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*Perspective*

# Antiestrogens and Selective Estrogen Receptor Modulators as Multifunctional Medicines. 2. Clinical Considerations and New Agents

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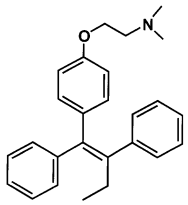
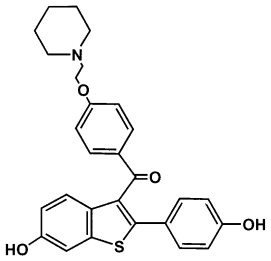
## Clinical Basis for Selective Estrogen Receptor Modulation

The critical step in the development of novel approaches to prevent disease is the translation of laboratory concepts to clinically useful interventions. The expanding database for selective estrogen receptor modulators (SERMs) focuses on the complementary studies of tamoxifen (**1**) for the treatment and prevention of breast cancer and of raloxifene (**2**) for the treatment and prevention of osteoporosis. Tamoxifen has been studied thoroughly in breast cancer patients for more than 30 years.[1−4] Additionally, the pharmacology and toxicology of raloxifene are now being rigorously evaluated because the primary target is the well woman. In part 2, the important clinical observations will be presented and the toxicological issues will be addressed as a basis for the development of new agents. Tamoxifen is the first clinically useful SERM, so a careful reevaluation of its drug actions is essential for the understanding of drug mechanisms and to avoid toxicological problems in the future with a new agent. Also, the widespread use of tamoxifen as a breast cancer treatment has resulted in renewed laboratory efforts to understand drug resistance. Tamoxifen-stimulated tumor growth is a unique mechanism of resistance, so an understanding of the molecular events that switch a drug from being predominantly antiestrogenic to being estrogenic could potentially open the door to a better understanding of the molecular mechanism of SERM action. This aspect of SERM pharmacology will be addressed at the end of the review with a description of new models of drug resistance to SERM action and potential mechanisms of drug resistance.



**(1)** tamoxifen



**(2)** raloxifene

**(a) Tamoxifen: Mixed Antiestrogen and Estrogen Actions.** Tamoxifen is currently (2002) the endocrine therapy of choice for the adjuvant treatment of ER-positive breast cancer. The drug is FDA-approved for the treatment of all stages of breast cancer and for the reduction of breast cancer incidence in high-risk pre- and postmenopausal women. Adjuvant tamoxifen therapy is optimally effective with a 5-year course of treatment

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and produces a profound increase in disease-free and overall survival.[4] Currently, 5 years of adjuvant tamoxifen is recommended to be optimal, since extending treatment beyond 5 years provides no further improvement.[5,6] There are reports of tamoxifen-stimulated tumor growth occurring during the treatment of advanced (metastatic stage IV) breast cancer,[7,8] but there is currently no evidence that extending tamoxifen beyond 5 years of adjuvant therapy increases the risk of tumor recurrence.

Tamoxifen has been used for the treatment of endometrial cancer.[9,10] However, tamoxifen causes an unusual increase in the stromal thickening in the uterus,[11,12] some endometrial hyperplasia, and an increase in polyps.[13] Although the unusual endometrial histopathology that occurs with tamoxifen causes concern, the histologic appearance is very different from that of full agonist estrogen action. Nevertheless, long-term adjuvant tamoxifen treatment is associated with a 4-fold increase in the incidence of endometrial carcinoma in postmenopausal women.[14−16] There is no reported increase in the risk of endometrial cancer in premenopausal women[15,17] probably because menstrual cycles persist during tamoxifen treatment for the majority of women. The link between tamoxifen and endometrial cancer will be considered in detail later in part 2.

Tamoxifen treatment increases a number of estrogen-induced circulating proteins, such as sex hormone binding globulin[18,19] and antithrombin III,[19] and alters the plasma protein profile.[20,21] Additionally tamoxifen has an estrogen-like action to reduce LH and FSH in postmenopausal women.[22]

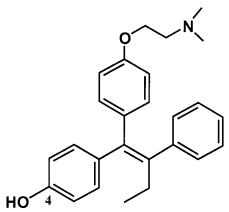
Tamoxifen has a consistent ability to decrease low-density lipoprotein (LDL) cholesterol but unlike estrogen does not cause an increase in high-density lipoprotein (HDL) cholesterol.[23−31] Although tamoxifen was originally classified as an antiestrogen, the drug does not predispose women to coronary heart disease.[32−34] Most studies find that tamoxifen does not protect against coronary heart disease, but the finding may be because only clinical trials with small numbers of patients at risk have been examined. Only retrospective results from the Scottish adjuvant tamoxifen trial of 5 years of adjuvant tamoxifen showed a decrease in fatal myocardial infarction.[35,36] Tamoxifen has not been tested prospectively for the prevention of coronary heart disease in high-risk women.

Tamoxifen maintains bone density in postmenopausal women[37−43] and causes a slight decrease in bone density in premenopausal women.[43] The drug has not been tested prospectively as a preventive for osteoporosis, but a nonsignificant decrease in hip, wrist, and spinal fractures has been noted as a secondary endpoint in the National Surgical Adjuvant Breast and Bowel Project chemoprevention trial.[15] Interestingly enough, tamoxifen produces significantly fewer fractures compared to the aromatase inhibitor anastrozole when used as an adjuvant therapy in postmenopausal women.[44] Although tamoxifen could be classified as a partial agonist in most estrogen-like parameters, the reduced estrogenicity is not reflected in a reduction in the incidence of blood clots relative to hormone replacement therapy (HRT).[15,45]

**(b) Tamoxifen and the Initiation of Carcinogenesis.** Tamoxifen had been used for more than a decade for the treatment of breast cancer (in Europe since the early 1970s and in the U.S. since 1978) without the reporting of serious side effects.[1] However, by the end of the 1980s, with the expanded use of tamoxifen as a long-term adjuvant therapy in node-negative breast cancer[46] and the proposed use of tamoxifen for chemoprevention in high-risk women,[47] there was a requirement to reexamine the toxicology of tamoxifen in greater detail.

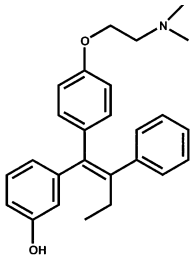
Tamoxifen initiates hepatocellular carcinoma in rats[48−53] by a non-ER-mediated mechanism. This finding was a major concern and naturally was linked to an increased incidence of endometrial cancer and two cases of hepatocellular carcinoma noted in women taking tamoxifen.[14,54−56] The laboratory finding of carcinogenicity, so late in the drugs' development, occurred because the rat had not previously been used to evaluate the long-term toxicology of tamoxifen prior to introduction as a breast cancer treatment. This was not a requirement. It was equally true that if tamoxifen had been tested and found to be carcinogenic, then adjuvant endocrine therapy, aromatase inhibitors, SERMs, and raloxifene would not have been pursued without proof of principle that tamoxifen was a SERM and saved lives.[57] The questions subsequently became the following. What is the mechanism of carcinogenicity in rat liver? Why is the metabolic handling of tamoxifen different in rats, mice, and humans? Is tamoxifen unique because the toxicology has not translated from the laboratory to the clinic?

Kupfer[58] first noted that tamoxifen could covalently bind to proteins. Tamoxifen is converted by cytochrome P450 to *N*-desmethyltamoxifen and 4-hydroxytamoxifen.[59,60] Originally, cytochrome P4503A was identified as the enzyme that catalyzes the activation and covalent binding of tamoxifen to rat and human liver microsomes.[61] It has also been shown that cytochromes P450 3A and 2D6 can catalyze the hydroxylation of 4-hydroxytamoxifen (**3**) and 3-hydroxytamoxifen (droloxi-

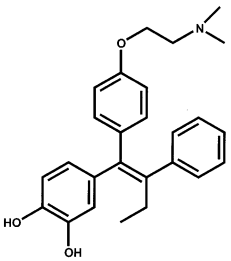



**(3)** 4-hydroxytamoxifen

fene) (**4**) to yield tamoxifen catechol[62] (**5**) and can



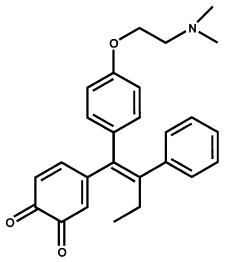

**(4)** droloxifene




**(5)**

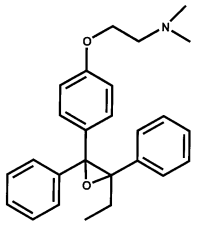
support the participation of tamoxifen catechol in covalent binding to proteins. This is consistent with the

suggestion that tamoxifen catechol[62] (**5**) has the potential to cause cytotoxicity in cells through the formation of tamoxifen-*o*-quinone (**6**).[63]



**(6)**

Han and Liehr[64] first noted an accumulation of DNA adducts in the liver of Sprague−Dawley rats following repeated adminstration of 20 mg/kg (cf. human dose of 0.3 mg/kg). The focus of subsequent investigations has been the identification of the actual DNA adducts. Several candidates have been proposed: an epoxide (**7**),[65,66] 4-hydroxytamoxifen (**3**),[67−69] metabolite E (**8**),[70]



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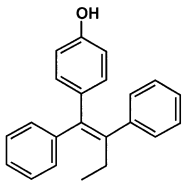
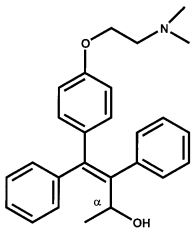


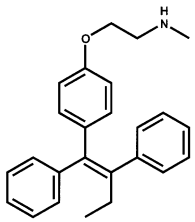
**(8)**

α-hydroxytamoxifen (**9**),[71−74] adducts derived from *N*-

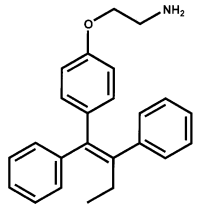


**(9)** α hydroxytamoxifen

desmethyl- (**10**) or *N*,*N*-didesmethyltamoxifen (**11**).[75]



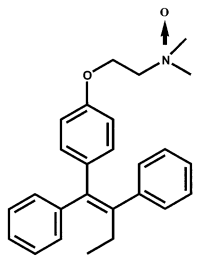
**(10)** N-desmethyltamoxifen



**(11)** N.N. didesmethyltamoxifen

Osborne and co-workers[76] prepared an acetoxy derivative of α-hydroxytamoxifen that is able to react with DNA to a greater extent (1 in 50 bases) than α-hydroxytamoxifen (1 in 105 DNA bases). The products were identical, using $^{32}$P postlabeling of adducts isolated from DNA derived from rat hepatocytes or the livers of rats treated with tamoxifen. The adduct has been identified at the nucleoside deoxyguanosine in which the α position of tamoxifen is linked convalently to the oxocyclic amino of deoxyguanosine. There has been particular interest in the direct identification of DNA adducts based on knowledge of the metabolism of tamoxifen to 4-hydroxytamoxifen (**3**), *N*-desmethyltamoxifen (**10**), and *N*,*N*-didesmethyltamoxifen (**11**).[75] 4-Hydroxytamoxifen could form the α-hydroxy metabolite that could alkylate DNA, but no adducts could be found in liver DNA of female Fisher 344 rats treated orally with 4-hydroxytamoxifen or 4-α-dihydroxytamoxifen.[77] Interestingly enough, cutaneously applied 4-hydroxytamoxifen is not carcinogenic in female rats but can reduce the incidence of spontaneous mammary and pituitary tumors.[78] Clearly, chemoprevention is possible with antiestrogens, but carcinogenesis is dose-, route-, and agent-related.

Not only is tamoxifen α-hydroxylated to form DNA adducts but it is probable that the *N*-oxide (**12**)[79] and



**(12)**

the *N*-desmethyl derivative[80,81] are formed to produce adducts. Hemminki's group has identified the α-hydroxylated *N*-desmethyltamoxifen and tamoxifen adducts to deoxyguanosine in rat liver by mass spectrometry.[82] However, the issue is whether these observations in the rat are relevant to the use of tamoxifen in humans.

Although DNA adducts are readily identified in rat and mouse hepatocytes (90 and 15 adducts per 108 nucleotides, respectively), DNA adducts were not detected in human hepatocytes following tamoxifen treatment.[83] Similarly, the pattern of DNA adducts found in the rat liver is not found in the liver obtained from patients treated with tamoxifen.[84] There is, however, some controversy about whether DNA adducts can be detected in the tissues from patients treated with tamoxifen. Phillips and co-workers do not find DNA adducts in endometrium and lymphocytes,[83,85,86] whereas Hemminki and others[87−90] do. The DNA adducts in human endometrial tissue is α-(*N*2-desoxyguanosinyl) tamoxifen.[90]

Overall, it appears that specific metabolic pathways in rat liver predispose that species to liver carcinogenesis.[91] α-Hydroxytamoxifen (**9**) is a poorer substrate for human sulfotransferase (that is apparently necessary for adduct formation[89]) than the rat form of the enzyme. Conversely, glucuronidation, which would detoxify α-hydroxytamoxifen, predominates in human hepatocytes.[92] Additionally, primates may produce inhibitory metabolites to suppress liver carcinogenesis.[93] Another way of considering carcinogenesis, particularly in the endometrium, is to evaluate specific genomic changes between patients who take tamoxifen and those who do not. None have been found.[94]

Overall, this area of drug evaluation is extremely important for understanding the relevance of species-related toxicity to clinical practice. Phillips has recently reviewed[95] the genotoxicity of tamoxifen. In his conclusion he raises the concept of whether tamoxifen is a genotoxic carcinogen in the rat but a nongenotoxic carcinogen in humans. This may make tamoxifen unique.

