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Perspective

Antiestrogens and Selective Estrogen Receptor Modulators as Multifunctional Medicines. 1. Receptor Interactions

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The essential role estrogen plays in reproductive endocrinology has been deciphered during the 20th century. Estrogen is also important for supporting physiologic homeostasis in a woman's body as evidenced by the progressive changes that occur at menopause when ovarian estrogen synthesis stops around the age of 50.

Knowledge of the biological actions of sex steroids (estrogens and progestins) resulted in the development of oral contraceptives[1] to prevent pregnancy and the application of estrogen replacement therapy (ERT) as a supplement to alleviate symptoms[2] and urogential atrophy[3] at the time of menopause. These are landmarks in drug development because, for the first time, the target populations were well women and a disease state was not being treated or prevented. In other words, the quality of life for the individual is improved by either planned parenthood or a short course of hormone replacement. However, the strong inverse relationship between age and bone mineral density in the decade following menopause[4,5] suggested that hormone replacement could elevate bone density and reduce the risk of fractures in elderly women. The protective effect of hormone replacement therapy (HRT), i.e., a combination of estrogen and progestin, on increasing bone mineral density is clearly demonstrated in the postmenopausal/progestin intervention (PEPI) trial.[6] Women taking placebo lost an average of 1.8% and 1.7% in bone mineral density of the spine and hip, respectively, over the 3 years of the study. In contrast, women taking HRT gained an average of between 3.5% and 5% in the spine and 1.7% in the hip. It is estimated that current users of HRT have a 50% and 25% reduced risk of vertebral and hip fractures, respectively.[7]

Hormone replacement therapy is used widely by postmenopausal women for the treatment and prevention of osteoporosis. Additionally, the epidemiologic link between the use of HRT and a reduction of coronary heart disease (CHD) and Alzheimer's disease[8−10] provoked a wider use of HRT by well women. Unfortunately, the benefits of HRT based on prospective clinical trials has not, as yet, been demonstrated for Alzheimer's or CHD[11−13] and the cardiovascular system. Indeed, the recent results from the Women's Health Initiative[14] demonstrate, in a placebo controlled trial of 160 000 postmenopausal women, an increase in heart disease (23%), stroke (38%), and blood clots (100%). Although the absolute changes per 1000 women were only increased from 3.0 (placebo) to 3.7 (HRT) for heart disease, 2.1 (placebo) to 2.9 (HRT) for stroke, and 1.3 (placebo) to 2.6 (HRT) for blood clots, the trend is all in the wrong direction.

Despite the unproven benefits of HRT in Alzheimer's and CHD, estrogen does encourage the development and growth of cancer in the breast and uterus. The Women's Health Initiative noted an increase in breast cancer of 26% with an absolute change per 1000 women of 3.0 (placebo) to 3.8 (HRT).[14] The link between ovarian hormones and breast cancer has been known throughout the 20th century. Oophorectomy of premenopausal patients with metastatic breast cancer caused tumor regression in approximately one-third.[15,16] The reason for this apparently arbitrary responsiveness to estrogen withdrawal was unknown until the discovery of the

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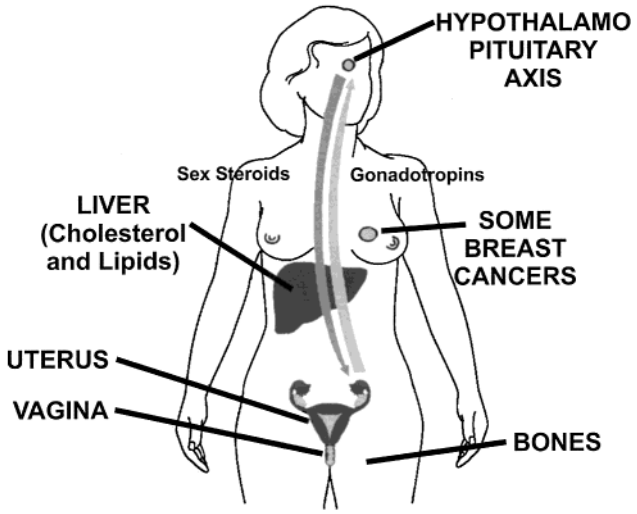
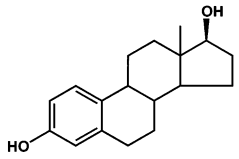


Figure 1. Location of estrogen target tissues around a woman's body. Estrogen receptors (ERs) in the hypothalamo-pituitary axis regulate the release of gonadotropins by both positive and negative feedback mechanisms. The gonadotropins, in turn, control the ovarian synthesis of estrogens and progestins that are essential for maintaining the menstrual cycle and for reproduction. Estrogens cause proliferation in the uterine and vaginal epithelium through the ER. Additionally, breast cancer growth is supported through the ER in some breast cancers. The ERs located in liver and bone cells regulate the circulating levels of cholesterol and lipids and bone density, respectively.

estrogen receptor (now referred to as ERα) and the application of the knowledge to predict the hormone responsiveness of breast cancer.

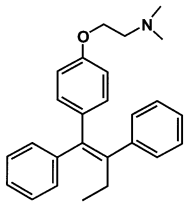
In the late 1950s, Jensen and Jacobson[17,18] synthesized the first high specific activity tritium labeled estradiol-17β (**1**). They showed that radiolabeled estra-



(1) estradiol-17β

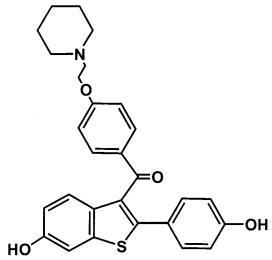
diol was bound to, and was retained by, estrogen target tissues (uterus, vagina, pituitary gland) but was not retained by nontarget tissues such as muscle or lung. They hypothesized that there must be a receptor molecule for estrogen in target tissues that initiates the cascade of biochemical events associated with estrogen action at that site. Subsequently, the ER was isolated as an extractable protein from rat uterus[19,20] and subcellular models of estrogen action were designed[21,22] and refined.[23,24] However, Jensen[25] took these concepts one step further by suggesting that if the ER were present in a breast tumor, then this would increase the probability of a response to endocrine ablative therapy (oophorectomy, adrenalectomy, hypophysectomy). This was shown to be true[26] and is the basis for the steroid receptor assay used routinely in the prediction of endocrine sensitivity of breast cancer. Overall, the discovery of the ER rationalized the target site-specific effect of estrogen around a woman's body (Figure 1).

The finding of ER in breast tumors also provided the rationale for the eventual development of antiestrogens[27] as a safe and simple alternative to ablative surgery for the treatment of breast cancer. However, the development of a simple, nonsteroidal antiestrogen tamoxifen (**2**) that blocks the estrogen-stimulated growth



(2) tamoxifen

of breast cancer[28] was to become the key to discovering selective estrogen receptor modulation. The nonsteroidal antiestrogens tamoxifen and raloxifene (**3**) prevent the



(3) raloxifene

development of carcinogen-induced rat mammary carcinoma[29] but were tested, in 1986, to determine whether they would have a detrimental effect on bone density. These studies were conducted with a view to using nonsteroidal antiestrogens for chemoprevention. Indeed, at that time, a pilot study of tamoxifen as a chemopreventive in high-risk women was initiated at the Royal Marsden Hospital in London.[30] Both tamoxifen[31−33] and raloxifene[31] maintained bone density in ovariectomized rats. These data were subsequently translated to the clinic where tamoxifen was found to preserve bone density in postmenopausal breast cancer patients.[34,35] Although tamoxifen was not considered for use as a drug to prevent osteoporosis, the safety of the drug in bone provided important assurances to advance with the testing of tamoxifen as a chemopreventive in high-risk women.[36] However, not all women who develop breast cancer have risk factors other than age. The following question therefore arose: Could breast cancer be prevented in postmenopausal women without identifiable risk factors? In 1990, on the basis of existing laboratory data,[29,31] a paradigm shift was proposed to develop tamoxifen analogues that would prevent osteoporosis and atherosclerosis and would prevent breast cancer as a beneficial side effect.[27] The result was raloxifene (**3**), which is now the first selective estrogen receptor modulator (SERM) to be available for the treatment and prevention of osteoporosis that is also being tested as a preventive for breast cancer and CDH.[37−39]

Part 1 of this review will describe the development of the scientific ideas about antiestrogen action and the evolution of our understanding of their molecular mechanisms of action. As in most areas of pharmacology, the use of antagonists and partial agonists has provided enormous insight into the mechanics of receptor func-

