Receptors
6. Steroid Enzymes
7. Angiogenesis
8. Cell-Cell Interactions
9. Apoptosis
10. Primary Prevention
11. Gene Therapy

Invited Speakers: J. Adamski, Germany; F. Aurucchio, Italy; M. Beato, Germany; F. Berrino, Italy; F. Boccardo, Italy; H. L. Bradlow, USA; P. Bruzzi, Italy; M. Buendia, France; G. Carruba, Italy; S. Chen, USA; L. Cohen, USA; C. Coombes, UK; J.-A. Gustaffson, Sweden; K. Horwitz, USA; R. Kaaks, France; C. Knabbe, Germany; K. Korach, USA; V. Luu-Thé, Canada; M. Marcelli, USA; R. Nicholson, UK; R. Montironi, Italy; J. Prieto, Spain; M. Schwab, Germany; J. Thjissen, The Netherlands; C. P. Tonini, Italy; G. C. Vecchio, Italy; E. Villa, Italy.

Poster Session: Participants may submit abstracts for a poster session. The deadline for Abstracts is March 1, 2001. The abstracts should fit in a 10- × 15-cm box. Travel: The nearest international airport is Palermo, Sicily. Bus service to Erice is available.