**(c) Tamoxifen and Endometrial Cancer.** Tamoxifen can encourage the growth of human endometrial cancer in athymic mice.[96,97] These laboratory findings and three case reports in patients[98] prompted a closer examination of the association between tamoxifen and the risk of endometrial cancer.[14,99] After 10 years of clinical reporting (1989−1999), it appears that tamoxifen causes a 3- to 4-fold increase in endometrial cancer in postmenopausal patients, but there is no association between tamoxifen use and endometrial cancer risk in premenopausal women.[15,100] Bernstein[17] has noted that postmenopausal women with preexisting risk factors for endometrial cancer (i.e., prior use of HRT and increased body weight) are the women at most risk for endometrial cancer during tamoxifen treatment. In other words, a postmenopausal woman without risk factors is not at substantially increased risk for endometrial cancer during tamoxifen therapy. The issue of the biological significance of tamoxifen action on uterine tissue is controversial. Tamoxifen increases stromal thickness,[101−103] and there is an increase in hyperplasia[104] and polyps,[13,105,106] but the question of techniques for safety monitoring remains unclear.[107−112] Guidelines have been offered to advise clinicians;[113,114] however, patients with symptoms of spotting need immediate follow-up.[115]

**(d) Raloxifene: Antiestrogen Action.** Raloxifene (**2**) has not been systematically evaluated as a breast cancer treatment. Early clinical appraisal of LY156758 (now called raloxifene) in the mid-1980s showed no activity in therapy-resistant advanced breast cancer,[116] although high-dose raloxifene (300 mg daily) recently showed modest activity in ER-positive breast cancer.[117] These data are consistent with the laboratory finding[118,119] that raloxifene is less effective than tamoxifen (**1**) in animal models of breast cancer. The fact that raloxifene has extremely poor (2%) bioavailability[120] because of rapid first-pass phase II metabolism suggests that long-acting agents are required for the treatment of breast cancer. Nevertheless, on the basis of the hypothesis that raloxifene could reduce the incidence of breast cancer as a beneficial side effect of the prevention of osteoporosis,[121] the placebo-controlled trials with raloxifene have been monitored for changes in breast cancer incidence. There are two separate databases to test the hypothesis. First, an ongoing single trial entitled Mulitple Outcomes of Raloxifene Evalutation (MORE) has randomized 7704 postmenopausal women (mean age of 66.5 years) who have osteoporosis (hip or spine bone density at least 2.5 SD below normal mean or have vertebral fractures) and no history of breast or endometrial cancer, into groups taking placebo or 60 or 120 mg of raloxifene daily. Results at 3 years with a total of 40 cases of confirmed breast cancer indicate a 70% reduction in risk of breast cancer.[122,123] The second database pools all placebo-controlled trials and includes 10 553 women monitored for an average 3 years. In this younger group of women, a 54% reduction in the incidence of breast cancer was observed in the raloxifene-treated women.[124,125]

Raloxifene is receiving a rigorous evaluation in the human uterus. In women prescreened to ensure the absence of preexisting endometrial abnormalities, raloxifene does not show an increased endometrial thickness.[126−130] Data from postmenopausal women suggest that raloxifene is not associated with vaginal bleeding or an increased endometrial thickness.[122,126] To date, raloxifene is not associated with an elevated risk of endometrial cancer but laboratory studies demonstrate that the drug will support the growth of a tamoxifen-stimulated endometrial cancer transplanted into athymic mice.[131,132] However, the growth response of human endometrial carcinoma to raloxifene under laboratory conditions is not as much as that of tamoxifen or toremifene.[133]

A consistent finding is an increased incidence of hot flashes and other climacteric symptoms with raloxifene,[134−136] which may be an expression of antiestrogenic action. Clearly, improvements in drug design and targeting should be focused on converting the antiestrogen action at this target to an estrogen-like action.

**(e) Raloxifene: Partial Estrogen-like Action.** Preliminary studies in 251 normal postmenopausal women randomized into groups taking placebo, raloxifene (200 mg daily), raloxifene (600 mg daily), or Premarin (0.625 mg daily) show decreases in serum alkaline phosphatase, serum osteocalcin, urinary pyridinoline, and urinary calcium excretion with raloxifene that were no different than with estrogen.[137] However, the doses of raloxifene were far higher than the 60 mg daily currently recommended for the prevention and treatment of osteoporosis. Evaluation of raloxifene (60 mg daily) on bone remodeling in early postmenopausal women, using calcium tracer kinetic methods, found that although remodeling suppression was greater for estrogen, the remodeling balance was the same for the two agents.[138] These results are consistent with the finding that raloxifene increases bone density by 2.4 ± 0.4% in the lumber spine and 2.4 ± 0.4% for the total hip.[126] Raloxifene has recently been shown to decrease spine fractures by 40%,[139] although there is no significant decrease in hip fractures.

Raloxifene decreases LDL cholesterol and homocysteine,[140] but HDL cholesterol remains unchanged.[126,141] Additionally, triglycerides do not rise during raloxifene treatment. Blood clots with raloxifene occur at the same frequency as observed with HRT. A recent analysis of the incidence of CHD in women at high risk during treatment with raloxifene for the prevention of osteoporosis noted a 40% decrease in those taking raloxifene.[142]

**(f) Carcinogenesis.** Toxicity studies in the rat do not show an increase in liver cancers, but a significant increase in ovarian cancers occurs (FDA hearings for the approval of raloxifene for the prevention of osteoporosis). This observation may not be relevant because the clinical use of raloxifene is confined to postmenopausal women.

## Current Clinical Evaluation of Tamoxifen and Raloxifene

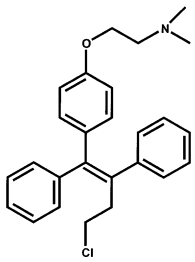
The study of tamoxifen and raloxifene (STAR) is a phase III double-blind trial that is randomizing 22 000

high-risk postmenopausal women to receive 5 years of either tamoxifen (20 mg daily) or raloxifene (60 mg daily). By the end of 2002, more than 14 000 women had been randomized. The results of the study, i.e., a comparison of breast cancer incidence, fractures, coronary heart disease, endometrial cancer, and other side effects, will be available in 2005.

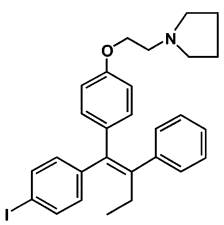
Currently, the MORE trial of raloxifene and placebo is being continued for an additional 4 years to evaluate accumulative fractures and breast cancer incidence with a total of 7 years of raloxifene. Since raloxifene must be given indefinitely to prevent osteoporosis, it is essential to evaluate breast safety. To address the question of whether SERMs reduce the risk of coronary heart disease, 10 101 women at risk have been recruited to receive placebo or raloxifene (60 mg daily) in the study entitled raloxifene use for the heart (RUTH).[143] Volunteers will receive 5 years of treatment. The study will be terminated after a minimum of 1670 participants experience a primary coronary end point. Data are also being collected on risk reduction for invasive breast cancer.

## Clinical Evidence from Tamoxifen Derivatives

During the past 15 years, several triphenylethylenes have been evaluated as breast cancer treatments with the goal of both reducing toxicity and increasing the response rate as an antitumor agent. Three drugs have extensive clinical testing, toremifene (**13**), droloxifene




**(13)** toremifene




**(14)** idoxifene

(**4**), and idoxifene (**14**), but only toremifene (Fareston) is available for the treatment of stage IV breast cancer in postmenopausal women and is currently being tested as an adjuvant therapy.[144]

Toremifene, or chlorotamoxifen (**13**), has been thoroughly investigated in the laboratory[145−148] and has antitumor activity in carcinogen-induced rat mammary cancer[148−150] but is less potent than tamoxifen. Originally, it was believed that toremifene would be active in ER-negative tumors,[148] but extensive studies in athymic mice demonstrated that this was unlikely to be true.[151] Toremifene has been tested extensively in phases I−III clinical trials.[152−155] As predicted from the reduced potency in animal studies, the dose required for activity is 60 mg of toremifene daily (tamoxifen is used at 20 mg daily). The side effects are similar to those of tamoxifen, and as with tamoxifen, the responses are observed in ER-positive tumors. However, because adjuvant therapy with tamoxifen is standard throughout the world, issues of cross-resistance of tamoxifen and toremifene are important considerations for the use of toremifene in recurrent breast cancer. Laboratory studies by Osborne and co-workers[156] have demonstrated that toremifene-stimulated tumors can develop from MCF-7 breast cancer cells transplanted into athymic mice. Toremifene is cross-resistant with tamoxifen in tamoxifen-stimulated breast cancer in the laboratory.[157] Similarly, crossover clinical trials demonstrate that there is little possibility of a second response to toremifene after tamoxifen failure.[158,159]

The interesting property of toremifene is the reduced liver carcinogenicity in the rat.[50,160] Toremifene produces fewer DNA adducts than tamoxifen;[50] however, there are reports of DNA damage[161] and the drug can still act as an estrogen-like tumor promoter in the rat.[52] The lower potential to produce DNA adducts probably reflects an inability of toremifene to produce the α-hydroxy metabolite observed with tamoxifen (**9**). The chlorine of toremifene would sterically prevent α-hydroxylation.

Issues of the incidence of endometrial cancer during toremifene therapy are controversial. Toremifene can support the growth of tamoxifen-stimulated endometrial cancers in athymic mice,[133] so it would not be unreasonable to predict a modest rise in endometrial cancer in patients treated long term with adjuvant toremifene. The general pharmacology of toremifene in the endometrium and uterus is the same as that of tamoxifen.[162] However, an analysis of side effects in adjuvant studies shows no increases in endometrial cancer with toremifene.[144]

Idoxifene (**14**) is a metabolically stable analogue of tamoxifen synthesized to avoid the toxicity reported with tamoxifen in rat liver.[163−165] Substitution of halogens in the 4 position of tamoxifen is known to reduce the antiestrogenic potency by preventing the conversion of the parent drug to 4-hydroxytamoxifen.[166] Additionally, it was argued[164,165] that by reduction of demethylation, liver toxicity would be reduced because increased local levels of formaldehyde would not occur. Unfortunately, the increased metabolic stability also increases toxicity, since the drug cannot easily be detoxified. Idoxifene accumulates so that high parent drug levels are observed that can cause death in mice[157] at doses that are safe for tamoxifen.

Idoxifene inhibits the growth of carcinogen-induced rat mammary tumors[167] and MCF-7 tumors grown in athymic mice.[168,169] Idoxifene has been reported to develop acquired antiestrogen resistance more slowly than tamoxifen.[168] However, there appears to be cross-resistance in laboratory models of tamoxifen-stimulated growth.[157] Idoxifene has been evaluated as a breast cancer treatment for postmenopausal patients,[170,171] but planned studies to evaluate idoxifene as a preventive for osteoporosis have not been pursued because of concerns about uterine prolapses. This side effect is not seen with tamoxifen.

Droloxifene, or 3-hydroxytamoxifen (**4**), is a mimic of a tamoxifen metabolite 3,4-dihydroxytamoxifen (**5**) that has weak estrogenic properties in the mouse[172,173] and weak antiestrogenic actions. The drug has antitumor activity in laboratory animals[174] but does not form DNA adducts under laboratory conditions or produce liver tumors in rats.[174,175] These data lead to the extensive clinical testing of droloxifene in stage IV breast cancer,[176] but drug development as an approved breast cancer treatment has not been pursued. As might be

anticipated for an agent that has rapid clearance because it is rapidly conjuged by phase II metabolizing enzymes,[177,178] doses of 60 mg daily were used to determine antitumor actions in clinical trial.

Droloxifene maintains bone density in rats,[179−181] but clinical trials for the prevention of osteoporosis have not been reported.

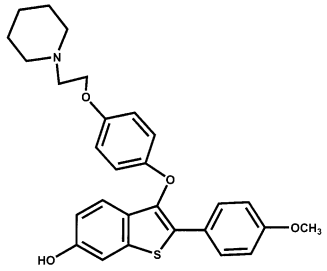
The effects of droloxifene on lipid parameters in postmenopausal women have recently been reported.[182] Droloxifene produced a greater reduction in low-density lipoprotein cholesterol and lipoprotein(a) than conjugated estrogen. However, like tamoxifen and raloxifene, droloxifene does not increase high-density lipoprotein cholesterol. Droloxifene also dramatically reduces fibrinogen.

### Modified Molecules as SERMs

Current interest in new SERM molecules has built on the experience of the prototypes with the goal of enhancing bioavailability or decreasing the prospects of drug resistance in the breast. All compounds under study have predominantly antiestrogenic effects in the rodent uterus with virtually no estrogen agonist properties.

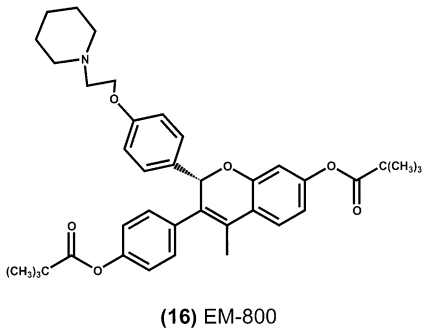
**(a) LY353389 (Arzoxifene).** The chemists at Eli Lilly, Indianapolis, IN, have contributed an enormous body of information about the structure−activity relationship of SERMs.[183−187] Replacement of the ketone group of raloxifene with an ether oxygen results in a 10-fold increase in antiestrogen potency both in vivo and in vitro.[188] A methoxy derivative, with improved bioavailability over raloxifene[189] is currently being evaluated as a breast cancer therapy in advanced disease.[190]

Arzoxifene (**15**) is partially cross-resistant with tamoxifen in models of drug-resistant breast and endometrial cancer.[191,192] A recent report[193] demonstrates that arzoxifene is superior to raloxifene as a chemopreventive in rat mammary carcinogenesis.