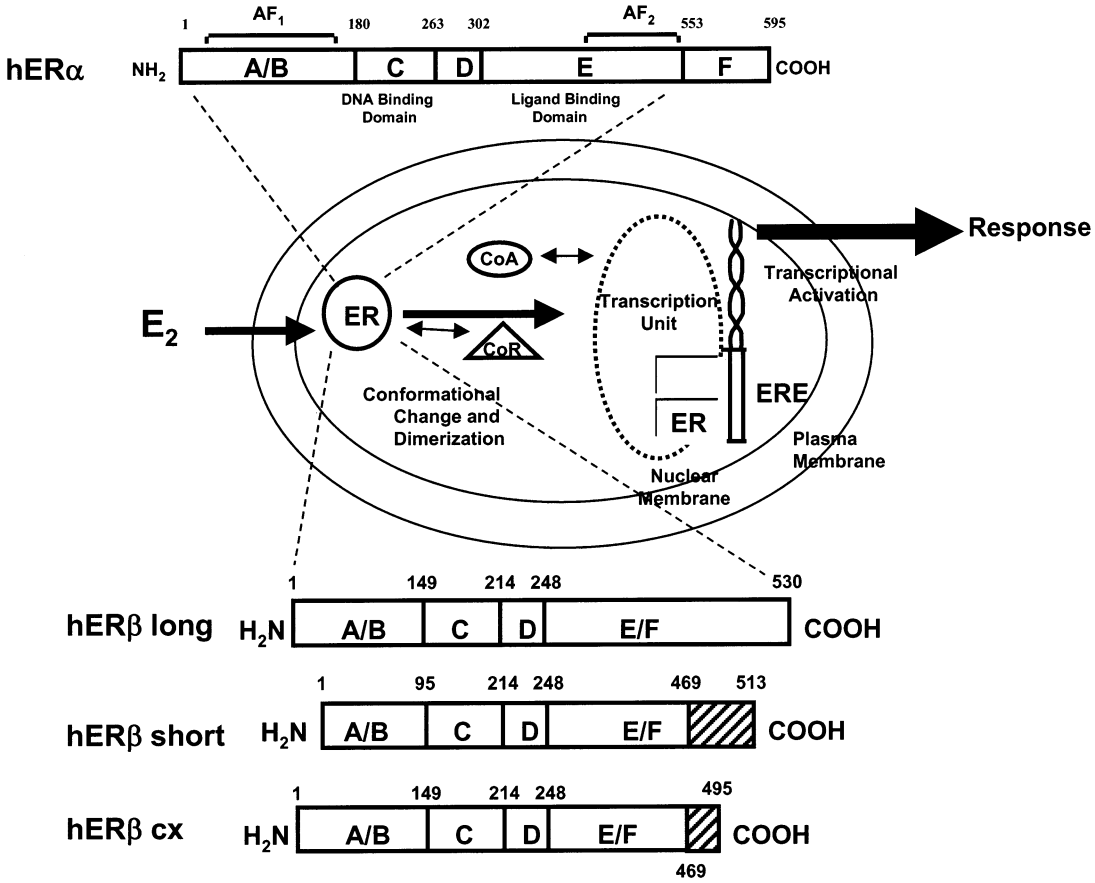


Figure 2. Structure and signal transduction pathway of estrogen receptors (ER) α and *β* (and variants) in an estrogen target tissue cell. The nuclear receptors can bind estradiol (E_2) and hetero- or homodimerize at an estrogen response element (ERE) in the promoter region of an estrogen responsive gene. Gene transcription occurs by binding coactivator molecules (CoA) to form a transcription unit. Corepressor molecules (CoR) are more likely to bind the unliganded receptor. The ERs are organized into different functional regions (A−F), but the most important are the C region, the DNA binding domain, and the E region ligand binding domain. These are two activating functions (AFs) on ERα referred to as AF-1 and AF-2. AF-2 is activated by E_2 binding but needs to synergize with AF-1 to develop a stable structure for optimal CoA binding to form the transcription unit.

tion and the associated molecules that must be recruited to complete a signal transduction pathway. The targets for SERM action are ERs, and developing molecular knowledge is now being utilized to dissect the multiple mechanisms of estrogen action. Through the application of this knowledge, new strategies of drug discovery can be exploited either to develop the ideal SERM, as a multifunctional medicine, or to target SERMs to specific organ sites. The clinical considerations and novel agents will be addressed in part 2.

Estrogen Receptors (ERs) α and *β*

The biological effects of estrogen are now known to be mediated by two receptors referred to as ERα and ER*β*. ERα was first identified and isolated in the 1960s.[17−22] The cloning of ERα in the mid-1980s[40−42] subsequently focused research efforts on the existence of only one ER protein (ERα) identical in all target tissues. Almost 3 decades after the initial discovery of ERα, ER*β* was identified in the rat, human, and mouse.[43−45] The discovery of ER*β* has already advanced our understanding of estrogen signaling and may explain the responses to estrogen in tissues in which ERα was not present.[46−48] Additionally, the existence of ERα and -*β* subtypes provides a possible explanation for the tissue selectivity of SERMs.

The two ERs share a functionally conserved structure (domains A−F, Figure 2) consisting of a variable amino terminal region that is involved in transactivation (A/B), a centrally located, well conserved DNA binding domain (C), a region involved in dimerization and in binding to Hsp90 (D), a ligand binding domain (LBD) (E), which synergizes with the transactivation functions in the A/B region, and a carboxy-terminal F region,[49,50] which appears to play a role in modulating transcriptional activation by ERα.[51,52] ER*β* is homologous to ERα at the ligand binding (58%) and DNA binding (95%) domains, whereas the A/B region, hinge domain, and F region are not well conserved.[45,50,53] Several forms of human ER*β* known as ER*β* short,[44] ER*β* long,[54] and ER*β*cx[55] have been identified (Figure 2).

The ERα isoform has two regions called activation functions (AFs) that contribute to transcriptional activity. AF-1 is located in the amino-terminal region within the A/B region and was originally believed to be constitutively active and ligand-independent.[49,56,57] It has been demonstrated that ligand-independent activation of ER via the AF-1 domain is closely related to the phosphorylation status of the receptor.[58−60] In particular, Ser-118 in the A/B region of ERα is important for activation through the Ras-MAP kinase (MAPK) signaling cascade[60,61] and Ser-106 and Ser-124 are two phosphorylation sites in the A/B region of ER*β* that are essential for ligand-independent activation of the ER*β* via the MAPK cascade.[62,63] In addition, both receptors

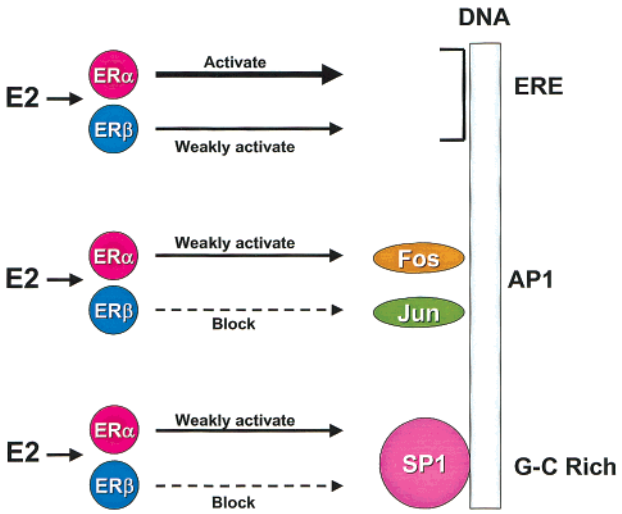


Figure 3. Potential options for the signal transduction pathway for the estradiol (E_2) estrogen receptor (ER) α or β complex. In a physiologic context E_2 (1 nM or the circulating level in a premenopausal woman, 272 pg/mL) will maximally activate through an ERα estrogen response element (ERE) pathway but the ERβ complex will be weakly active.[81,357] In contrast, the E_2 ERα complex can weakly activate AP-1 sites but the transcription pathway is blocked by E_2 at the ERβ complex.[74] Similarly, the E_2 ERα complex can bind the Sp1 and weakly activate genes but the E_2 ERβ cannot.[78]

contain a second activation domain, AF-2, which is present at the carboxy terminus and is ligand-dependent.[56,64] Mutational analysis has demonstrated the importance of this region for ER transactivation[65−67] because AF-2 can interact with a number of transcriptional coactivators in a ligand-dependent manner.[68−71]

AF-1 and AF-2 of ERα can activate transcription independently, but in most cases they synergize with one another in a promoter- and cell-context-specific manner.[56,67] In the classical model, it is believed that ER activates gene expression by binding to estrogen response elements (EREs) in responsive genes through the synergistic action of AF-1 and AF-2.[72] ERβ also activates transcription of target genes through EREs.[44,73] However, it has been demonstrated that while estrogen can induce an AP-1 site in a reporter construct through ERα, it is inactive via ERβ[74] (Figure 3). Interestingly, SERM ERβ complexes induce gene activation through an AP-1 site[74−76] and through the human retinoic acid receptor α-1 promoter.[77]

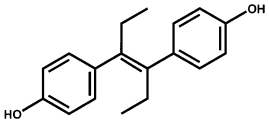
Estradiol has recently been shown to induce expression of several genes via ER-Sp1 protein interactions with GC-rich promoter elements. Sp1 rather than ER binds to the DNA.[78] Exchange of AF-1 between ERα and ERβ shows that it is the AF-1 site of ERα that is responsible for the activation at an Sp1 element independent of the ER subtype (Figure 3). The region between aa 79−117 of this domain is important.[78]

The two receptors ERα and ERβ may form functional heterodimers on DNA[54,62,73] that can bind the coactivator, SRC-1, and stimulate transcription of a target gene.[79,80] The ability of ERα and ERβ to form heterodimers suggests that ER may function through different dimeric states, and it is possible that the dimers could be activated by selective ligands.

Hall and McDonnell[81] have proposed a working model to explain the crosstalk between ERα and ERβ. The model is based on the fact that unliganded ERβ can bind to target gene promoters in a ligand-independent manner within cells.[79] Thus, in a low estrogen environment (0.1 nM or 27.2 pg/mL) that does not saturate ER, unliganded ERβ blocks ERα action when present in equimolar concentrations in the target cell. As the concentration of estrogen rises, the inhibitory effects of ERβ are removed and the liganded receptors are indistinguishable. However, it is difficult to see, under physiologic conditions, where estrogen is abundant how the inhibitory mechanism of low levels of ERβ would come into play. Nevertheless, if patients were receiving aromatase inhibitors to treat or prevent breast cancer,[82] thereby producing a very low estrogen environment, then the inhibitory role of ERβ could become relevant as an antitumor mechanism during chemoprevention before ERα becomes dominant.

Molecular Mechanism for Estrogen Action

The existence of two rather than one ER indicates that the mechanism of action of estrogen and antiestrogens (SERMs) is even more complex than previously thought.[83] Estrogen, upon binding to its high-affinity receptor (or receptors), triggers expression of multiple genes involved in the regulation of cell proliferation and differentiation. Estrogen binding causes the ER to dissociate from heat shock protein, dimerize, and bind to specific DNA sequences and stimulates the transcription of responsive genes. Apparently, dimerization is inhibited by elements in the F region of ERα that are neutralized by ligand binding.[52] It is now established that the ER itself is not the direct controller of transcription; it requires an interaction with a complex of coregulatory proteins (coactivators or corepressors) that act as signaling intermediates between the ER and the general transcriptional machinery[84,85] (Figure 2). The crystal structure of the LBD of ERα was determined with estradiol[86,87] and the synthetic nonsteroidal estrogen diethylstilbestrol (**4**, DES).[88] The crystal structure



(4) diethylstilbestrol

represents a dimer,[86] and it must be pointed out that the F region is removed for these studies, which would be consistent with the inhibitory role of this region.[52] Both high-affinity ligands interact with the same amino acids to locate the estrogens correctly (Figure 4). A key feature of the agonist−receptor structure is the ability of the ligand to be enveloped in a hydrophobic pocket that is closed by helix 12 (an essential site for AF-2 activation through coactivator binding) in the ligand binding site of ERα (Figure 5). Helix 12 positioning over the hydroplobic pocket is critical for the recruitment of coactivators to the AF-2 site (Figure 5B) and subsequent initiation of RNA polymerase activity. The repositioning of helix 12 after ligand binding has been proposed as an important mechanism for full estrogen action at ERα.[86,89,90]

Recently, the crystal structure of a triple cysteine to serine (381, 417, and 530) mutant ERα LBD complexed with estradiol was reported.[91] The mutant ERα−estra-

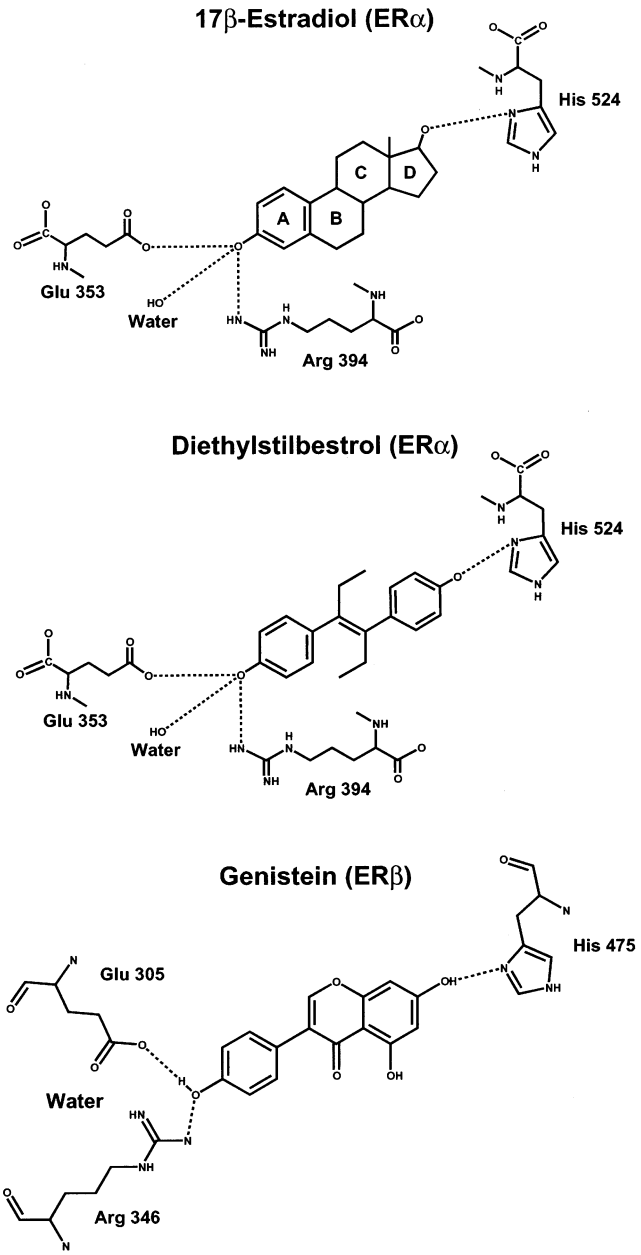


Figure 4. Binding of ligands in the hydrophobic pocket of the E region of human ERα and -*β*. The phenolic A ring of estradiol is located in the ligand binding domain by Glu353 and Arg394 and tethered by his524.[86,88] The mixed agonist/antagonist genistein is located and tethered by complementary amino acids in the ligand binding domain of ER*β*.[92]

diol complex has partial agonist properties, and estradiol has a lower binding affinity. This is because helix 12 does not seal the steroid in the hydrophobic pocket as it is repositioned to a structure similar to that predicted with raloxifene and ERα.[86] The study illustrates the importance of helix 12 positioning for agonist and antagonist actions. These concepts will be discussed in detail following the section on antiestrogenic ligands.

The crystal structure of the ligand binding domain of ER*β* has not been resolved with a natural or synthetic estrogen; however, the crystal structure of the phytoestrogen genistein (**5**), a partial agonist, has recently been reported.[92] The paraphenolic hydroxyl of genistein interacts with the side chains of Glu 305 in ER*β*,

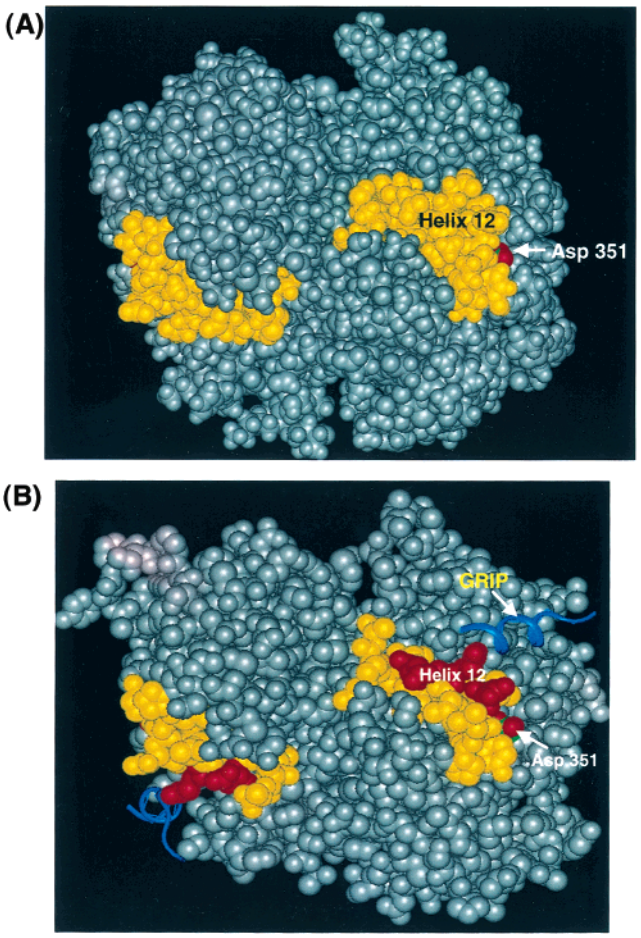
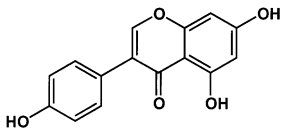


Figure 5. External surface of the dimerized ERα ligand binding domain complex liganded with either (A) estradiol-17*β* or (B) diethylstilbestrol. Helix 12 (shown in yellow) seals the ligands into hydrophobic binding pocket to activate the coactivator binding site referred to at AF-2. The binding domain of the coactivator GRIP (shown in blue in part B) has been cocrystallized with the diethylstilbestrol ERα binding domain. The amino acids indicated in red on helix 12 (B) can be mutated to nonpolar amino acids (D538A/E524A/D545A) to silence AF-2. The aspartate at 351 interacts with the underside of helix 12. Reprinted with permission from *Cancer Research* (page 3636, Figure 8).[304] Copyright 2001 American Association for Cancer Research. (Information is based on the original reports.[86,88])

equivalent to 353 in ERα, and arginine 346, equivalent to 394 in ERα. The flavone portion of genistein makes



(5) genistein

a hydrogen bond with His 475, equivalent to 524 in ERα (Figure 4). Although the genistein molecule causes a repositioning of helix 12, there are fundamental differences in the length, positioning, and interactions made by helix 12 compared with the "antagonist" orientation induced by raloxifene.[92]

Nuclear steroid receptors must associate with other nuclear proteins to form a transcription complex.[85] For example, it has been shown that AF-1 and AF-2 domains of ERα bind to TATA binding protein (TBP)

in vitro.[93] Moreover, ER can interact with components of the transcription factor IID (TFIID) complex[94] and TFIIB.[95] Interactions between ER and TFIID-associated factor (TAF), $TAF_{II}30$, have also been described.[96] However, while all these interactions are necessary, they are not sufficient to mediate transcription. The multiprotein complex formed by ligand-activated ER composed of multiple factors must not only provide the machinery to transcribe the appropriate gene but also facilitate a mechanism for opening the wound and quiescent gene[97] in the correct place.

Several coactivators have been described for ER on the basis of their ability to interact with agonist-bound receptor but not with an antagonist-bound receptor. ERAP 160[98] and RIP 140[69,99] were identified using the LBD of ER as bait. Interestingly, RIP 140 does not interact with the basal factors of the transcription machinery, suggesting a role other than a bridging function for this coactivator.[99] RIP 140 is able to interact with the LBD of ERα liganded with estrogens or antiestrogens, but sequences outside the LBD compromise binding.[52] SRC-1, on the other hand, was isolated from a human cDNA library using the LBD of hPR as bait.[100] However, studies demonstrate that SRC-1 and ERAP 160 are variants of the same family of proteins. The family members are now referred to as SRC-1 (also termed p160/NCoA-1/ERAP-160), SRC-2 (also termed TIF-2/GRIP-1), and SRC-3 (also termed A1B1/RAC-3/TRAM-1). The majority of receptor-interacting factors have been identified by using LBD of nuclear receptor as a bait. This, therefore, explains why the AF-2 site is believed to be the most important site for the recruitment of coactivators. However, it has been demonstrated that SRC family members are not only a transcriptional mediator for the ligand-dependent AF-2 of ER[101] but also involved in ligand-independent interaction with AF-1[101] and with ERβ through phosphorylation of AF-1 via MAP kinase.[62] Functional interaction of ER and SRC-1 occurs in the absence of exogenous ligand through cyclin D bridging.[102] In addition, the SRC-1 complex contains SRA (steroid receptor RNA activator), which mediates transactivation via AF-1.[103] Moreover, ERα/ERβ heterodimers are able to bind the SRC-1 and stimulate transcription of a reporter gene.[80]

Another protein involved in coactivator complex with SRC-1 is p300/CBP, a 300 kDa protein related to the cAMP response element-binding protein, CBP.[104−106] It has been demonstrated that SRC-1 and p300/CBP contain intrinsic acetyltransferase activity and can interact with other histone acetyltransferases (HATs). Acetylation by the SRC-1 complex of histones bound at specific promoters could be a mechanism by which the AFs of ER and associated coactivators activate transcription of specific genes by enhancing formation of a stable preinitiation complex.[97]

The SRC-3 gene is located on the long arm of chromosome 20[107] and becomes amplified in some ER and PR positive breast[108] and ovarian cancers. The protein contains three LXXLL motifs that are necessary to activate transcription through the recruitment of CBP and is colocalized in human tissues and cancer with SRC-1 and -2.[109] Indeed, disruption of the SRC-3 gene in mice shows that the coactivation is required for normal growth, puberty, mammary gland development, and female reproductive function.[110] SRC-3 function has been studied extensively in MCF-7 breast cancer cells.[111] Not only can SRC-3 enhance transcription and growth through the steroid receptor[112] but also the protein itself can become phosphorylated through the MAPK pathway, which enhances CBP recruitment and associated histone acetyl transferase activity.[113] Thus, coactivators as well as steroid receptors are targets for cell surface signaling pathways (see part 2). A recent report of a SRC-3 Δ3 (actually Δ4) isoforms in breast cancer[114] demonstrated enhanced transcriptional activity for the mutant protein compared with full-length SRC-3 when either estradiol or EGF was the activating stimulus.