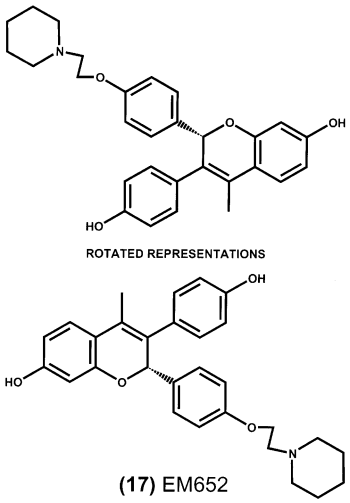


**(15)** arzoxifene

**(b) EM652.** EM-800 (**16**) is a chromene prodrug[194] for the active agent EM-652 (**17**) that is now called SCH57068. The agent is routinely drawn to show the similarity of side chain position to the pure antiestrogen ICI 182,780 (**18**); however, the compound is a SERM.



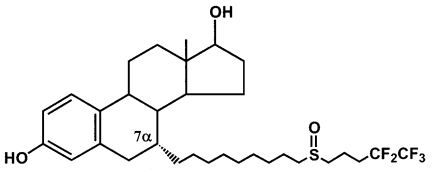
**(16)** EM-800



ROTATED REPRESENTATIONS

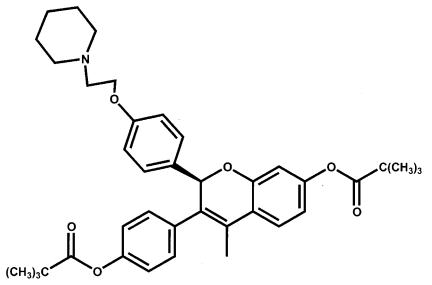


**(17)** EM652



**(18)** ICI 182,780 (fulvestrant)

The advantage with EM-800 and EM-652 is that they are both pure (*S*) enantiomers. Resolution of the active (*S*) enantiomer from the less active (*R*) enantiomers (EM776 (**19**) and EM651 (**20**), respectively) confers higher binding affinity for the ER. A comparison of the potent benzopyran described by Sharma and colleagues[195] referred to as EM312 by Gautier et al.[194] with EM652 on the proliferation of ZR-75-1 and T47D cells shows that EM652 is 9 and 28 times more potent, respectively.[194]



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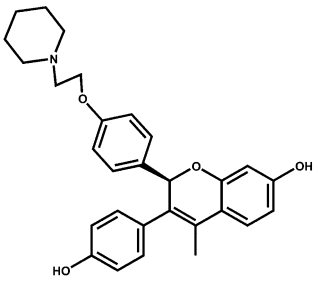
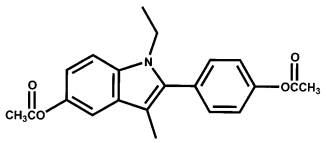


**(20)**

The compound EM800 and its active metabolite EM652[194] are orally active agents with virtually no uterotropic activity. EM800 is an orally active antitumor agent in the DMBA model,[196,197] and long-term studies in the mouse show clear-cut antiestrogenic activity[198] with little or no estrogenic activity compared with either tamoxifen or toremifene.[199,200] The drug is extremely potent against breast and endometrial cancer cells in culture[201,202] and prevents the growth of estrogen-stimulated tumor xenografts in athymic mice.[203] However, unlike ICI 182,780 (**18**), which has an expected negative effect on bone density,[204] EM800 does not decrease bone density in the rat.[205]

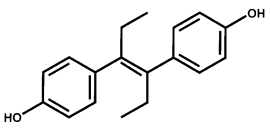
EM652 is misclassified as an orally active pure antiestrogen[200,206,207] and as such could be tested as a second-line therapy following tamoxifen failure. Although the location of the antiestrogenic side chain of EM652 (**17**) is reminiscent of the steroidal pure antiestrogens (**18**), the side chain would seem to be too short for optimal activity.[208] On the basis of the structural similarity of EM652 with other benzopyrans and raloxifene analogues (**2**, **15**) one would predict that EM652 would be a SERM with potential cross-resistance with tamoxifen. A recent report[209] demonstrates that EM652 and raloxifene both have the antiestrogen side chain interacting with aa 351 in the ER. The D351Y ER mutant converts both EM652 and raloxifene to an estrogenic complex, whereas ICI 182,780 is unaffected. On the basis of these data, there is a high likelihood that SCH57068 will fail as a second-line therapy after drug resistance to tamoxifen develops. Unfortunately, only a clinical trial can prove this prediction. Nevertheless, SCH57068 has beneficial effects on bones[210] and lipids so that an application as a SERM would seem to be more appropriate.

**(c) ERA-923.** The structure−function relationships of indole-based antiestrogens have been investigated thoroughly. Early investigations by Von Angerer demonstrated antitumor activity for 2-(hydroxyphenyl)-indoles, but compounds possessed estrogenic activity in the mouse uterus. A study of structure−activity relationships[211] within a series of 2-phenylindoles showed that antitumor activity can be retained with reduced estrogenic activity in the uterus. One of the compounds, zindoxifene (**21**), initially looked promising[211,212] but



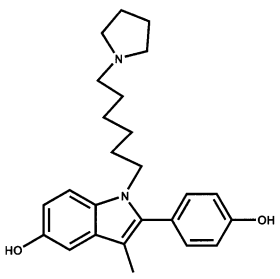
**(21)** Zindoxifene

proved to be inactive as an antitumor agent in phase II clinical trials.[213] Not surprisingly, the deacetylated metabolite of zindoxifene, which has a remarkable structural similarity to DES (**22**), is estrogen-like in



**(22)** diethylstilbestrol

stimulating prolactin synthesis in cells from the pituitary gland and initiating the growth of MCF-7 cells in culture.[214] However, substitution of the indole nitrogen with long aminoalkyl side chains results in the loss of estrogen-like activities in mouse uterus[215] and resulted in the claim that novel pure estrogen antagonists could be synthesized. The compound ZK119010 (**23**)[216] is a



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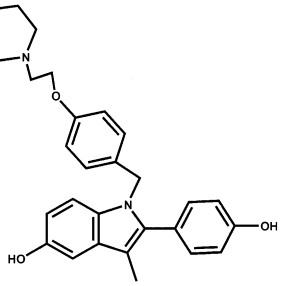
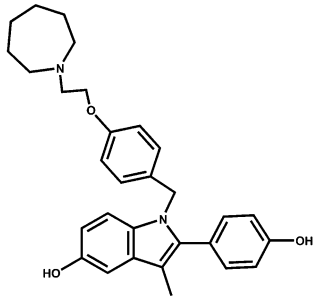


**(24)**

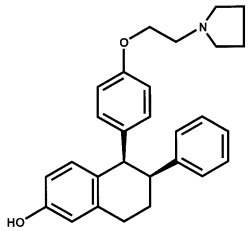
potent estrogen antagonist in rats and mice and apparently formed the basis of molecular modeling to discover ERA-923 (**24**) and TSE−4247 (**25**).[217] Compound ERA-



**(25)**

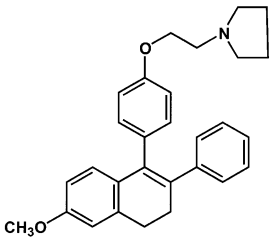
923 is a potent antiestrogen in vivo using mouse xenograft models[218] and is currently in phase II clinical trials for the treatment of hormone-dependent breast cancer. Compound TSE-4247 is effective at protecting bone loss and reducing total cholesterol in ovariectomized rats. It is being advanced to treat postmenopausal osteoporosis.

**(d) CP336156.** A diaryltetrahydronaphthalene derivative referred to as CP 336156 (**26**, lasofoxifene)[219]



**(26)** lasofoxifene

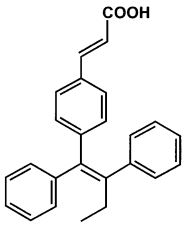
has been reported to have high binding affinity for ER and have potent activity in preserving bone density in the rat.[220,221] The structure of CP336156 is reminiscent of nafoxidine (**27**) (see part 1) if it were to be demethylated in vivo. There are two diastereometric salts. CP336156 is the *l* enantiomer that has 20 times the binding affinity of the *d* enantiomer. Studies demonstrated that the *l* enantiomer had twice the bioavailablity of the *d* enantiomer. The authors ascribed the difference to enantioselective glucuronidation of the *d*



**(27)** nafoxidine

isomer.[219] A recent evaluation of CP336156 (**26**) in the prevention and treatment of rat mammary tumors induced by *N*-nitroso-*N*-methylurea shows activity similar to that of tamoxifen.[222]

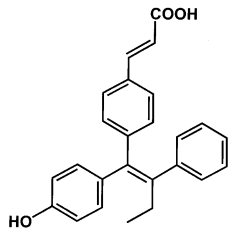
**(e) GW5638.** GW5638 (**28**) has had an interesting



**(28)** GW 5638

development. The compound was discovered by Willson and colleagues in 1994 at Glaxo Wellcome in North Carolina and was reported to be an effective agent for the preservation of bone density but with minimal uterotropic activity.[223] The compound does not have the usual tertiary amino antiestrogenic side chain but a shorter allylcarboxylic group on a triphenylethylene carrier molecule. McDonnell[224] demonstrated that the molecule has a unique classification as a SERM; i.e., the folding of the ER around the negatively charged side chain clearly produces subtle, but significant, differences on the surface of the ER.[225] This hypothesis has been confirmed using molecular modeling.[226] The carboxylic side chain emerges from the SERM ER complex and repels aa 351, thereby changing coregulator binding (Figure 1), as hypothesized. This interesting new SERM is currently being rigorously tested in new and established animal models of drug resistance to tamoxifen to establish a lack of cross-resistance to tamoxifen and low potential to enhance endometrial cancer growth. GW5638 is apparently non-cross-resistant with tamoxifen in the tamoxifen-stimulated MCF-7 model in athymic mice.[227,228] Extensive clinical trials would be appropriate if further laboratory data are obtained. This is particularly true for rat liver carcinogenesis, since GW5638 is a tamoxifen analogue and could potentially undergo α-hydroxylation. Although the link between rat liver carcinogenesis and carcinogenesis in humans is not established,[95] it would be wise to determine whether GW5638 is a liver carcinogen in the rat. If it is, then a simple molecular modification could be made to permit a broader clinical use.

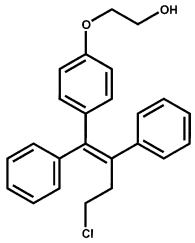
One intriguing claim is that GW5638 (or rather its hydroxylated metabolite GW7604 (**29**)) is really a pure antiestrogen because it has the ability to reduce ER levels and has no uterotropic activity.[225] GW5638 does reduce ER levels because it enhances ubiquitination,[229] but the SERM action does not classify the compound



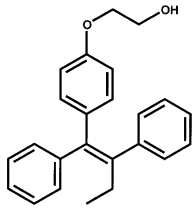
**(29)** GW 7604

as a pure antiestrogen. It is possible that the two exposed negative changes on the GW7605 surface could alter the positioning of helix 12, thereby enhancing receptor destruction.

**(f) Deaminohydroxytoremifene (FC-1271a).** Deaminohydroxytoremifene (**30**) is related to the deaminated metabolite Y of tamoxifen.[230] Metabolite Y (**31**) has a



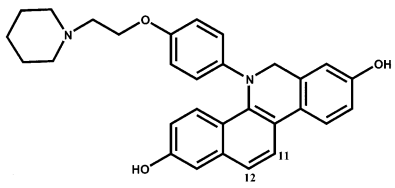
**(30)**



**(31)**

very low binding affinity for the ER[230,231] and has weak antiestrogenic properties compared with tamoxifen. Similarly, deaminohydroxytoremifene has very weak estrogenic and antiestrogenic properties in vivo[232] but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. Preliminary clinical data in healthy men and postmenopausal women demonstrate pharmacokinetics suitable for daily dosing between 25 and 200 mg.[233] Overall, the concept of developing a bone-specific agent is reasonable, but the inability to compete with estrogen at the breast or uterus may not result in the prevention of breast and endometrial cancer as beneficial side effects.

**(g) Achiral SERM.** The photochemical synthesis of a new SERM class has recently been reported.[234] The lead compound (**32**) does not stimulate the immature



**(32)**

rat uterus between 0.1 and 10 mg/kg but lowers circulating cholesterol. The compound has a low $K_i$ of 0.28 nM for ERα. The SERM has a structural similarity to EM652 (**17**).