Gee and co-workers[115] have demonstrated that specific peptides designed from SRC-1 can interact with ERα and ERβ differentially to stabilize agonist ER complexes. The authors suggest that their data support the proposition that different levels of coactivator molecules in target sites could modulate the estrogenicity of agonist ER complexes. Additionally, Klinge[116] has demonstrated that DNA allosterically modulates the LBD and creates a tighter fit between the ligand and the ER. Hall and co-workers[117] have followed up this lead to show that different EREs can influence the activities of E_2 and various xenoestrogens through both ERα and ERβ. These important observations suggest that ERE-induced alterations in protein structure can influence coregulator recruitment and provide an explanation for the differential sensitivity of genes to estrogenic stimuli. Similarly, the chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptor binding to EREs is increased by the addition of E_2 ERα. The converse is also true; COUP-TF enhances ERE binding of E_2 ER. However, pull-down assays demonstrate no direct enhanced interaction between COUP-TF and E_2 ER. Transient transfection of an expression vector for COUP-TF demonstrates repression of E_2-induced luciferase reporter gene expression.[118] Thus, the collaborative efforts of the ligand, the particular receptor, the ERE, and the concentration and availability of coactivators or corepressors in a tissue can all contribute to the activation of genes in a target tissue.

The description of the two ERs in estrogen target tissues provides opportunities to develop drug molecules not only to modulate ER action at the ligand binding domain but also to identify secondary targets to modulate coactivator binding. To date, the drug discovery process has focused on ligands (see following sections) to modulate ER action at the E (LBD) region of ERα. However, it is now clear that the ligand can coordinate the agonist and antagonist actions of the ER complex by changing the surface shape of the ER.[119−124] Deletion mutagenesis and in situ analysis indicate that nuclear colocalization of E_2 ERα complexes requires a central SRC-1 domain containing LXXLL motifs.[125] Indeed, fluorescence resonance energy transfer (FRET) can be used to monitor the interaction between the E_2 bound ER and LXXLL peptides expressed in cells in fusion with spectral variants of the green fluorescent protein.[126] The peptides bind in the hydrophobic cleft on the surface of the ER, but interaction is blocked by a single amino acid mutation (K362A). As would be anticipated, the antiestrogens 4-hydroxytamoxifen and

ICI182,780 both prevent FRET because the protein conformation is disrupted with alterations in helix 12 (see section b, A Complex Problem from the Outside). In contrast, a number of planar natural and synthetic estrogens that are sealed within the LBD by helix 12 can be identified with FRET. It is possible that, in the future, novel ligands could block the binding of specific coactivators or that targeted compounds could specifically decrease the synthesis of coactivators or increase the synthesis of corepressor molecules.

Another dimension in the modulation of estrogen action is the family of orphan receptors referred to as estrogen receptor related receptors (ERRs). There are currently known to be at least three family members, ERRα,[127−129] ERRβ,[127,130] and ERRγ,[130−132] that share significant amino acid sequence identity with ERα and ERβ and exhibit similar but distinct biochemical and transcriptional activities as the ERs. Each of the ERRs binds and activates transcription via consensus palindromic EREs[133−136] or binds to an ERRE.[129,132,134,137−140] However, the ERRs do not bind natural estrogens.[127,141] Instead, the ERRs may serve as constitutive regulators, interacting with transcriptional coactivators inside cells in the absence of ligands.[134,140,142] Bulky amino acid side chains inside the ligand binding pocket of ERRs substitute for the analogous ligand-induced interactions observed in ERα.[142,143] However, the synthetic estrogen DES (**4**) is an antagonist because it also disrupts coactivator interactions with ERRs.[144]

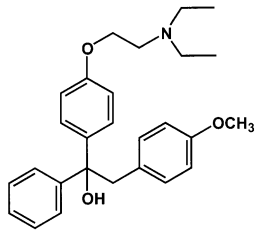
The transcriptional activity of each ERR depends on the promoter and the particular cell line in which it is assayed as well as the presence of ERs.[132−137,139−141,143−151] Although ERRα stimulates ERE-dependent transcription in the absence of ERα in HeLa cells, it down-modulates estradiol-stimulated transcription in ERα-positive human mammary carcinoma MCF-7 cells via an active mechanism of repression.[133] ERRα can also modulate transcription of at least some genes that are estrogen-responsive and/or implicated in breast cancer such as pS2,[145] aromatase,[149] osteopontin,[147,148] and lactoferrin.[146,152] Overall, the ERRs may play an important role by modulating or substituting for ER-dependent activities.

Modulating the ER System

On the basis of the background of estrogen action and its target site effects, part 1 of the review will now trace the evolution of ideas about antiestrogen action from the 1960s.[153−155] The discovery of antiestrogens in the late 1950s, at a time when research on contraception was at its height, resulted in the extensive examination of structure−function relationships using antifertility endpoints in rodents. The failure of the initial goal to develop novel contraceptives ultimately evolved into antiestrogens or subsequently SERMs seeking an appropriate application in medicine. Although the drug group was originally referred to as antiestrogens, the pharmacologic classification, based on rodent uterine weight assays, was as partial agonists with antiestrogenic properties in mammary gland and breast tissue. The application of the idea that an antiestrogen (tamoxifen) could be used as a treatment and preventive for breast cancer[27,156] has had a profound effect on the prognosis of patients with breast cancer or who are at risk and has opened the door to the era of multifunctional medicines.

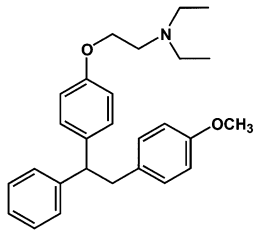
Antiestrogens: Key Compounds

Lerner and co-workers[157] described the pharmacological properties of the first systemically active, nonsteroidal antiestrogen ethamoxytriphetol (MER 25) (**6**). The



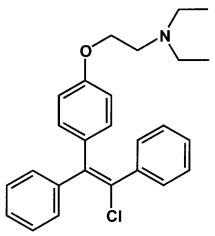
(6) MER 25

compound is virtually devoid of estrogenic activity in mice, rats, rabbits, chickens, and monkeys and is an estrogen antagonist but with low potency. An exciting pharmacological property of MER 25 and the related compound MRL 37 (**7**) is their antifertility actions in



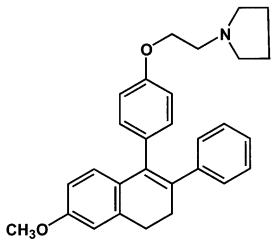
(7) MRL 37

laboratory animals,[158−160] an observation that stimulated a search for more potent agents for clinical applications.[161] Clomiphene (originally known as chloramiphene or MRL 41)[162,163] (**8**), nafoxidine (U-11,-

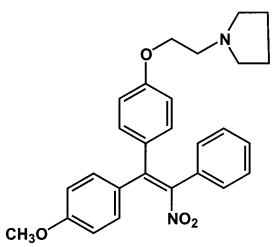


(8) enclomiphene

100A)[164,165] (**9**), nitromifene (CI628 or CN-55,945-27)[166] (**10**), and tamoxifen (ICI 46,474) (**2**)[167,168] were all the



(9) nafoxidine

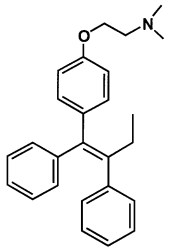


(10) nitromiphene

result of that search, but clinical application as postcoital contraceptives was found to be inappropriate. The drugs induced ovulation in subfertile women.[169] As a result of these clinical findings, clomiphene[170,171] and

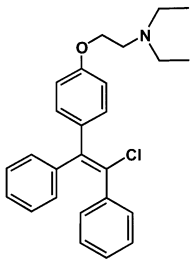
initially tamoxifen[172,173] were approved as profertility drugs for the induction of ovulation.

A pivotal observation in the 1960s was the description of opposing biological activities for the *E* and *Z* isomers of substituted triphenylethylenes. Tamoxifen (ICI 46,-474) is the *Z* isomer of *p*-dimethyaminoethoxy-1,2-diphenylbut-1-ene (**2**) and is an antiestrogen in the rat.[167,174] In contrast, ICI 47,699 (**11**), the *E* isomer, is



(11) ICI 47,699

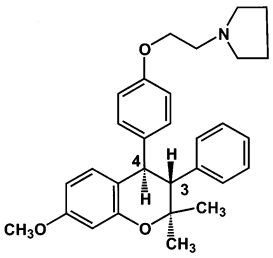
an estrogen. Tamoxifen and its *E* isomer ICI 47,699 were identified by nuclear magnetic resonance,[175] and the structure of ICI 47,699 was confirmed as the *E* isomer by X-ray crystallography.[176,177] Similarly, clomiphene is a mixture of geometric isomers with opposing biological properties. Unfortunately, the isomers were originally given the wrong designations as isomers A and B [178] to identify the *E* and *Z* isomers, respectively. These were subsequently renamed enclomiphene (**8**) and zuclomiphene (**12**) for the antiestrogenic *E* isomers and



(12) zuclomiphene

estrogenic *Z* isomer, respectively.[179,180] The crystal structures and stereochemical features of nafoxidine (**9**), tamoxifen (**2**), and enclomiphene (**8**) have been compared and found to be almost identical.[181]

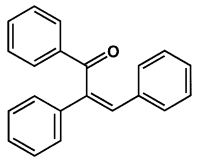
In the late 1960s, enthusiasm for continued contraceptive research by the pharmaceutical industry waned. In India, however, structure−activity relationship studies continued with an investigation of indene and



(13) centchroman

chroman derivatives related to nafoxidine. Centchroman, *trans*-1-{2-{4-(7-methoxy-2,2-dimethyl-3-phenyl-3,4-dihydro-2*H*-1-benzpyran-4-yl)phenoxy}ethyl}pyrrolidine hydrochloride (**13**), has been tested in India as the first nonsteroidal contraceptive.[182] The compound and its analogues have been studied extensively in animals,[183−185] and the contraceptive action in humans is attributed to its antiestrogenic actions as well as its weak estrogenic properties. Centchroman is a racemate; however, its enantiomers are known to have different ER binding affinities as well as different estrogenic and antiestrogenic potencies.[185] The L-enantiomer is more potent than the D-enantiomer,[185] and on the basis of X-ray crystallography, it is suggested that L-centchroman has the 3*R*,4*R* configuration[186] (**13**).

Studies at the Central Drug Research Institute, Lucknow, India, have played an important role in the direction of new drug development. Simple acyclic 1,2,3-triarylpropenones (**14**) were shown to possess antifertil-



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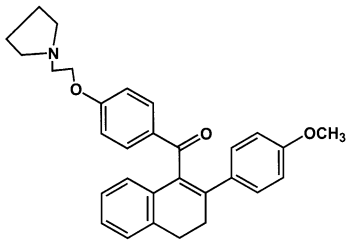
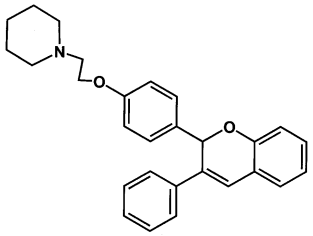


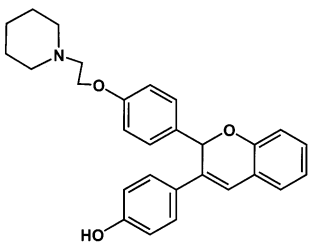
(15) trioxifene

ity activity, but the *Z* isomers are more potent than the *E* isomers.[187−189] This observation led to the discovery, by scientists at Eli Lilly, of trioxifene (**15**) with diminished estrogen agonist activity when compared with tamoxifen (**2**).[190] However, the hydroxylated derivatives of 1,2,3-triaryl-2-buten-1-ones have proved to be very instructive when their structure−activity relationships are compared with those of the benzopyrans. There is an absolute requirement for a correctly positioned antiestrogenic side chain in the para position, and the agonist potencies of 2,3-diaryl-2*H*-benzopyran analogues decrease while antagonist potencies correspondingly increase in going from dimethylaminoethoxy to the



(16)

pyrrolidinoethoxy to the piperidinoethoxy (**16**).[191] The



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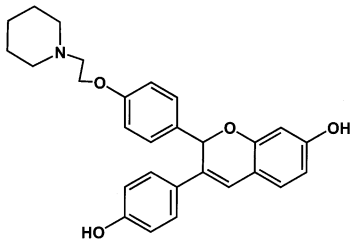
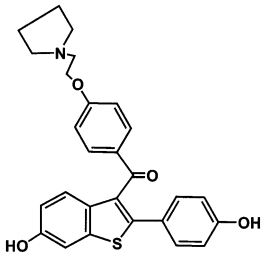
introduction of one (**17**) or two (**18**) strategically placed



(18)

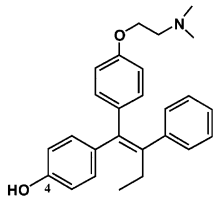
phenolic groups in the benzopyrans enhances ER binding, but the resulting compounds are completely devoid of estrogen agonist activity in the immature mouse uterine weight test while retaining antiestrogenic activity that is superior to LY117018[191,192] (**19**), a raloxifene



(19) LY117018

analogue. The structure−function relationships of a huge number of naphthalene,[193] indene,[194] benzofuran,[195] and benzopyran[191,196,197] derivatives have been evaluated for both antiestrogenic and antifertility properties in rats and mice and form the foundation for medicinal chemistry in this area.

The introduction of tamoxifen (**2**) as the first successful antiestrogen for the treatment of breast cancer[28,198,199] provided the incentive for a renewed investigation of the structure−activity relationships of the drug group. This time, the targeted application was as antitumor agents to treat hormone-dependent cancers. Trioxifene (LY133314)[190] (**15**) is a compound related to nafoxidine (**9**) but diverges from the general triphenylethylene structure by the introduction of a ketone bridge that links the phenyl ring with the *p*-alkylaminoethoxy side chain to the rest of the carrier molecule. The general pharmacology of trioxifene is very similar to that of tamoxifen.[200,201] Phase II clinical trials have shown activity in the treatment of breast cancer[202,203] but without any of the negative side effects (ichtheosis, photophobia) previously noted with nafoxidine.[204,205] However, trioxifene had no advantage over tamoxifen and was abandoned for development as a treatment of breast cancer in the early 1980s.

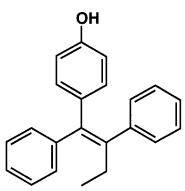


(20) 4-hydroxytamoxifen

The finding that a metabolite of tamoxifen, 4-hydroxytamoxifen (also referred to as monohydroxytamoxifen or metabolite B; **20**), is a potent antiestrogen in the rat[206] with a binding affinity for the ER equivalent to that of estradiol[206−208] stimulated a search for compounds with potential use as new research tools and anticancer agents. It could be argued that the ideal antitumor agent should have negligible estrogen agonist activity and be a potent antagonist with high affinity for the ER. To this end, two novel antiestrogens were described some 20 years ago: LY117018[192] (**19**) and LY156758[209] (**3**)**.** Both compounds have a high affinity for the ER and low estrogenic activity in uterine tests in vivo. Antitumor activity is observed in vivo and in vitro;[210,211] however, the hydroxylated compounds are rapidly conjugated through phase II metabolism and excreted. As a result, higher doses must be administrated in vivo to obtain an efficacy equivalent to that with tamoxifen.[29,212] In contrast, tamoxifen has a low binding affinity for the ER, but the compound is a prodrug that accumulates by constantly being converted to the active metabolite 4-hydroxytamoxifen (**20**).[207,213] Clinical trials with LY156758, then known as keoxifene but now known as raloxifene (**3**), show either no activity or modest activity as a breast cancer therapy,[214,215] so drug development as an antitumor agent was abandoned in the late 1980s. However, the recognition of selective estrogen receptor modulation and the possibility of developing multifunctional medicines[216−218] has resulted in the successful development of raloxifene to treat and prevent osteoporosis.[156] The successful development of raloxifene is a direct result of a novel finding that nonsteroidal antiestrogens can maintain bone density[27,31] but may not increase the risk of breast cancer like HRT.[14,219−221] This concept, originally stated to describe SERMs in 1990,[27] has encouraged the development of drugs to prevent osteoporosis and CHD but with the beneficial side effect of preventing breast and endometrial cancer in postmenopausal women in general.

Recognition of Selective Estrogen Receptor Modulation

Throughout the 1970s, the antiestrogenic activity of nonsteroidal compounds was equated with their potential as antitumor agents for breast cancer.[153] However, the finding that triphenylethylene-type antiestrogens expressed increased estrogenic properties in mouse uterus and vagina compared with the rat[174,222] raised questions about the reasons for the species specificity. Tamoxifen, for example, appeared to be an estrogen in the mouse but a partial agonist with antiestrogenic properties in the rat[167,223−225] and an antiestrogen for the treatment of breast cancer in the human.[198,199] One obvious explanation was species-specific metabolism that converts antiestrogens to estrogens via novel



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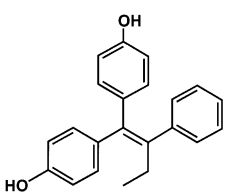


(22)

metabolic pathways. In other words, the antiestrogenic side chain of tamoxifen (**2**) or 4-hydroxytamoxifen (**20**) could be cleaved to produce estrogenic triphenylethylenes, metabolite E (**21**)[226] or bisphenol (**22**), respectively. However, no species-specific metabolic routes to known estrogens have been identified,[227,228] but knowledge of the mouse model created a new dimension for study, which ultimately led to the recognition of the target site-specific actions of triphenylethylene-type antiestrogens and raloxifene.

Before the potential mechanisms of action for SERMs and the clinical proof of principle are considered (see part 2), it is reasonable first to describe the biological evidence that established the new drug group. The ER-positive breast cancer cell line MCF-7[229] can be heterotransplanted into immune-deficient athymic mice, but the cells can only grow into tumors with estrogen support. Paradoxically, tamoxifen, an estrogen in the mouse,[167,222] does not support tumor growth[230] but stimulates uterine growth with the same spectrum of tamoxifen metabolites present in both the mouse uterus and implanted human tumor.[231] To explain the selective actions of tamoxifen in different targets of the same host, it was suggested that the tamoxifen ER complex could be interpreted as a stimulatory or inhibitory signal at different target sites.[231] A similar conclusion can be drawn from the observation that long-term tamoxifen treatment prevents mouse mammary tumorigenesis in high-incidence strains[232,233] despite the fact that uterine weight is dramatically increased. Tamoxifen is an antitumor agent in mouse mammary tissue despite being classified as an estrogen in the uterus and vagina.

The concept that antiestrogens had target site specificity was consolidated with experimental evidence from two further models from the laboratory that translated to the clinic. First, tamoxifen and raloxifene both maintain bone density in the ovariectomized rat[31] but both compounds act as antiestrogens in the rat uterus[167,209] and prevent rat mammary carcinogenesis.[29,234−236] The biological principle has been confirmed adequately in the laboratory[237−240] and translated to the clinic. Tamoxifen[34,35,241−245] and raloxifene[246,247] maintain bone density in postmenopausal women, and raloxifene prevents fractures of the spine.[248] Tamoxifen reduces the incidence of breast cancer in high-risk women,[36] whereas raloxifene reduces breast cancer incidence in women treated to prevent osteoporosis.[249,250] Second, the finding that a human endometrial carcinoma, transplanted into athymic mice, would partially grow during tamoxifen treatment[251] allowed the following question to be asked: If a human breast and endometrial carcinoma were simultaneously transplanted into an athymic mouse, would tamoxifen block estrogen-stimulated growth in the breast tumor but facilitate the growth of the endometrial cancer? The answer is yes, since tamoxifen demonstrated target site specificity with the two human tumors. Estrogen-stimulated breast tumor growth was blocked, but endometrial tumor growth was enhanced.[252] Since the range of tamoxifen metabolites was the same in the breast and endometrial tumors, the tamoxifen ER complex was selectively blocking or promoting growth pathways. These data also suggested the possibility of an increased risk of endometrial cancer detected during long-term tamoxifen therapy. It is known that the uterus harbors 5 times the amount of occult disease as is reported clinically.[253] Clearly any "estrogen-like" molecule will enhance growth and promote selection of hormone-responsive disease. After a decade of investigation, there is now known to be a 3- to 4-fold increase in the incidence of endometrial cancer in postmenopausal women treated with tamoxifen.[36,254]

The acronym SERM (selective estrogen receptor modulator) was invented by Eli Lilly in the 1990s to describe the multiplicity of effects from molecules that interact with the ER at different sites. The selective actions of tamoxifen and raloxifene have successfully been expoloited by the pharmaceutical industry with the application of tamoxifen as a preventive in women only at high risk for breast cancer but, most importantly, with the innovation of a new drug group (SERMs) to prevent osteoporosis.

SERMs produce a spectrum of agonist and antagonist actions at different target tissues. The emerging understanding of approaches to modulate the ER complex at its target sites is a fascinating story of work in progress. The complementary and interdependent facets of the signal transduction pathways, controlled by the structure−activity relationships of the ER ligands, are being used to identify a range of novel targets for new drug discovery programs.