## Pure Antiestrogens

By definition, a compound that is a pure or complete antiestrogen in all laboratory tests is unlikely to be selectively active in humans. To produce antiestrogen

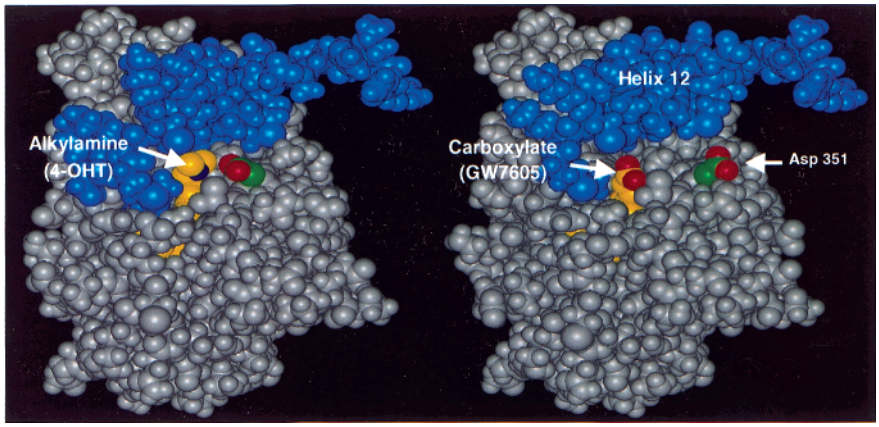
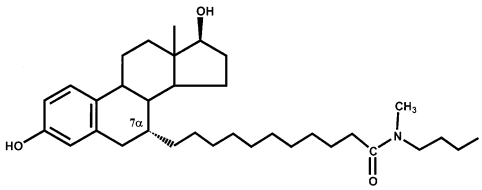



**Figure 1.** Comparison of the external surfaces of the 4-hydroxytamoxifen (4OHT left) ERα ligand binding domain complex with the GW7604 complex. The shift in aspartate 351 because of repulsion by the exposed carboxylate of GW7604 is hypothesized to reduce coactivator binding or enhance corepressor binding. Reprinted with permission from *Endocrinology* (page 844, Figure 8B).[226] Copyright 2001 The Endocrine Society.

action at all sites, pure antiestrogens have a unique mechanism of action. The compounds have zero intrinsic activity by preventing the formation of a transcription complex at target genes, and the ligand enhances the ability of the ER complex to be destroyed. The use of pure antiestrogens for the adjuvant treatment of breast cancer is appealing if the benefits in lives saved are not confounded by increases in osteoporosis and coronary heart disease. Although pure antiestrogens were first described by Wakeling and Bowler[235] 15 years ago, there is remarkably little information about adverse effects of these drugs on bones and lipids. Drug development has been slow. The concern about increased risk of osteoporosis and coronary heart disease, as well as problems with drug delivery, has encouraged the development of aromatase inhibition as an alternative strategy for "antiestrogen action" without the endometrial complications observed with tamoxifen. Nevertheless, there is clearly a strategic role for the pure antiestrogen fulvestrant (**18**) in the treatment of advanced breast cancer[236,237] when the patient may or may not have received 5 years of adjuvant tamoxifen. Additionally, a pure antiestrogen could find a role in the adjuvant treatment of high risk (four or more lymph node positive) breast cancer. It is clear, however, that the application of a pure antiestrogen will compete with the established methods of estrogen deprivation with aromatase inhibitors (postmenopausal) or LHRH superagonists (premenopausal). One aromatase inhibitor, anastrozole, is currently completing adjuvant clinical trials where it is being tested either alone versus tamoxifen or in combination with tamoxifen. The result in the ATAC (anastrozole vs tamoxifen vs the combination) trial shows a significant enhancement of disease-free survival for anastrozole compared to that for tamoxifen, but the combination is only as good as the tamoxifen alone.[44] This result would be anticipated because the partial agonist tamoxifen will occupy the waiting ER and produce a partial agonist effect at target tissues. Conceptually, the fact that there are only low circulating levels of estradiol and estrogen in postmenopausal women that could ever reverse tamoxifen would predict that there could be no advantage in adding an antiestrogen to an aromatase inhibitor. The situation is unlike premenopausal women who have high circulating levels of estrogens in response to tamoxifen that could blunt antiestrogen action.[22,238,239] In this situation, the addition of an LHRH superagonist such as leuprolide or goserolin seems to be a valid treatment strategy to cause a medical oophorectomy.[240,241]

Since the pure antiestrogens are currently being evaluated in clinical trial, it is important to consider the pharmacology of established compounds to provide guidelines for assessing the veracity of the claims for any new drug. The first compound to claim the title of pure antiestrogen ICI 164,384 (**33**)[235] was a derivative




**(33)** ICI 164,384

of estradiol, but numerous nonsteroidal compounds claim "pure antiestrogen" status. This is not a surprise because MER-25 (see part 1), the first nonsteroidal antiestrogen,[242] was almost completely devoid of estrogen-like properties. However, the fact that MER-25 had a very low affinity for ER strongly suggests that the molecule could not maintain a transcription complex by binding coactivators.[243] If the goal of targeted therapeutics is to reduce toxicity by developing highly potent drugs, then high-affinity compounds with novel mechanisms of action and efficient pharmacokinetics must be discovered. This goal has been partly achieved with steroidal compounds, but drug delivery is a concern.

## Steroidal Compounds

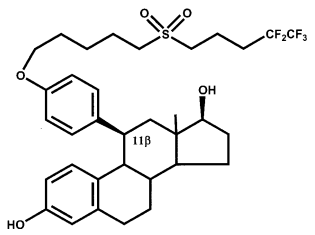
The pure antiestrogens were discovered by Wakeling and colleagues.[235] The lead compound, ICI 164,384 (**33**)- is a 7α-substituted derivative of estradiol-17$\beta$ that has no detectable estrogen-like properties in vivo or in vitro.[244] The compound was identified in a search for drugs that do not possess the estrogen-like effects of tamoxifen and that would, as a result, be more effective antitumor agents.

Originally, compounds substituted in the 6 and 7 positions were investigated as potential alkylating

agents,[245] but the observation that the ER could be purified on resin columns containing estradiol-17$\beta$ with a 7$\alpha$-carbon chain linker of 10 atoms[246] opened the door to the drug discovery process. The structure−activity relationships are well established: 7$\beta$ substitutions are ineffective at producing antiestrogenic activity and the length of the carbon chain determines optimal activity for 7$\alpha$ substitutions.[208] The compound ICI 182,780 (**18**) is more potent than ICI 164,384[247] and contains terminal fluorine atoms to retard side chain metabolism to estrogen. Although the pure antiestrogen ER complex can be classified as having zero intrinsic activity, there is another dimension to the mechanism of the pure antiestrogens that appears to be unique. Initially, it was believed that pure antiestrogens prevent the dimerizations of receptor complexes, thereby preventing the binding to EREs.[248] Clearly, if receptor complexes do not bind to any ERE, then no gene can be activated and the compound would be "a pure antiestrogen". However, investigators[249,250] have subsequently demonstrated that the pure antiestrogen ER complex does bind to EREs but the transcription unit is inactive. What appears to be unique about pure antiestrogens is the observation that they provoke the rapid destruction of ER in breast cancer cells in culture,[251] mouse uterus,[252] and breast tumors in situ.[253] The ER is synthesized in the cytoplasm and transported to the nucleus where it functions as a transcription factor. A pure antiestrogen binds to the newly synthesized receptor in the cytoplasm and prevents transport to the nucleus.[254] The paralyzed ER complex is destroyed rapidly[254] by proteasomes.[255] Although normal target cells could be affected in the long term, the loss of a key transcription factor in a breast tumor cell will immediately prevent cell survival and result in tumor regression.

The steroidal pure antiestrogens ICI 164,384 and ICI 182,780 are not cross-resistant with tamoxifen in laboratory models of tamoxifen-stimulated breast[131,256] and endometrial cancer[257] grown in athymic mice. However, drug resistance to ICI 182,780 does occur in cell culture.[258] ICI 182,780 is active as a second-line agent, following tamoxifen failure for the treatment of advanced breast cancer.[259,260] The drug is approved in the U.S. and Great Britain as a second-line therapy for advanced breast cancer.

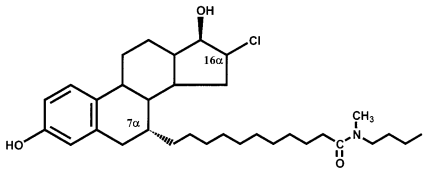
The discovery of ICI 164,384 and ICI 182,780 stimulated a search for other potential agents. The compound RU 58,668 (**34**) is substituted in the 11$\beta$ position with




**(34)** RU 58,668

a side chain of comparable length and physical chemistry as that used for ICI 182,780.[261,262] Studies in vivo and in vitro confirm that RU 58,668 is appropriately classified,[263] but rigorous mechanistic studies have not been published.

The compound EM-319 (**35**) is essentially ICI 164,-

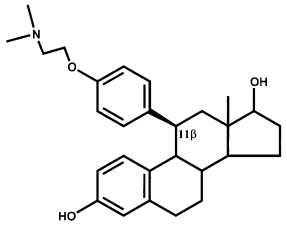



**(35)**

384 but builds on the idea that complete endocrine blockade may ultimately be more effective. A chlorine at the 16$\alpha$ position confers activity as an aromatase inhibitor.[264−267] Clearly a compound of this type with dual activity may be useful. One would predict that an agent with both the properties of an aromatase inhibitor and antiestrogen will have fewer side effects than two separate drugs used in combination. Nevertheless, current clinical studies indicate that a pure antiestrogen and an aromatase inhibitor have equivalent activity in advanced breast cancer.[236,237]

The problems with the pure antiestrogen described thus far are bioavailability and the route of administration. The steroidal derivatives are extremely hydrophobic so that oral administration is unacceptable. The drug fulvestrant (ICI 182,780, Faslodex) is administered as a 1 month slow-release depot injection containing 250 mg. Although this method of administration can be valuable to ensure compliance, patient convenience with monthly visits to the hospital may prove to be unacceptable in some countries. This pharmaceutical dilemma should act as an incentive to discover simple, orally active drugs.

**Interaction of ICI 164,384 with ER.** The crystals of rat ER$\beta$ LBD and ICI 164,384 are internally disordered and cannot be resolved unless treated with *p*-chloromercuribenzenesulfonic acid.[268] This results in a distorted homodimer structure. There are several similarities and differences of the crystal structure of ICI 164,384 when compared to that observed with raloxifene in ER$\alpha$ or ER$\beta$.[269,270] The bulky para-substituted phenyl side chains of raloxifene and 4-hydroxytamoxifen occupy a narrow channel in the ER, pushing H12 aside to silence AF-2. ICI 164,384 adopts a similar binding mode by flipping 180° about its longest hydroxyl to hydroxyl axis. The 7$\alpha$-substituted group is now adjacent to the 11$\beta$ channel, so the side chain can exit the binding cavity (Figure 2). This molecular solution has been suggested previously to describe the antiestrogenic activity of the 11$\beta$-substituted estrogen Ru 39,411 (**36**) and ICI 164,384 (**33**).[271] The unique




**(36)** RU 39,411

aspect of X-ray crystallography is the finding that the long hydrophobic side chain prevents the binding of H12 to the surface of the LBD. Although the side chain exits

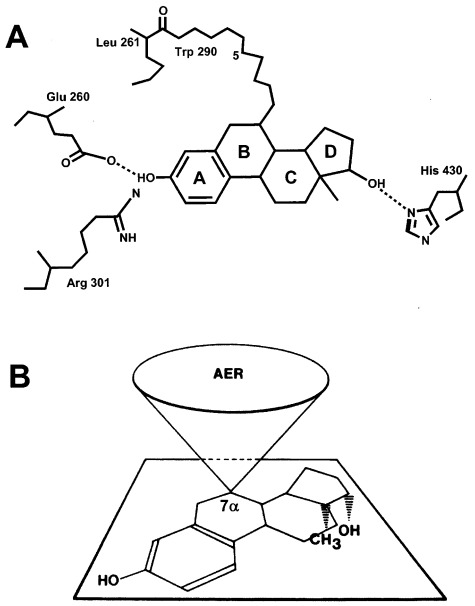



**Figure 2.** Binding mode of the pure antiestrogen ICI 164,-384 in the rat ER$\beta$ ligand binding domain cavity (A). The steroid flips 180° about its longest hydroxyl to hydroxyl axis so that the 7α hydrophobic side chain exits the complex in a manner analogous to that of nonsteroidal antiestrogens (see Figure 7 in part 1).[268] (B) Potential solution to the fit of ICI 164,384 had been proposed previously with the side chain having a large area of influence on many possible binding options in the "antiestrogenic region" (AER).[271]

the binding pocket in a manner identical to that observed with raloxifene, the side chain is bent by 90° at its fifth carbon and binds against the indole face of the Trp290. The antiestrogenic side chain is 6 Å longer than the side chain of raloxifene so that it extends deep into the groove between H3 and H5. As a result, H12 cannot dock on the surface of the LBD. This unique structure presumably results in the premature destruction of the complex by the proteasomes.

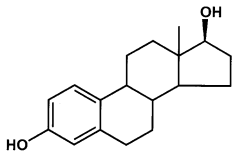
### Targeting Specific Receptors: ERα and ER$\beta$

The relative physiologic importance of ERα, ER$\beta$, and their subcellular interactions has been documented using ERα knockout mice.[272] The most commonly recognized estrogenic responses (uterine weight, vaginal cornification, etc.) are obliterated in ERα knockout mice, which can be considered to be ER$\beta$-dominant.[273−275] However, the ovary secretes high levels of estrogen. The ER$\beta$ knockout, by contrast, has ovarian anomalies.[276]

Although knockout animals can provide important clues to the integration of physiological systems during development, pharmacological agents can emphasize the importance of a receptor system in the adult or during a disease process. Indeed, much of the pharmacology is based on the development of selective agonists or antagonists to treat disease, e.g., α and $\beta$ adrenergic blockers, muscarinic and nicotinic antagonists, and H1 and H2 antihistamines. Clearly, specific agonists or antagonists for ERα and ER$\beta$ might potentially be important therapeutic agents. The complex situation with selective targeting of ERα and ER$\beta$ is that the distribution of the receptors may be exclusively ERα or ER$\beta$ at some target sites but other sites may contain a combination of receptor sites leading to compensatory mechanisms. As a start, the drug discovery process is exploiting relative receptor affinity as a means of establishing drug selectivity. If the ligand has a 1000-fold excess affinity for one receptor over another, this may provide therapeutic selectivity if pharmacokinetics can be controlled.