Partial Agonism and Antagonism

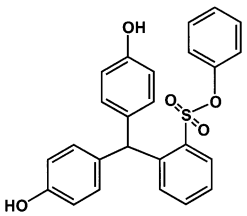
(a) Insight from the Inside. Prior to the cloning of ER,[40−42] the approach to drug discovery was to identify an assay system based either on an animal or a cell and to perform structure−activity relationship studies with a lead compound. The endpoint was a reproducible biological response that could be quantitated by modulating the receptor complex.

In their simplest form, the theories of drug interaction with receptors are based on the fundamental studies by Clark[255] and Gaddum[256] who suggested that the response to a drug is proportional to the number of receptors occupied. However, the occupation theory was modified by Stephenson[257] and Ariens and Simonis[258] into two steps: (1) receptor binding (dependent on affinity) and (2) the production of a response (dependent on the intrinsic activity α or efficacy of the drug−receptor complex). Thus, within a group of drugs that are all full agonists (i.e., intrinsic activity $\alpha = 1$) but that have progressively smaller affinity constants, their sigmoidal log dose−response curves will be progressively shifted to the right. However, for a group of drugs with intrinsic activities progressively less than 1.0, the maximal responses in their log dose−response curves will become progressively lower. These compounds are known as partial agonists. When administered with a full agonist, partial agonists do not produce an additive effect but rather an antagonist effect. Within these definitions, an ideal pure antagonist would have high affinity for the receptor but the complex would have zero intrinsic activity ($\alpha = 0$). A SERM would have different intrinsic activities at different sites but preferably zero intrinsic activity in breast and uterus. Naturally, with the identification of the ER more than 40 years ago, it became important to apply the basic concepts of receptor theory and pharmacology to understand the multiplicity

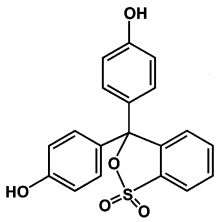
of actions of ER ligands at the molecular level. Knowledge in this area has resulted from the steady application of pharmacologic principles to endocrinology.

Antiestrogen action in the rat uterus appears to comply with the concepts implicit in the occupation theory. The intrinsic activity of the antiestrogen ER complex is lower than the estradiol ER complex; only a minority of receptors are used to produce for agonist activity[259] and partial agonists produce a good dose-related inhibition of agonist activity, eventually occupying all of the receptor pool.[223,260] However, it was reasonable to suppose that different subcellular effects are produced by different forms of the ER complex that result in a range of intrinsic activities.

Unfortunately, in the early 1980s, there was no precise assay system to study the structure−activity relationships of antiestrogens. It was then axiomatic that the complex pharmacology of antiestrogens in vivo resulted from different proportions of estrogenic and antiestrogenic metabolites interacting at single or multiple receptor sites within a given target tissue of a particular species. The problem was to dissect the events that occurred in vivo. A well-defined cell system in vitro was required so that the actions of parent compounds and their metabolites could be identified, thereby avoiding the complications of bioavailability, pharmacokinetics, pharmacodynamics, and metabolism. Breast cancer cells lines were available, but there were concerns about their origins and pedigree.[261] The ER-positive breast cancer cell lines MCF-7,[262,263] ZR75,[264] and T47D[265] had been described, but there was, at that time, little evidence for the direct stimulatory effects of estrogen on cell growth, although antiestrogens could prevent cell growth and this could be reversed by estrogen.[266,267] The control cells grew as rapidly as estrogen-treated cells in vitro, and there was speculation that breast cancer growth was, in fact, an indirect action of estrogen in vivo.[268] It was the discovery by the Katzenellenbogens in the latter part of the 1980s[269,270] that an estrogen was already present as a contaminant (**23**) in the phenol



(**23**)



(**24**) phenol red

red indicator (**24**) that clarified issues about the direct action of estrogen on growth. It is clear that breast cancer cells are so exquisitely sensitive to the actions of occult estrogens in the media that the addition of exogenous estrogen does not increase the growth of maximally growing cells. Thus, reliable studies on the structure−activity relationships of antiestrogen were only possible toward the end of the 1980s, i.e., more than 10 years after the original culture of ER-positive breast cancer cells.[262] On the other hand, the fact that the first ER-positive cell lines were initially propagated in estrogen-containing (i.e., phenol red) media maintained their phenotype for decades and prevented their eventual drift toward hormone-independent growth in response to estrogen withdrawal.

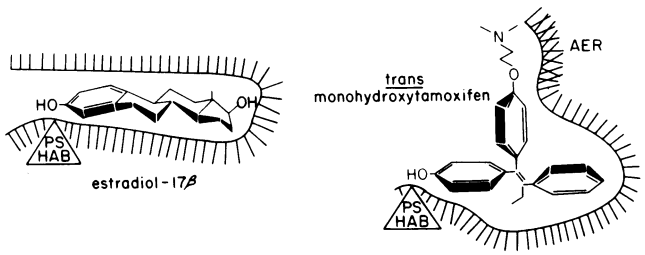


Figure 6. Hypothetical models for estrogenic and antiestrogenic ligand binding to the estrogen receptor. Estradiol is anchored at a phenolic site (PS) with high affinity binding (HAB). This is now known to be aa's Glu353 and Arg394.[86] *trans*-Monohydroxytamoxifen (now referred to as 4-hydroxytamoxifen) has the same high affinity binding site,[88,206] but the antiestrogenic ligand binds to the receptor site so that the dimethylminoethoxyphenyl side chain can interact with a hypothetical antiestrogenic region (AER). This region has subsequently been identified with a natural mutation of the ER at D351.[294] The aspartate may regulate the surface charge in the region[121,304] to modulate the binding of coactivators or corepressors.[304,308] Reprinted with permission from *Journal of Biological Chemistry* (page 4744, Figure 1).[274] Copyright 1983 The American Society for Biochemistry and Molecular Biology.

In contrast, by the end of the 1970s, primary cultures of rat pituitary cells were shown to respond to physiologic concentrations of estradiol with a specific increase in prolactin synthesis.[271] The reason that estradiol could increase gene activation of prolactin over control was subsequently believed to be because the higher protein concentration in media adsorbed the estrogenic contaminant in phenol red.[272] It also became clear that the concentration of an estrogenic stimulus required to stimulate growth was 1−2 logs less than the concentration required to monitor a gene product.[273] This model system initially proved to be ideal for evaluating the structure−activity relationships of nonsteroidal antiestrogens[153,274−279] and for proposing reasonable mechanical models for the action of estrogen and antiestrogen ER complexes at a single gene target. These ideas were eventually found to be conceptually accurate, but the complexity of hormone action was not anticipated.

Belleau's macromolecular perturbation theory was originally developed from a consideration of the structure−activity relationships of acetylcholine and a series of partial agonist/antagonists at the muscarinic receptor.[280] The concepts were applied to the regulation of prolactin synthesis by the ER to describe agonist, partial agonist, and antagonist actions.[276] With the receptor in a resting state, it can bind an agonist that produces a specific conformational perturbation and a stimulant action. Binding with an antagonist would produce a nonspecific conformational perturbation, and a partial agonist would produce an equilibrium mixture of the two states. A correctly positioned antiestrogenic side chain is essential for antiestrogen action, and the length was predicted to dictate the conformation change in the ER protein (Figure 6). It was proposed that the side chain controlled the subsequent activation of the ER interacting with a hypothetical "antiestrogen region" on the ER. Changes in the side chain length[193] or basicity[281] were predicted to produce a range of complexes with

different intrinsic activities that would result in different partial agonist activities.[274,276,277]

The structure−activity relationship studies in the early 1980s were complemented by work on the binding of radiolabeled estradiol and 4-hydroxytamoxifen to human ERs before and after incubation with an ER-specific polyclonal antibody.[282] The model for estrogen action proposed that estradiol (**1**) bound in the ligand binding domain and intrinsic activity were developed by locking the ligand into the complex by changes in the tertiary structure. Ligand-locking was a term used to describe transformation to the biologically active complex. By contrast, 4-hydroxytamoxifen (**20**) was wedged into the ligand binding domain because of multipoint attachment through the side chain. The polyclonal antibody did not affect ligand interaction after estrogen was locked or after the 4-hydroxytamoxifen was wedged; however, preincubation of the antibody with uncharged receptor prevented ligand locking for estradiol so that the ligand rapidly dissociated from the open cavity whereas 4-hydroxytamoxifen was still wedged into the cavity.

These studies with primary cultures at pituitary gland cells[274,275] and human breast tumor ER[282] were subsequently complemented with structure−activity relationship studies with tamoxifen analogues in breast cancer cells[283−285] using the complex endpoint of cell replication as a model system for the signal transduction pathway. The stated goal was to study ligand−ER interactions as a prelude to the interpretation of crystallography data from the ER complex. The problem was to obtain enough purified ER to produce crystals to resolve the structures by X-ray crystallography. The development of monoclonal antibodies to ER[286] and the cloning of the ER[40−42] opened the door for the crystallization of the ligand binding domain of ER with estrogens and antiestrogens and led (as an aside) to the identification of the "antiestrogen region" on ERα that controls intrinsic activity.

Chambon's group[56] was the first to address the issue of the target side specificity of 4-hydroxytamoxifen using recombinant human ER. They reported that the estrogen-like actions of 4-hydroxytamoxifen were cell-type- and promoter-context-dependent, which produced ligand-independent activity of the AF-1 site. This in turn, they stated, could explain the target site-specific estrogen-like actions observed with tamoxifen in animals and human tissue.[31,287] In contrast, a pure antiestrogen (zero intinsic activity) had no estrogen-like actions in model systems or in vivo.[56] However, the fact that a pure antiestrogen could produce complete antiestrogenic activity by also silencing AF-1 suggested that AF-1 activity could be ligand-specific, at least under controlled conditions.

The crystallization of the LBD of the ER with estrogens and antiestrogens has provided enormous insight into the change in protein shape that prevents antiestrogens from building a transcription complex at AF-2.[86,88] Both raloxifene and 4-hydroxytamoxifen fit into the hydrophobic pocket of the ligand binding domain, but the antiestrogen side chain prevents the reorientation of helix 12 that must seal the ligand into the receptor before coactivators can bind and produce a transcription complex. The high-affinity antiestrogens

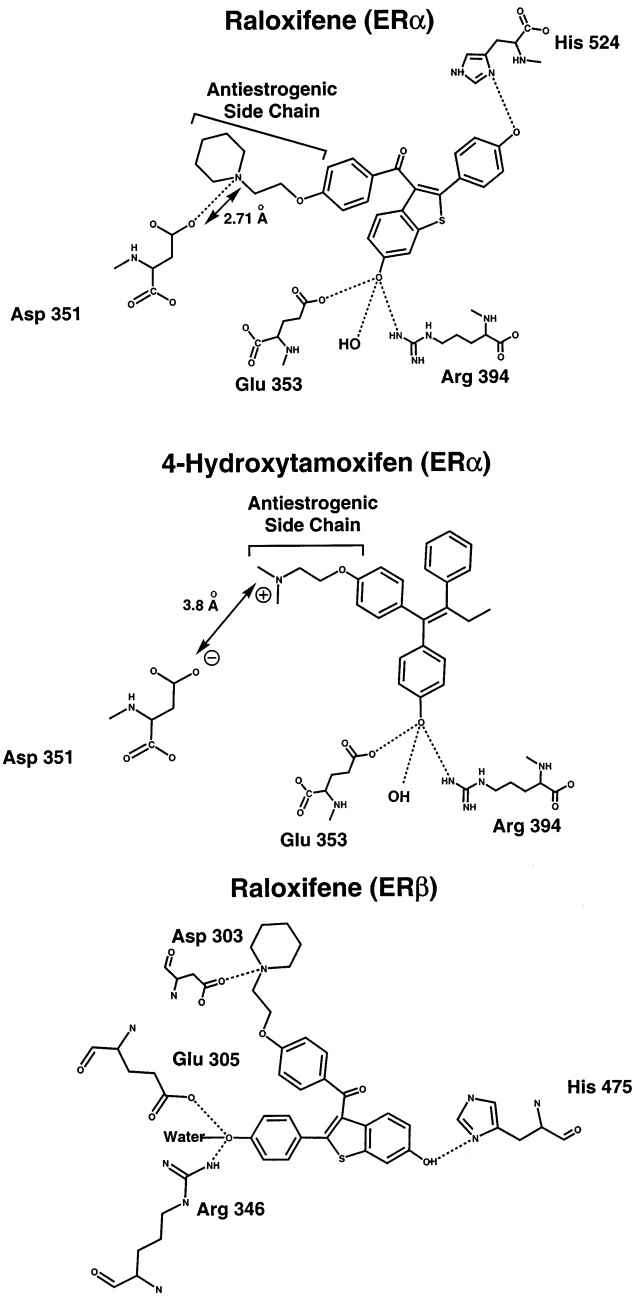


Figure 7. Binding of antiestrogenic ligands in the hydrophobic pocket of the E region of human ERα and rat ER*β*. The phenolic ring of 4-hydroxytamoxifen and raloxifene is located in the ligand binding domain by Glu353 and Arg394,[86,88] analogous to the A ring phenolic hydroxyl of estradiol-17*β* (Figure 4). The equivalent amino acids in rat ER*β* tether raloxifene in the ligand binding domain.[92] The antiestrogenic side chain of both 4-hydroxytamoxifen and raloxifene penetrates the receptor complex and either has an intimate (raloxifene ERα) or loose (4-hydroxytamoxifen) interaction with the surface amino acid D351. An equivalent interaction occurs in the side chain of raloxifene and D303 in rat ER*β*.

both interact through phenolic hydroxyls with Glu 353 and Arg 394 to locate the ligands correctly in the binding domain[86] (Figure 7). This is the high-affinity phenolic site proposed earlier[274] (Figure 6). However, the side chain, which is critical for antiestrogenic activity, interacts with Asp 351 in the case of raloxifene,[86] but this is only a weak interaction in the case of 4-hydroxytamoxifen[88] (Figure 7). It is possible that the charge distribution around aa 351 is part of the "antiestrogenic

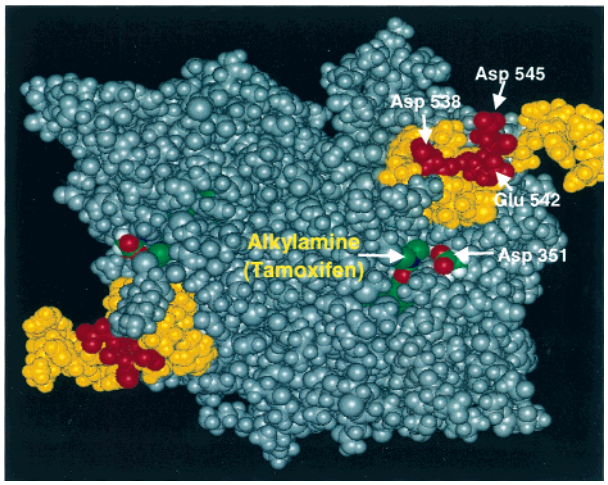


Figure 8. External surface of the dimerized ERα ligand binding domain complex liganded with 4-hydroxytamoxifen. The dimethyaminoethoxyphenyl side chain of 4-hydroxytamoxifen (Figure 7) causes the helix 12 (yellow) to be pushed away and occupy the GRIP binding site, thereby silencing AF-2 (Figure 5). The dimethyaminoethoxy side chain of 4-hydroxytamoxifen exits near asp(D) 351. The amino acids Asp538, Glu542, and Asp545 on helix 12 are necessary to maintain the estrogen-like actions of the 4-hydroxytamoxifen ERα complex.[304]

region" and the bulky dimethylaminoethoxyphenyl side chain prevents the locking of the receptor, i.e., the sealing of the ligand binding domain by helix 12.[218] This interpretation would be consistent with the X-ray crystallography results because the binding of the coactivator GRIP into the putative AF-2 region (see Figure 5B) is now blocked by the repositioning of helix 12.[88] The external surface of the 4-hydroxytamoxifen ERα complex is illustrated in Figure 8.

This molecular model provides an elegant solution to AF-2 silencing, but as noted above, there are subtle differences between the positioning of the 4-hydroxytamoxifen and raloxifene in the LBD (Figure 7) that may ultimately affect the intrinsic activity of the antiestrogen ERα complex. These clues now provide a link between the unusual pharmacology of the antiestrogens and the structure−function relationships of their ERα complexes.

(b) A Complex Problem from the Outside. McDonnell's group[119] extended the early hypothetical models for antiestrogen−ER interaction[153] and presented convincing evidence that the shapes of different antiestrogen ER complexes are not all the same.[119] An artificially mutated ER assay methodology in human liver cancer cells (HEPG2) was used to classify new antiestrogens based on their interaction with an engineered C3 promoter target.[288] However, it is the realization that a third component of the signal transduction system is involved[289] that has provided the most important clues for deciphering the mechanisms of SERMs. Coactivators and corepressors are key components that can modulate gene activation. Clearly the shape of an antiestrogenic ER complex will dictate how, or if, any other protein will bind to form the transcription unit (Figure 2).

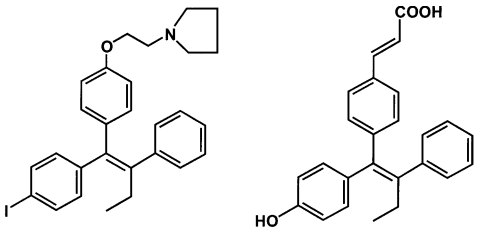
The fit of the SERMs in the ligand binding domain are similar enough that one could infer that both raloxifene and 4-hydroxytamoxifen simply silence AF-2[86,88] (Figure 7), but the intrinsic activity of the complexes is very different in vitro[290−292] and in the rodent uterus.[209] Clearly, a more precise explanation for antiestrogen action is required.

An advance has occurred through a variety of interdependent events: the study of drug resistance to tamoxifen, the development of a novel breast cancer system to determine the intrinsic activity of SERM ER complexes at a relevant target gene in situ, and molecular modeling with the published structures of the LBD of SERM ER complexes to interpret biological results.

A natural mutant ER D 351Y was isolated in 1994 from a tamoxifen-stimulated tumor line developed by the continuous passage of an MCF-7 tumor in tamoxifen-treated athymic mice.[293,294] Although a mutant receptor is not considered to be the mechanism of drug resistance for tamoxifen in breast cancer,[295,296] the recognition of the importance of D351Y has opened the door for an appreciation of drug−receptor interactions that control the intrinsic efficacy of the SERM ER complex. The cDNA for wildtype[297] and D351Y[298] ER have been stably transfected into MDA-MB-231 ER-negative breast cancer cells, and the intrinsic activity of SERM complexes have been determined at a transforming growth factor α (TGFα) gene target in situ.[299] The ER-negative breast cancer cells were chosen because it was thought they would be replete with transcription factors that dominate replication after the demise of the ER signal transduction pathway. It was reasoned that the reintroduction of the ER would allow the exploitation of the excess of transcription factors and amplify the actions of a SERM ER complex. Also, a complex promoter target for an estrogen-responsive gene was believed necessary to avoid generating inappropriate results with simple ERE reporter gene constructs. This was a critical decision because transient transfection, using simple ERE reporters, does not produce the optimal actions[300,301] observed in complex stably transfected systems.[119,121,302]

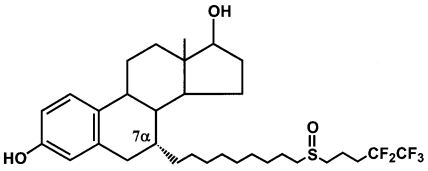
The MDA-MB-231 breast cancer assay provided a unique classification system for discriminating between tamoxifen and raloxifene at a relevant gene target in situ. The 4-hydroxytamoxifen wild-type ER complex is estrogen-like at the TGFα target and the D351Y enhances the estrogen-like properties of tamoxifen and its derivatives.[291] However, raloxifene is an antiestrogen in the assay and only has estrogen-like properties in the context of the MDA-MB-231 cells with the D351Y ER[290,303] mutation in the ligand binding domain. The cellular context amplifies the estrogen-like properties of the tamoxifen ER complex at AF-1,[121] but more importantly raloxifene silences both AF-2 and AF-1 but AF-1 can be reactivated allosterically by the D351Y ER.[304] Although the mechanistic role of the D351Y mutation in ER was obscure prior to resolution of the crystal structure of the raloxifene ER LBD,[86] the demonstration of ER modulation was valuable for identifying the first natural mutation that would change the pharmacology of an antiestrogenic to an estrogen-like complex.

Wijayaratne and co-workers[120] have recently completed a comprehensive analysis of ERα complexed with 4-hydroxytamoxifen (**20**), iodoxifene (**25**), raloxifene (**3**), GW7604 (**26**), and ICI 182,780 (**27**) using a novel set of



(25) idoxifene **(26)** GW 7604

peptides from phage display that recognize different surfaces on ERα. The question to be addressed is how do these observations translate into a molecular mechanism?