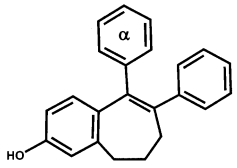
The other complicating situation is that agonist ligands at ERα and ER$\beta$ may have selectivity of action based on the perturbation of their respective complexes. In other words, all estrogens may not be the same and different classes of estrogens that exist by complexing with ERα may produce agonist and antagonist actions at ER$\beta$ sites based on the structure of the complex. The reason for this is the recognition that ER$\beta$ has an impaired AF-1 domain compared with ERα[277] so that the necessary synergy with AF-2 is dramatically reduced. Progress in receptor selectivity will be evaluated on the basis of both structure−function relationships and relative receptor affinity.

Estrogens can alter the folding of the ERα complex into two discrete shapes. The essentially planar estrogens, e.g., estradiol (**37**) or DES (**21**), are class 1 (type




**(37)** estradiol-17β

1), whereas angular estrogens based on triphenylethylene[278] (**38**) or novel piperazine (**39**) and imidazolines

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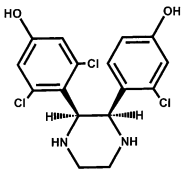
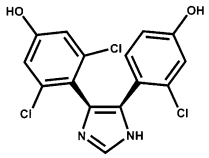



**(39)**

(**40**) are class 2 (type 2) estrogens.[279,280] Class 1 estro-




**(40)**

gens will use the AF-2 coactivator binding site (see Figure 5 in part 1) that synergizes with AF-1 to produce optimal estrogen-like actions. In contrast, an angular estrogen such as a triphenylethylene will bump into L540 on the underside of helix 12 and the ligand is more likely to fit into the 4-hydroxytamoxifen ER structure (Figure 3), thereby exposing the surface D351 once the helix 12 is repositioned in the AF-2 silenced conformation (Figure 4). The estrogen-like actions of an angular estrogen must then occur at the AF-2b coactivator site through D351 and select charged amino acids on the surface of helix 12.[279] AF-1 is essential for this activation process because unlike AF-2, AF-2b cannot act independently.[281] This concept, with a reliance on AF-1 for

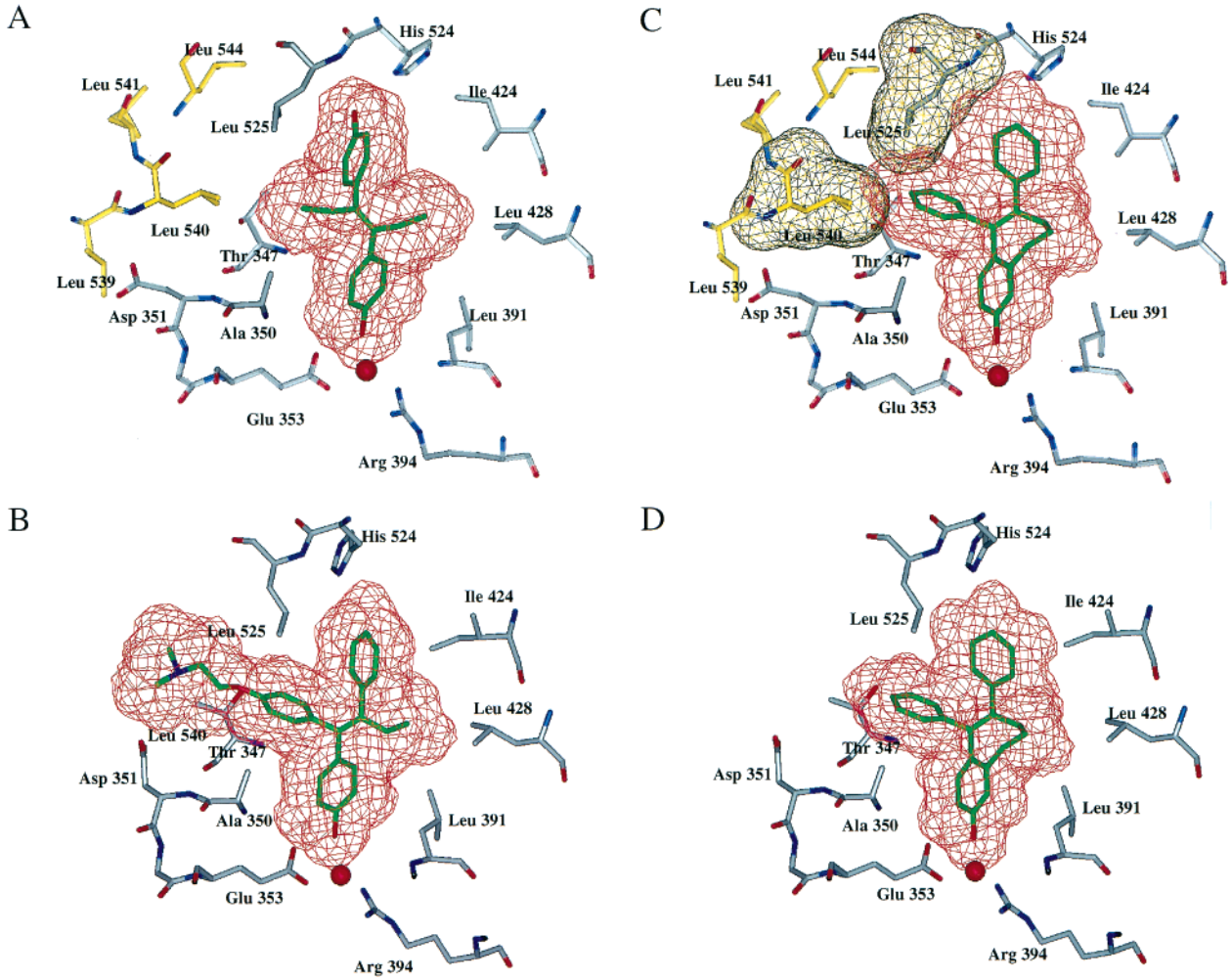
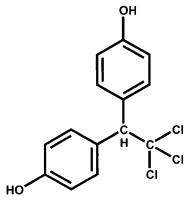



**Figure 3.** Cross-section views of the ERα ligand binding site. van der Waals surfaces of ligands are represented as a red grid. Helix 12 carbon atoms are shown in yellow. The remaining protein carbon atoms are in gray, and ligand carbon atoms are in green. Oxygen and nitrogens atoms are shown in red and blue, respectively. An ordered water molecule between E353 and R394, and the ligands is shown as a red ball. (A) DES (**22**) bound form. (B) 4-Hydroxytamoxifen (**3**) bound form. (C) Novel triphenylethylene compound (**38**) is docked onto the DES form of the receptor. van der Waals surfaces of L540 and L525 in helix 12 are shown as a yellow grid, which sterically clash with the compound. (D) Novel triphenylethylene compound (**38**) is docked into the 4-hydroxytamoxifen form of the receptor. The conformation of the benzocycloheptene ring on **38** was derived from the small-molecule X-ray structure.[278] Reprinted with permission from *Cancer Research* (page 6621, Figure 3).[279] Copyright 2001 American Association for Cancer Research.

ERα activity with angular estrogens (class 2), can be extrapolated to ligands binding to ER$\beta$.

Gaido and co-workers[282] have noted a differential interaction of the insecticide metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE, **41**) with




**(41)**

ERα and ER$\beta$. HPTE is an agonist at ERα but an antagonist at ER$\beta$.

On the basis of the new mechanistic approach to estrogen action at ERα,[279] it could be supposed that angular estrogens require a dominant AF-1 cell context for activity[283] in ER$\beta$. The AF-1 activity of ER$\beta$ is impaired;[277] therefore, the changes in AF-2 structure observed with SERMs in X-ray crystallographic studies[270] would indicate that the angular estrogens will produce antagonist rather than agonist actions. This would explain the action of HPTE as an antagonist at ER$\beta$.[282] This concept needs to be rigorously tested because angular traditional estrogens may be found to produce antagonist actions if only ER$\beta$ is present in a target site or if there is enough ER$\beta$ to overwhelm the ERα signal transduction pathway.

Endocrine disrupters could potentially produce opposing effects at different receptor sites if the target organ is dominant for one receptor or the other. However, receptor affinity will ultimately decide physiology if the environmental hazard is only parts per million. Nevertheless, the laboratory observation about opposing biologic actions at different receptors is valuable for expanding the ER classification model.

As an aside, the concept of the molecular classification of estrogens[279] that produce partial agonists might also apply to past reports by Pento and Magarian[284−286] with

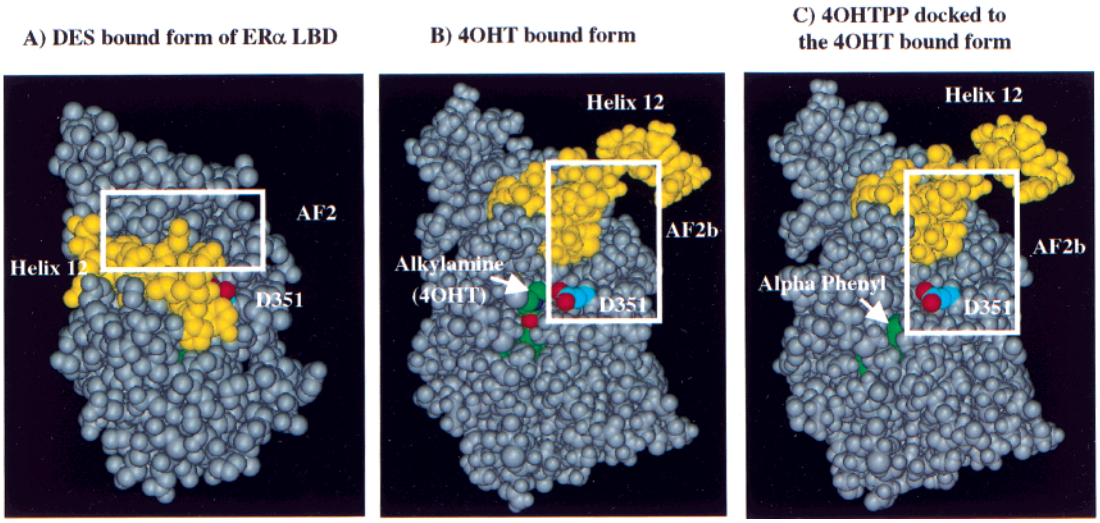
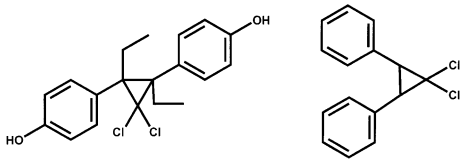



**Figure 4.** Surface location of D351 and different conformations of helix 12 in various ER complexes: (A) DES (**22**) bound form; (B) 4-hydroxytamoxifen (**3**) bound form; (C) novel triphenyl compound (**38**) docked in the 4-hydroxytamoxifen bound form. The α-phenyl of **38** forces the repositioning of helix 12 to expose the AF-2b site (D351 plus select amino acids on helix 12). Reprinted with permission from *Cancer Research* (page 6622, Figure 4).[279] Copyright 2001 American Association for Cancer Reserach.

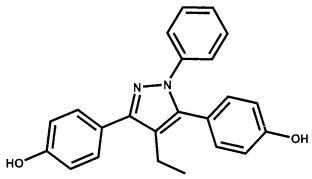
substituted cyclopropane derivatives. A trans-substituted compound (**42**) is an estrogen, but the cis-substituted derivative known as analogue II (**43**) is weakly




(42) (43)

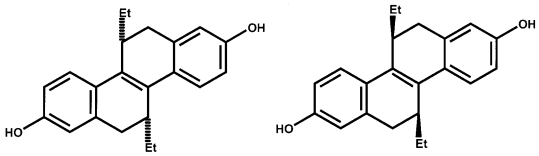
estrogenic in mouse uterus (not surprising because the compound has no hydroxyls) but had antiestrogenic properties. Removal of the chlorine atoms destroys activity.

Benita and John Katzenellebogen are exploring the structure−function relationship of a broad range of ER ligands.[287] In a preliminary study, they showed that an aryl-substituted pyrazole (**44**) has 120 times the potency




(44)

for stimulating ERα versus ER$\beta$. The compound, however, is an ERα agonist. In contrast, the *R*,*R* enantiomer of tetrahydrochrysene (THC, **45**) is an antagonist at

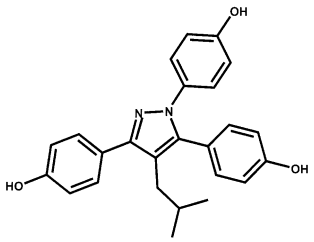



(45) (46)

ER$\beta$. This is because the *S*,*S*-THC enantiomer (**46**) is an agonist at ERα and ER$\beta$, whereas the *R*,*R*-THC enantiomer is an antagonist at ER$\beta$ with only weak affinity for ERα.

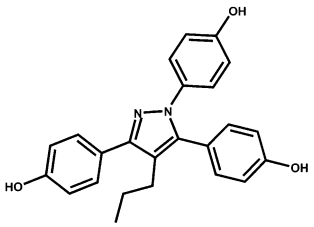
The ERα and ER$\beta$ ligand binding domain and *R*,*R*-THC have recently been crystallized,[288] and the results provide an interesting insight into novel drug mechanisms. While *R*,*R*-THC is sealed inside the ERα ligand binding domain by helix 12, this does not occur with *R*,*R*-THC when bound to ER$\beta$. The ligand prevents the closure of helix 12 over the ligand binding pocket, and the complex adopts a antiestrogenic conformation despite the fact that it has no bulky side chain as required for all other antagonists.[268,270] Apparently, *R*,*R*-THC stabilizes a nonproductive conformation of key residues actually inside the ligand binding pocket, thereby preventing the progression to an active agonist complex. This passive form of antagonism could clearly be exploited by further drug design to target tissue sites that are dominant for ER$\beta$.

The Katzenellenbogen laboratory[289] has identified an interesting series of substituted pyrazoles and furans with estrogenic specificity for ERα. They originally found that certain pyrazoles with a 1,3,5-triaryl-4 alkyl pattern were very selective for ERα with regard to affinity, potency, and efficacy. In particular, one pyrazole (**47**) had the highest ERα binding affinity but



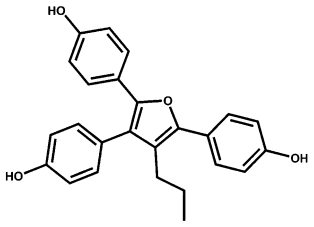

(47)

another pyrazole (**48**) had the greatest ERα subtype selectivity.[290,291] In a related study, substituted furans



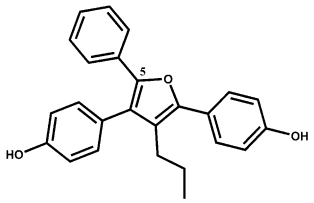
**(48)**

were investigated.[289] A triphenolic derivative (**49**) had



**(49)**

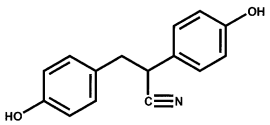
excellent selectivity for ERα with potent agonist activity, whereas no activity was observed (agonist or antagonist) with ER*β*. Interestingly, removal of the hydroxyl at the 5 phenolic substitution (**50**) resulted in estrogen-like



**(50)**

action at ER*β*. It is possible that the helix 12 of ER*β* can now effectively seal[270] the compound into the ligand binding domain to activate AF-2. Unfortunately, there are no X-ray crystallographic data of a full estrogen agonist liganded with human ER*β*, so the concept cannot be confirmed.

Interesting leads to ER*β* specificity are also being found with other simple molecules.[292] The compound DPN (2,3-bis(4-hydroxyphenyl)propionitrile (**51**) has a



**(51)**

70-fold higher binding affinity and 170-fold higher potency in activating ER*β* compared to ERα. Replacement of the CN group with acetylene or a polar function, which mimics the linear geometry and polarity of the −C≡N group, demonstrates that it is essential for ER*β* selectivity. Furthermore, addition of a nitrate substitution *β* to the first in DPN or an *o*-methyl on the aromatic rings increases ER*β* selectivity.[292]

Currently, a search for completely ERα and -*β* selective agents is a priority so that new medicines can be applied to disease treatment without the concern of multiple receptor interactions. Clearly, if a drug is an antagonist at ER*β* but an agonist at ERα, then this might prove to be unsuitable if the specific blocking of one receptor is required. In a heterogeneous distribution, ERα agonist complexes would overwhelm the antagonist ER*β* complexes. However, if the drug discovery assay is an artificial signal transduction pathway based on an estrogen response element, then complex physiology could be missed. It has been suggested that tamoxifen-stimulated tumors occur through an overexpression of ER*β* at the expense of ERα.[293−295] The theory proposes that tamoxifen ER*β* complexes activate AP-1 responsive genes through a protein−protein interaction. The idea might open up a small but significant escape route for cell cycle blockade. However, the current compounds appear to have been tested only on an ERE reporter systems. It is therefore unclear whether the *R,R*-THC (**45**) will also block the AP-1 response. Nevertheless, the developing knowledge of the crystal structure of the ER*β* binding domain[270,288] will provide valuable clues for further SAR studies.

## Drug Resistance to SERMs

**(a) Clinical Concepts.** Estrogen receptor is expressed in approximately 70−80% of breast cancer, but endocrine therapy with tamoxifen is only effective in half of ER-positive breast cancer patients. There is intrinsic resistance to tamoxifen because the ER is no longer the dominant growth mechanism. Evidence suggests that either there is crosstalk between the ER and members of the erbB growth factor receptor family of membrane receptors to activate the cell cycle or the tyrosine kinase pathway is dominant and ER has become vestigial.[296] One member of the EGFR family erbB2 (HER2/neu) is also associated with a lack of response to endocrine therapy (primarily tamoxifen) in ER-positive breast cancer patients,[297−304] but the concept remains controversial.[305−309] Nevertheless, compelling evidence that HER2/neu can subvert hormone responsive growth completely in ER-positive tumors comes from the laboratory. Stable transfection of MCF-7 breast cancer cells with the HER2/neu gene results in spontaneous growth in athymic mice that is not regulated by tamoxifen.[310] Additionally, blocking HER2/neu and mitogen-activated protein kinase (MAP kinase) signaling pathways in engineered ER-positive cells can enhance tamoxifen action and abrogate antiestrogen resistance.[311]

The second form of resistance to SERMs is acquired during SERM therapy. Tamoxifen is effective in approximately half of the patients with ER-positive tumors. Disease-free survival is increased and survival advantages are noted following 5 years of adjuvant treatment.[4] Acquired resistance occurs following a tumor response when the disease subsequently progresses despite continuing tamoxifen treatment. Recurrences can be ER-negative, but a unique form of tamoxifen drug resistance is tamoxifen-stimulated tumor growth that remains ER-positive.[7,8,312] It is important to stress that the documentation of tamoxifen-stimulated tumor growth has uniformly been observed in stage IV disease where tumor response can be monitored during tamoxifen treatment. Studies with adjuvant therapy show that 5 years is superior to 2 years of treatment in lives saved and patients should

not stop at 2 years for fear of acquired resistance.[4] Some studies of more than 5 years of adjuvant tamoxifen are ongoing in the U.K., but current published results show no advantages for longer treatment but an increase in side effects.[5,6] Either the aromatase inhibitors[44,313] or pure antiestrogens[236,237]are positioned strategically to be the endocrine treatments of choice by replacing tamoxifen once adjuvant testing is complete.

Clearly, all new SERMs targeted to be developed exclusively as breast cancer treatments will have to be tested against an aromatase inhibitor rather than tamoxifen. Nevertheless, the fact that all ER-positive patients have been exposed to 5 years of adjuvant tamoxifen and the fact that tamoxifen and raloxifene are used in high-risk women to prevent breast cancer and osteoporosis respectively, suggest that issues of cross-resistance among SERMs must be considered. Clearly, exposure to one SERM may invalidate the application of a suitable and safe replacement for tamoxifen if tamoxifen-exposed populations are used. Most importantly, cross-resistance must be considered when raloxifene is used to prevent osteoporosis following adjuvant tamoxifen or conversely if tamoxifen is to be used to treat a woman who develops ER-positive breast cancer during raloxifene treatment for osteoporosis. Validated laboratory models can provide an important insight for the appropriate testing of new SERMs. Not only is this to prevent the initiation of an inappropriate but expensive clinical trial that is doomed to failure but also, and more importantly, appropriate laboratory testing can avoid recruitment of women for a study that will not provide benefits.

Drug resistance to SERMs is an extremely important therapeutic issue and critical for the future development of SERMs for prevention or of antiestrogens for breast cancer treatment. Millions of women have been exposed to tamoxifen and raloxifene. A clear definition of the mechanisms involved in SERM resistance will not only provide new targets for the treatment of breast cancer but also may provide clues to understand SERM action around a woman's body. This knowledge will prove to be invaluable to prevent the development of numerous diseases associated with menopause and may open the door to new therapeutic opportunities with other receptor families.

**(b) Laboratory Models.** Tamoxifen and raloxifene-like compounds have been used to develop drug-resistant MCF-7 and other breast cancer cell lines.[314−326] The cells have been used to identify SERM-specific gene products[314−326] and are routinely used in industry to obtain gene expression fingerprints[327] or to perform gene arrays. It is reasoned that the identification of novel signal transduction pathways will provide new targets for drug discovery. Although progress is possible, the value of resistant cell lines may be limited because there is no confirmation that the cells can develop into tumors. Tumorgenesis requires not only a shift in the cellular equilibrium away from apoptosis toward extended survival but also an increased growth that depends on the organized recruitment of oxygen and sources of nutrition. Estrogen can regulate the vascular endothelial growth factor (VEGF) gene;[328] therefore, it would not be surprising if other components of the angiogenic signaling pathway required hormonal influences. Estrogen can also enhance angiogenesis perhaps through the ER$\beta$ pathway in myoendothelial cells.[329] In contrast, the cell culture environment provides uniform oxygen and nutrition, so a critical part of tumorigenesis is never initiated. It has been argued that SERMs can have antiangiogenic effects;[330−335] therefore, a multitarget mechanism of action might occur at both the cancer cell and the tumor growth support systems. Studies in vivo can potentially be used to identify multiple targets for SERM or antiestrogen action.

Until recently, only two model systems were available in vivo to address issues either of the mechanism of action of SERM resistance or of the prediction of cross-resistance to novel SERMs in the laboratory prior to clinical trial. Human endometrial cancers will grow in athymic mice. ER-positive tumors are estrogen-dependent for growth, whereas ER-negative tumors are hormone-independent.[336] Tamoxifen can encourage endometrial cancer growth but not as effectively as estrogen.[96] The fact that tamoxifen prevents the growth of estrogen-stimulated breast cancer but encourages the growth of endometrial cancer in the same athymic mouse[97] raised the possibility that tamoxifen would increase the incidence of endometrial cancer in women.[14,16] The finding of a modest increase in endometrial cancer incidence in postmenopausal women demonstrated the value of the laboratory model. Most breast cancer research on the mechanisms of drug resistance to tamoxifen has used the MCF-7 breast cancer cell line.[337] MCF-7 breast cancer cells can be inoculated into athymic mice and form estrogen-stimulated solid tumors, whereas tamoxifen blocks estrogen-stimulated tumor growth.[338,339] However, continuous treatment with tamoxifen causes the growth of tumors despite tamoxifen treatment.[340] These tumors are transplantable into new athymic mice, and tamoxifen treatment is required for growth.[341,342]

Recently, new ER-positive SERM-resistant models have been reported that might provide further insight into cross-resistance and the mechanism of tamoxifen-stimulated tumor growth. Specific lines of T47D breast cancer cells have been reported to be exquisitely sensitive to estrogen action[343,344] and have been used extensively to study the SAR of nonsteroidal antiestrogens.[345−347] The ER-positive cell line is p53 mutant, and estrogen deprivation for long periods in vitro results in the development of an ER-negative clone C42.[344,348] In contrast, T47D cells implanted into athymic mice retain ER during long-term tamoxifen treatment and can form tamoxifen-stimulated tumors that are transplantable to subsequent generations of mice.[349]

Raloxifene-resistant breast and endometrial tumor models have recently been reported to grow in response to raloxifene or tamoxifen treatment in athymic mice.[350,351]

**(c) Cross Resistance.** Models in vivo provide an invaluable opportunity to study whether human tumors that are resistant to tamoxifen can subsequently respond to a second SERM or antiestrogen. Several SERMs have been tested in the laboratory, and some clinical correlations are available. Toremifene is cross-resistant with tamoxifen in MCF-7 tumors.[156,157] In the clinic, toremifene is cross-resistant with tamoxifen.[158,159] In contrast, raloxifene (**2**) and its analogues LY117018

(see part 1) and arzoxifene (**15**) are cross-resistant with tamoxifen in MCF-7 tumor models[132] but are not cross-resistant in T47D tumors.[192] Raloxifene has some activity in ER-positive breast cancer, but the patients who have been exposed to tamoxifen previously are unlikely to respond.[117] Toremifene (**13**), raloxifene, and arzoxifene are cross-resistant with tamoxifen in the EnCa101 tumor model,[132,133,191] but as yet increases in endometrial cancer have not been noted in clinical studies.[108,144] In these clinical studies, however, patients had no prior exposure to tamoxifen.

There is very little information about the xenograft testing of new SERMs prior to clinical trial, but this should be considered. However, a set of principles is emerging from the laboratory that may guide the design of clinical trials. High doses of SERMs appear to promote the early development of drug resistance, and SERM-stimulated tumors grow more rapidly with high doses than with low doses.[349] This is counterintuitive to the chemotherapy community but may result in bell-shaped dose−response curves during clinical trial. In other words, low doses of a new SERM may perform better than higher doses following tamoxifen. Additionally, any exposure to tamoxifen may reactivate quiescent drug resistance pathways, so an evaluation of a new SERM as a second-line therapy might be inappropriate.[191]

In contrast, the pure antiestrogens are proving to be uniformly non-cross-resistant in tamoxifen-stimulated tumor models. The lead compound ICI 164,384 was first shown not to be cross-resistant with tamoxifen in MCF-7 and EnCa101 models.[121,131,257] These data advanced the development of ICI 182,780 for testing in analogous models.[133,157,256] Current laboratory studies are evaluating the potential of ICI 182,780 to develop drug resistance.[324]

**(d) Potential Mechanism of Resistance.** The possibility that SERMs or antiestrogens are metabolized or converted locally to estrogens has been evaluated[156,352,353] and discounted as a mechanism of SERM-stimulated growth. Additionally, the development of mutant receptors or exon deletions, though attractive as a hypothesis, has not proven to be generally applicable. Indeed, it is unclear whether the accumulation of exon deletions in steroid receptor systems is not a surrogate marker of malignancy and disease progression. Many proteins may be mistranslated because of a general loss of accurate splicing mechanisms.

The unique aspect of drug resistance to SERMs is the initial switch of a mechanism from an antagonist or blocking action by the two ERs to a stimulatory signal. This acquired resistance requires the SERM to occupy the ER because SERM-stimulated tumor growth does not occur either in laboratory models[341] or in the clinic[8] if treatment is withdrawn. The SERM ER complex is clearly a critical focus to mediate a signal transduction pathway that regulates replication.

The molecular mechanism of SERM resistance is probably extremely complex with a compensatory web of interconnected signal transduction pathways that result in cell replication or an inhibition of apoptosis. The general principle is to amplify the weak agonist activity of the SERM ER complex so that selected clones of cells will survive. Although there would be growth advantages to decreasing corepressors or increasing coactivators to amplify the SERM ER signal, it is fair to say that work in this area is currently "work in progress". It is clear from knockout studies with SRC-1 [354] that loss of one coactivator does not inhibit hormone action in the uterus. There is considerable redundancy for coregulators, so survival can occur by recruiting additional coregulators from other members of the coactivator family.

Much recent work on SERM-stimulated signal transduction is focused on the ER$\alpha/\beta$ system and nontraditional (i.e., AP-I) signal transduction pathways. The promiscuous activation of inappropriate genes could lead to the amplification of survival pathways. Additionally, the amplification of surface signaling kinases could activate the quiescent SERM ER complex through novel phosphorylations, thereby subverting a blockade of the cell cycle (Figure 5). The goal of current research is to develop an integrated view of survival pathways so that new treatment and prevention strategies can be advanced to build on current practices. Although many other potential mechanisms of SERM-stimulated growth may occur, only the ideas involving ER$\beta$ (Figures 2 and 3 in part 1) and HER2/neu (Figure 5) will be presented in detail.

The new knowledge about ER$\beta$, signal transduction pathway, and the finding that ER$\beta$ can enhance the estrogen-like effects of tamoxifen or raloxifene through an AP-1[355,356] pathway (Figure 3 in part 1) has become an attractive hypothesis to enhance tumor growth by SERMs. There is evidence that tamoxifen-resistant breast tumors have increased AP-1 dependent transcription and phosphorylated cJun and phosphorylated Jun $NH_2$ terminal kinase activity.[357] The argument can be made that because both ER$\alpha$ and ER$\beta$ SERM complexes activate AP-1 sites in HeLa cells but AF-1 represses ER$\alpha$ activation[355,358] by estrogen, then a signal transduction pathway for SERMs is possible. The concept is supported by mutagenesis studies that by switching AF-1 from ER$\beta$ to ER$\alpha$ then the activation at AP-1 can be increased by raloxifene.[358] Conversely, deletion of AF-1 in ER$\beta$ destroys ligand activation at AP-1.[358] Interestingly enough, replacing the F region from ER$\alpha$ with that of ER$\beta$ causes loss of estrogen inhibitory action at AP-1 sites while still allowing significant tamoxifen activation. Furthermore, deletion of region F from ER$\alpha$ enhances raloxifene activation.[358] Clearly, it is extremely appealing to suggest that a reversal of the signal transduction pathway for agonists and antagonists at ER could be involved in SERM resistance; however, the concept might also be important for understanding SERM action at various target sites around the body. In this regard, there is an interesting reversed response with the regulation of the human quinone reductase gene[359,360] in MCF-7 cells made resistant to 4-hydroxytamoxifen.[361] Tamoxifen enhances gene transcription, but estrogen stops gene transcription. It is suggested that the physiologic benefits of tamoxifen as a chemopreventive could result not only from antiestrogenic actions but also from the activation of detoxifying enzymes. Carcinogens would therefore be unable to become DNA adducts.

Overall, the key to this mechanism of drug resistance to SERMs seems to be the dominant role played by AP-1

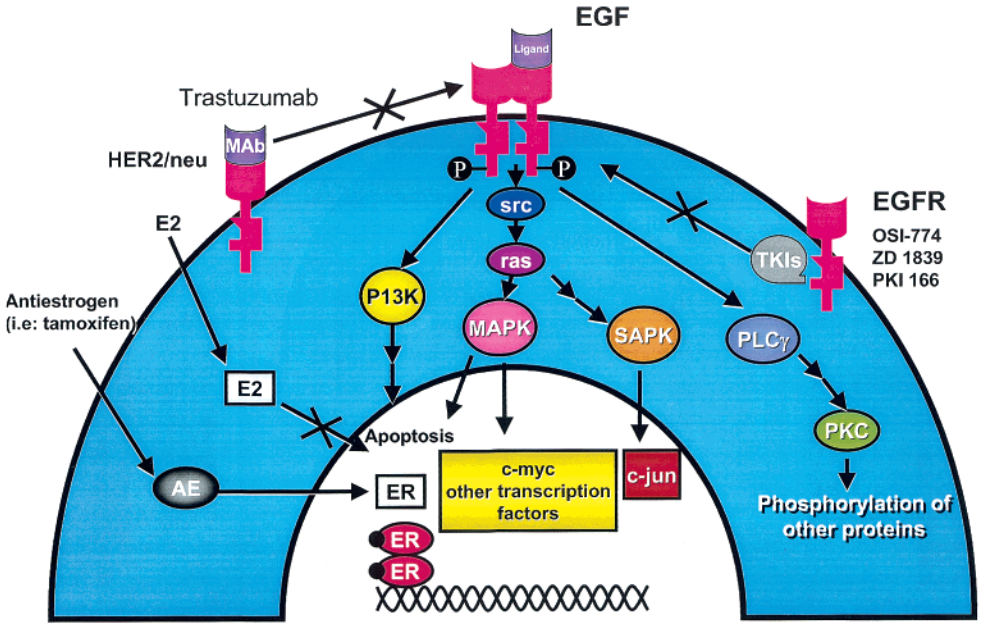



**Figure 5.** Possible signal transduction pathways from the cell surface that can subvert the antiestrogenic actions of tamoxifen at the estrogen receptor (ER) by phosphorylating the ER or other coregulatory proteins. The action of HER2/neu to dimerize and initiate a phosphorylation cascade with the epidermal growth factor receptor (EGFR) can be prevented by the antibody trastuzemab (HERceptin). Alternatively, the tyrosine kinase inhibitors (TKIs) OSI-774, ZD1839 (IRESSA), and PKI 166 can block the phosphorylation cascades.

sites interacting with ER$\beta$ or ER$\alpha$ complexes. Although tamoxifen-resistant tumors retain ER$\alpha$ and some ER$\beta$ can be detected in resistant tumors,[293] there is no dramatic increase in ER$\beta$ levels that could account for tamoxifen-stimulated growth. Less than 1% of ER$\beta$ is noted in breast tumors compared with ER$\alpha$.[362] If ER$\alpha$ is dominant at target sites of interest, the key to selectivity may be direct gene regulation. Interestingly enough, studies of the regulation of the IGF-1 gene by nonsteroidal antiestrogens may be instructive for identifying novel SERM regulated pathways.[363] The IGF-1 promoter can be differentially regulated by ER$\alpha$, but not ER$\beta$, in Hep3B liver cancer cells. Estradiol blocks the actions of raloxifene and raloxifene-like molecules. The CAAT/enhancer binding protein sites and an AP-1 site on the promoter may play a role in regulation.[363] It is therefore possible that a spectrum of gene targets could be activated in tumors by the appropriate conformations of a SERM ER$\alpha$ complex alone.

The possibility that antiestrogen action can be subverted by a signaling cascade from cell surface growth factor receptors and c-onc proteins is an area of intense investigation (Figure 5). If antiestrogen−estrogen receptor complexes can block cell cycle progression,[364−369] then a modified receptor complex could stimulate rather than impede the cell cycle. Deciphering the signal transduction pathways that could potentially link SERM ER complexes with enhanced subcellular phosphorylation events is extremely important for developing new treatment strategies for breast cancer, thereby avoiding or at least delaying drug resistance.

C-src encodes a 60kdal tyrosine-specific protein kinase[370−373] that indirectly stimulates the inositol lipid pathway to release diacylglycerol, which ultimately activates protein kinase C.[374] About 80% of breast and mammary cancer have increased activity for the src tyrosine kinase.[375−377] Two potential pathways (Figure 6) for the activation of the tamoxifen ER complex by

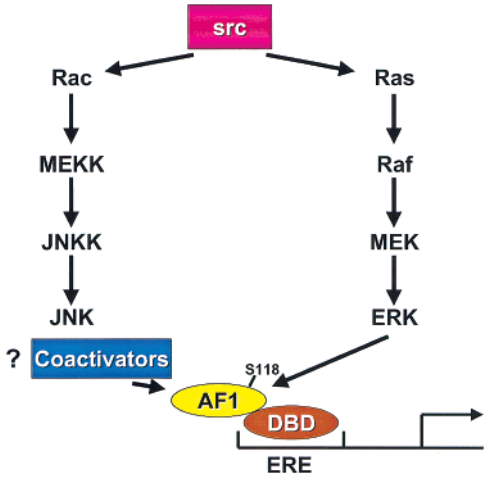



**Figure 6.** Subdivision of phosphorylation cascades that can influence the estrogen-like action of the tamoxifen ER complex.[378] It is possible that the ERK pathway phosphorylates S118 on the AF-1 site of ERE, whereas JNK may phosphorylate other regulatory proteins that are possibly coactivators.

v-src have been described either through serine 118 phosphorylation of AF-1 by the MAP kinases ERK and ERK2 or through indirect activation of AF-1 through the JNK subgroup of MAP kinases (Figure 6).[378] Indeed, coexpression of a constitutively active MEKK 1, but not Raf, into ovarian or uterine cells significantly increased the agonist activity of 4-hydroxytamoxifen to the level observed with estradiol.[379]

Recently, there has been an important finding that a small fraction of membrane-bound ERs can regulate the cell cycle and apoptosis through activation of the src/ERK pathway.[380]

src can mediate the sustained activation of MAPK in various cells,[381,382] which can rapidly affect cell replication and other subcellular events via membrane ERs.[383,384] Interestingly, ER$\alpha$ still appears to respond

to the antiestrogenic effects of tamoxifen (**1**) and ICI 182,780 (**18**) but ER$\beta$-regulated MAPK becomes refractory to inhibition.[384] It is, however, not clear how membrane ERs could modulate hormone responsive cancers and drug resistance, but it is possible that the target site specific effects of SERMs could use these pathways.

Estrogen can activate AKT by causing the binding of ER$\alpha$ to the regulatory subunit of PI3kinase.[385] AKT may modulate coactivator/corepressor functions by phosplorylating serine 167 in ER$\alpha$ (the site is not present in ER$\beta$). Since AKT protects breast cancer cells from tamoxifen-induced apoptosis, then this could be a mechanism of drug resistance.[386]

In contrast, the p38 MAP kinase is involved in cellular processes involving apoptosis[387] and is activated by environmental stress and cytokines.[388] The ERK kinases are activated by mitogenic stimuli, but the p38 pathway appears to phosphorylate p53,[389] which is required to control cell cycle progression and apoptosis. Indeed, MCF-7 cells grow in response to $E_2$ but do not switch on p38 MAP kinase whereas 4OHT induces both p38 MAP kinase[390] and apoptosis. In contrast, cells that are resistant to 4OHT do not switch on p38MAP kinase but $E_2$ switches both p38MAP kinase and apoptosis.

The acquired resistance to tamoxifen described earlier in the section b, Laboratory Models, suggests that the key event for drug resistance is the switch from tamoxifen-induced apoptosis to apoptosis resistant growth. An insight into a potential mechanism that deserves further study involves the nuclear hormone receptor intermediary protein forkhead homologue in rhabdosarcoma (FKHR). The protein participates in several signal transduction pathways regulated by the AKT protein kinases.[391,392] FKHR was recently identified by a yeast two-hybrid screen of a cDNA library from a tamoxifen-resistant tumor using an ER$\alpha$ bait.[393] FKHR will arrest the cell cycle in MCF-7 cells, but estradiol abrogates the effect.[393,394] The related protein FKHRL1 can induce apoptosis, but this process can be prevented by $E_2$ ER$\alpha$ complexes.[394] At present, it is not clear whether this apoptotic pathway is perturbed in tamoxifen-stimulated breast tumors.

Although src was the first oncogene to be recognized with tyrosine kinase activity, the whole family of cell surface growth factor receptors including EGF, HER2/neu (ErbB-2), HER3, and HER4 work together to initiate phosphorylation pathways.[296] Overexpression of HER2/neu results in autophosphorylation and an induction of signaling pathways involving ras,[395−397] and the level of cyclin D is increased.[398] The fact that cyclin D1 can shorten G1 and reverse an antiestrogen-induced arrest suggests both a direct role for HER2/neu in reversing antiestrogen action at the cell cycle via cyclin D and a secondary role by phosphorylation of the SERM ER complex. One can appreciate how the system is consolidated or amplified during long-term antiestrogenic treatment because estrogen down-regulates HER2/neu synthesis by binding coactivators[399] such as SRC-1. SERMs will enhance HER2/neu synthesis by releasing SRC-1 from the ER complex and ultimately encourage the expression of a signal transduction pathway that will subvert antiestrogen action. These observations not only enhance the rationale to employ tyrosine kinase inhibitors following tamoxifen failure but also raise the question of why tamoxifen is so successful as an adjuvant therapy following 5 years of treatment. One would imagine that continuous therapy with a competitive inhibitor of estrogen action is required to prevent growth, but it appears that an optimal duration is sufficient.

### Supersensitization with SERMs

The original concept behind the use of an antiestrogen as an adjuvant treatment for breast cancer was that the antitumor effect was tumoristatic rather than tumoricidal.[400] Since antiestrogens are competitive inhibitors of estrogen action,[401] then it was reasoned that they must be given indefinitely. This concept presaged the application of long-term adjuvant therapy that is now established by clinical trial.[4] However, stopping tamoxifen after 5 years of adjuvant therapy invariably does not result in disease recurrence. Indeed, the patients treated with tamoxifen maintain disease control for at least a decade after tamoxifen is stopped. Clearly, other novel anticancer mechanisms are operating because one would presume that a women's own estrogen would reactivate the ER.

No model systems mimicked the clinical situation of 5 years of adjuvant tamoxifen exposure until MCF-7 breast tumors, with acquired resistance to tamoxifen, were passaged for 5 years in tamoxifen-treated athymic mice.[402,403] Remarkably, tumors that originally grew in response to both tamoxifen and estradiol[341] grew exclusively with tamoxifen. Tamoxifen caused a supersensitivity to physiologic estrogen concentrations, resulting in rapid tumor regression[403] through an apoptotic pathway. These data suggest that 5 years of tamoxifen (or estrogen withdrawal) sensitize micrometastases to the apoptotic effects of a women's own estrogen[403,404] once tamoxifen has stopped. This physiologic response provides a second antitumor mechanism that could possibly be exploited therapeutically. Additionally, these laboratory data could have clinical significance with the aromatase inhibition or pure antiestrogens following the long-term use of SERMs. It might be possible to envisage a cyclic approach to long-term adjuvant treatment with SERMs or aromatase inhibitors. Each 5-year cycle with an inhibitor of estrogen action could be interspersed with courses of HRT. An intermittent estrogenic, rather than a continuing antiestrogenic, environment might be a more beneficial strategy for the patient.

With the concept of the beneficial therapeutic action of estrogen in mind, it is important to note that continued estrogen treatment of animals implanted with tamoxifen-resistant tumors results in the regrowth of some tumors that *again* respond to tamoxifen as an antiestrogen to block tumor-stimulated growth.[403] It therefore appears that, at least for MCF-7 cells, there is a cyclical responsiveness to estrogen and tamoxifen (Figure 7) that may have parallels in clinical practice. Interestingly enough, high-dose estrogen therapy can be beneficial following other endocrine therapies.[405,406] Additionally, there are anecdotal reports of patients who recur several years after stopping 5 years of adjuvant tamoxifen that then respond to tamoxifen again.

The trend toward long-term SERM therapy with tamoxifen and raloxifene as well as the current fashion

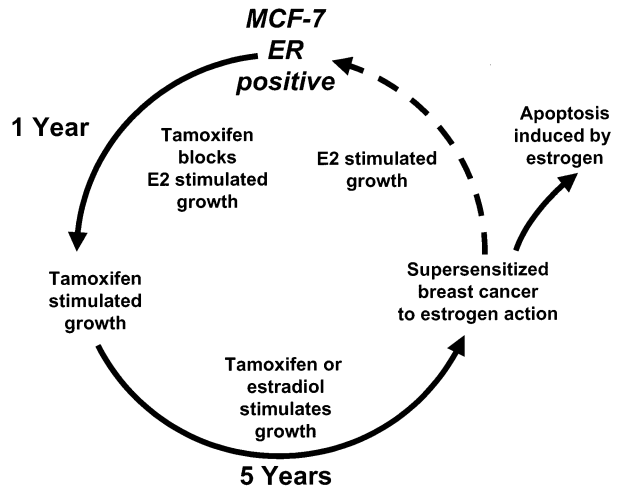



**Figure 7.** Cyclical sensitivity of ER-positive breast cancer cells to tamoxifen and estradiol ($E_2$).[403] Initially, tamoxifen blocks $E_2$-stimulated growth, but eventually tamoxifen and estradiol stimulated growth occurs. This form of drug resistance appears to occur 1−4 years after tamoxifen treatment under laboratory conditions. After 5 years of tamoxifen, the tumors are supersensitive to the apoptotic effects of $E_2$ and tumors regress. A few tumors are, however, reactivated by $E_2$, but tamoxifen again acts as an antiestrogen.

to employ long-term aromatase inhibition for breast cancer treatment[44] raises the question of whether the phenomenon observed with tamoxifen-resistant MCF-7 tumors in athymic mice is unique or can be applied to other systems and endocrine therapies. The recent report by Santen and co-workers[407] that some lines of MCF-7 cells in vitro become supersensitive to the apoptotic effects of estrogen if cells are estrogen-deprived for long periods suggests that estrogen withdrawal as well as tamoxifen is able to produce the phenomenon. Similarly, long-term exposure of ECC-1 ER-positive endometrial cancer cells or MCF-7 breast cancer cells to raloxifene followed by transplantation into athymic mice results in tumors that are tamoxifen- or raloxifene-stimulated for growth but are prevented from growing by estrogen.[350,351]

The mechanism for estrogen-induced apoptosis is unknown despite the interesting finding that a cDNA homologue of Requiem, a mediator of apoptosis, was noted in an MCF-7 variant.[408] One observation worthy of investigation is the finding that T47D breast cancer cells that normally only grow into tumors in athymic mice with estrogen treatment grow spontaneously if stably transfected with PKC α.[409] However, estrogen prevents growth, and physiologic estrogen induces apoptosis in growing tumors. In this model, there is no effect of estrogen on apoptosis of transfected T47D cells[410] in cell culture and the inhibitory action of estrogen is only observed in vitro.[409] Clearly, the animal model data suggest a complex interplay between epithelial and stromal cells that leads to persistent apoptosis and tumor regression.

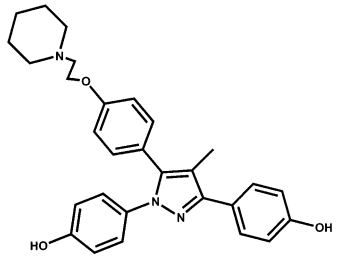
It is hoped that the new concept of supersensitizing cells to estrogen by estrogen withdrawal using aromatase inhibitors or SERMs will encourage new clinical trials for breast cancer treatment and result in the identification of new targets for drug action. A new treatment strategy to build on the survival advantages observed with tamoxifen (and potentially aromatase inhibitors) may result in improved responses for patients and avoid the use of chemotherapy alone. Indeed, the joint value of chemotherapies known to induce apoptosis and low-dose estrogen may in fact provide a revolutionary new synergistic approach to the treatment of SERM and aromatase resistant disease.

## Evolution of Antiestrogens and Future Perspectives

The clinical development of nonsteroidal antiestrogens over the past 40 years has resulted in the first agents (clomiphene and tamoxifen) for the induction of ovulation in subfertile women, the first antiestrogen (tamoxifen) specifically for the treatment of ER-positive breast cancer, the first chemopreventive (tamoxifen) to reduce the incidence of breast cancer in high risk pre- and postmenopausal, and the first SERM (raloxifene) for the treatment and prevention of osteoporosis but with breast and uterine safety. In each case, essentially orphan or failed drugs were successfully developed despite initial pessimistic opinions about the wisdom of investing in clinical trials. However, success in each case was the result of a close collaboration between academia and industry to find suitable applications for novel molecules that were remarkably nontoxic.

The actual success of antiestrogenic medicines depended on the changing fashions in research. The policy shift from an emphasis on contraception research in the 1950s and 1960s to cancer research and treatment in the 1970s and now to women's health in the 1990s was critical for progress in drug development. What was remarkable was the fact that a failure to develop tamoxifen or a decision to develop only a pure antiestrogen (if one had been available) in the early 1970s would probably have resulted in limited advances in endocrine therapeutics. There would be no aromatase inhibitors and no proof of principle that the chemoprevention of breast cancer can be acheived, and the SERMs would not have been discovered.

The idea of targeting a specific ER class or tissue is the goal for future drug development. The challenge for the medicinal chemist is to decipher the complex pharmacology of new ligands at the ERα and ER$\beta$ sites. Obviously, agents that discriminate on the basis of receptor affinity will be selective, but if estradiol still binds to the other receptor under physiologic conditions, this could confound target site specificity at sites that contain both receptors. A series of antagonists selective for ERα has recently been reported based on a pyrazole carrier (**48**). One example, methylpiperidinopyrozole (**52**), is 1000-fold more active at blocking the action of




**(52)**

estradiol at ERα than at ER$\beta$.[411] A vigorous effort to

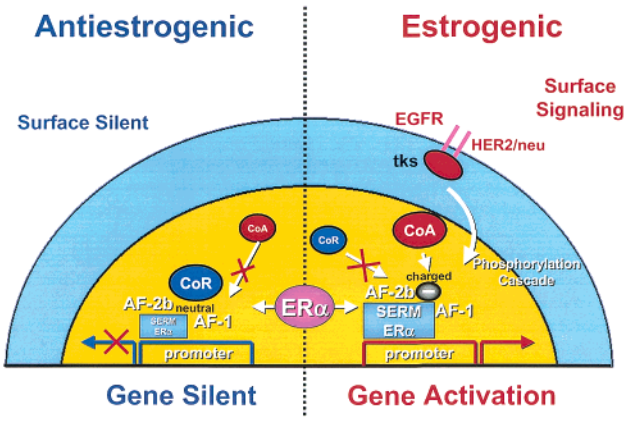



**Figure 8.** Integrated mechanism for the target site specific action of SERMs in breast or uterine cancer. The two extremes of antiestrogenic or full estrogenic actions are shown. Estrogen-like actions could occur in cells expressing an excess of coactivators (CoAs) and/or a decrease in corepressors (CoRs). The charged surface of a tamoxifen ER complex at AF-2b prevents CoR binding. The estrogenic action would be amplified by surface signaling with dimers of epidermal growth factor receptor (EGFR) and HER2/neu activating tyrosine kinases (tks). The phosphorylation cascade can activate AF-1 on ERα directly or can activate the excess of CoAs in a high-ER environment. Reduced levels of ER prevent the signal transduction pathway and promote antiestrogenic actions in a surface silent cell. Reprinted from *Cancer Cell* (Vol. 1, page 216 (Figure 1) of "The secrets of selective estrogen receptor modulation: cell specific coregulation" by V. C. Jordan), with permission from Elsevier Science.[414] Copyright 2002 Elsevier Science.

examine the basic biology of the ERα and ER$\beta$ systems is required to determine ratios and exclusivity at target sites. It is possible that an ER$\beta$ agonist or antagonist could have selective therapeutic value at a site only controlled by ER$\beta$.

An unusual observation that requires explanation is the fact that SERMs only activate ER$\beta$ through AP-1 sites at enormous concentrations, i.e., far higher that the affinity of the SERM for the receptor. Micromolar concentrations are required for raloxifene[412] to activate ER$\beta$, and these are unlikely to be observed in patients because of poor bioavailability. It does, however, raise the possibility that multiple binding sites for SERMs occur on the ER. The idea has been proposed earlier by Jensen where 4-hydroxytamoxifen can bind not only at the LBD but also at higher concentrations, at a second site that ensures antiestrogenic action at ERα.[413] It may be that multiple binding sites on ER$\beta$ promote AP-1 signal transduction pathways. The crystallization of the full ER or other members of the steroid hormone receptor superfamily may reveal new ligand binding domains on the surface of the complex for exploitation with novel therapeutic agents.

For the future, a rigorous examination of target site specificity will ensure the development of multifunctional medicines. As a potential guide, an integrated model for the extremes of SERM pharmacology is offered to explain the often jumbled facets of SERM molecular biology (Figure 8). Although these extremes may partly explain antitumor actions in the breast, drug-resistant growth, or endometrial cancer, it is not entirely clear how bone density is preserved or how circulating lipids are processed by SERMs. An effort is required to understand receptor-mediated mechanisms to allow a precise targeting of organ sites so that effective novel preventives for osteoporosis and CHD can be developed.

The idea that differently shaped estrogen ERα/$\beta$ complexes may program different target site specific effects is being explored. The alterations in the intermolecular signaling pathways for AF-1 with AF-2 or AF-2b may answer questions about the ability of a spectrum of differently shaped estrogens that produce cancers only at select target tissue sites. Clearly, the creation of a menu of coactivators and corepressors that interact at target sites with AF-1, AF-2, and AF-2b will aid the understanding of different SERM targeting.

In closing, the recognition of selective estrogen receptor modulation has created a new way of thinking about the selective actions of androgen receptors, thyroid receptors, glucocorticoid receptors, and the PPAR$\gamma$ receptor. The advances with tamoxifen and the SERMs in therapeutics are actively being extrapolated to other receptor systems to develop a new wave of pharmacological agents. New opportunities to evaluate unknown or orphan receptors, in the future, may provide insight into disease states never previously considered.

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## Biography

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