(27) ICI 182,780 (fulvestrant)

The ERα complex is modulated through a precise interaction of aa 351 and the antiestrogenic side chain of a SERM, which in some way allosterically communicates with AF-1. The antiestrogenic side chains of tamoxifen and raloxifene both exit the crystal structure of ERα at D351. However, the amine of the side chain of tamoxifen is further away by 1 Å than that of raloxifene[86,88] (Figure 9). The SERM ERα complex has been interrogated on the basis of the hypothesis that the shielding or neutralization of D351 by the side chain of raloxifene is responsible for the difference in the intrinsic activity of the raloxifene and tamoxifen ERα complexes. Replacing the aspartate with glycine results in a tamoxifen D351G ERα complex that has lost estrogen-like activity while retaining antiestrogenic properties.[121,300] The D351G mutation decreases the affinity of raloxifene for ERα, thereby illustrating the important role of the interaction of the piperidine side chain and D351.

The role of the side chain of tamoxifen in the intrinsic activity of the tamoxifen ERα complex is further illustrated with the compound GW7604 (**26**), an acrylic acid derivative. The GW7604 ER complex is less estrogen-like than the tamoxifen ERα complex, and the surface structure is distinct.[120,305] The carboxylic acid of GW7604 repels D351, thereby altering the surface charge of the SERM ERα complex (Figure 10).

Similarly, the raloxifene ERα complex can be modulated through both D351 and the antiestrogenic side chain. A D351E mutation that extends the interactive distance from 2.7 Å in raloxifene D351 to 3.5−5 Å in E351 increases the estrogen-like action of the raloxifene ERα complex[306] (Figure 11). Similarly, a D351Y mutation enhances the estrogen-like actions of raloxifene.[303,304] The charge extends beyond the influence of the side chain of raloxifene (Figure 12), but removal of the charge with D351F results in a loss of estrogen-like properties.[306] The critical role of the intimate relationship between the antiestrogenic side chain of raloxifene and D351 is confirmed with the raloxifene derivative

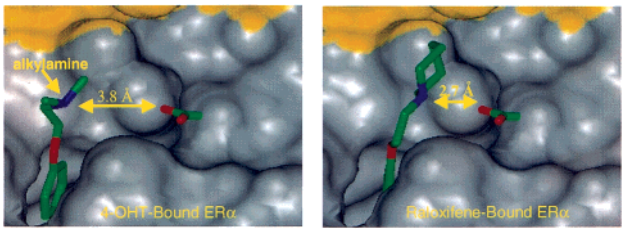


Figure 9. The relationship of the dimethylamine (alkylamine) side chain of 4-hydroxytamoxifen and D351 is different from the shielding of D351 noted with the piperidine side chain of raloxifene. The dimethylamine side chain of 4-hydroxytamoxifen is about 1 Å further away from D351 than the relationship noted with raloxifene.[86,88]

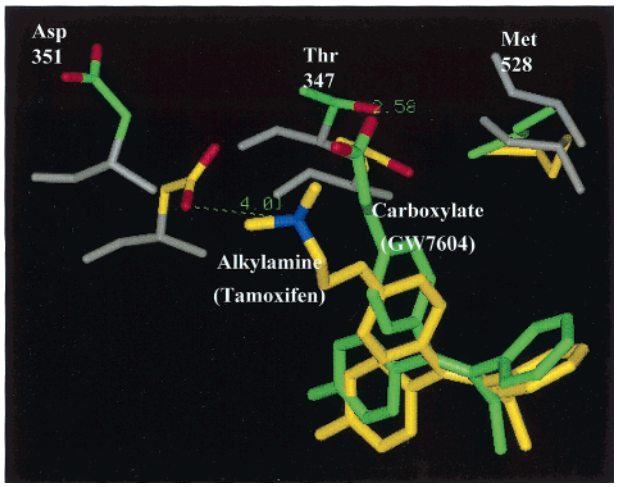
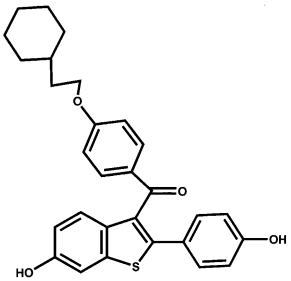


Figure 10. Comparison of the interaction of relevant amino acids with GW7604 and 4-hydroxytamoxifen in the ligand binding domain of ERα. The carboxylic acid of GW7604 repels aspartate 351. Reprinted with permission from *Endocrinology* (page 844, Figure 8A).[305] Copyright 2001 The Endocrine Society.

R1H where the piperidine ring of raloxifene (**3**) is replaced by a cyclohexane (**28**). The ligand loses anti-



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estrogenic properties and is a full agonist.[306,307]

Overall, the structure−function relationship for the allosteric silencing and reactivation of AF-1 in ERα could be interpreted by one of two equally plausible mechanisms: either the charge at 351 prevents the binding of a corepressor that would actually suppress the activation of AF-1[308] or the charge at 351 encourages the binding of coactivators at the novel site.[304] To the first suggestion, the recent finding of a new protein called RTA for repressor of tamoxifen transcriptional activity is of particular interest because it contains an RNA recognition motif and interacts with AF-1 of ERα. Norris and co-workers[309] have shown that RTA inhibits

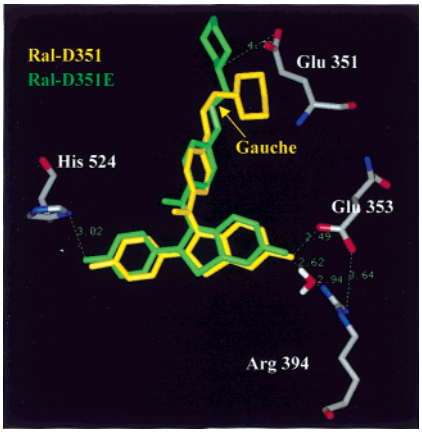


Figure 11. Change in the interaction of the piperidine side chain of raloxifene from the shielding position with aspartate 351 (gaule) to the extended interaction of 3.5−5.0 Å with glutamic acid 351. It is hypothesized that the extension of the bonding length enhances the probability of estrogen-like actions based on an available negative charge. Reprinted with permission from *Journal of Biological Chemistry* (page 9196, Figure 9A).[306] Copyright 2002 The American Society for Biochemistry and Molecular Biology.

the agonist actions of tamoxifen at ERα but mutation at the RNA binding domain acts as a dominant negative to enhance the agonist activity of all SERMs. To the second possibility, Shang and Brown[310] demonstrate an increase in the coactivator SRC-1 that can activate genes with a tamoxifen ER complex in endometrial cells but not breast cells. Interestingly enough, they show that modulation of SRC-1 can modulate gene transcription irrespective of cell context.

McDonnell's group[122] has used a phage display technique to identify two separate coactivator binding sites responsible for the expression of the estrogen-like effects of the estradiol or tamoxifen ER complex. The coactivator binding site on the estradiol ER complex could be the previously described AF2, but the novel site on the tamoxifen ER complex could be the same as the site on SERM complexes referred to as AF2b.[121,304] The site is more complex than the AF2a site previously noted,[311] which extends from aa 324 to aa 351. This is because acidic amino acids on helix 12 also play an essential regulatory role in the estrogen-like action of tamoxifen. Mutations D538A/E542A/D545A reduce the intrinsic activity of the tamoxifen ER complex,[121,304] thus indicating that the expression of full estrogen-like actions requires a triple interaction of aa351, helix 12, and AF-1.

Recent evidence of ERRs suggests that the protein has some interesting analogous structure−function relationship to ERα. Site-directed mutagenesis of ERRα-1 shows that F329A (analogous to A350 in ERα) acts as a dominant negative and loses constitutive activity.[143] Similarly ERRγ is blocked by 4-hydroxytamoxifen.[136,142,144]

(c) Turning the ER Off. If the ER modulates estrogenic responses in target tissues,[83] then the up- or down-regulation of the ER could have important consequences for gene activation. There appear to be two independent mechanisms for altering ER levels: (1) at the level of gene transcription and (2) at the level of protein turnover. Additionally, estradiol can down-regulate ERα mRNA in MCF-7 breast cancer cells but

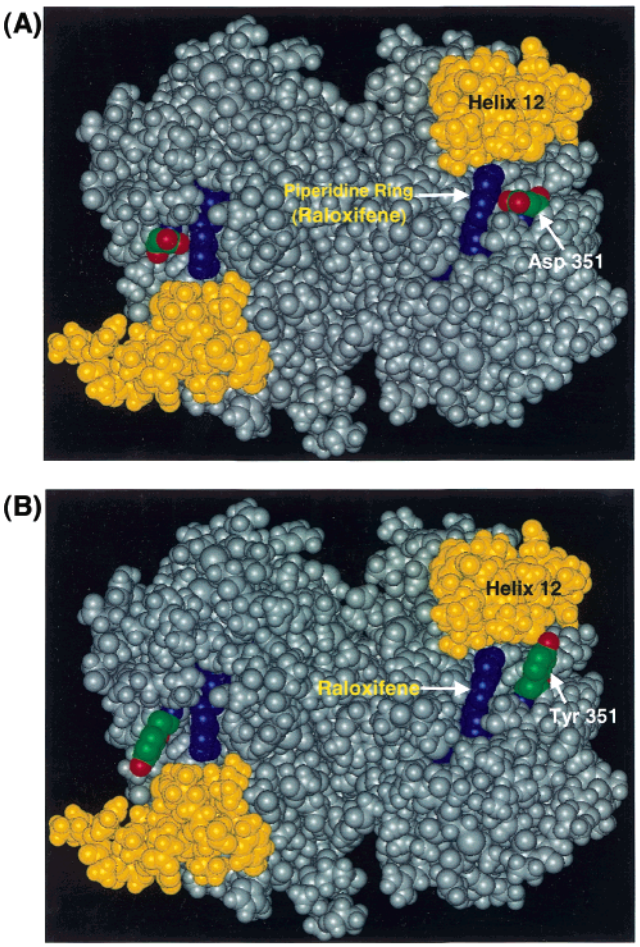
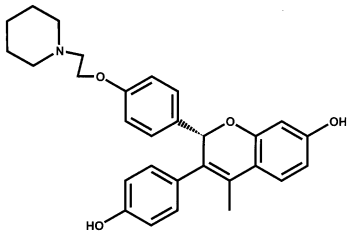


Figure 12. Comparison of the external surfaces of the wild type and D351Y ERα ligand binding domains complexed with raloxifene. The piperidine side chain of raloxifene pushes helix 12 away to silence AF-2 (Figure 4).[86] The piperidine side chain shields asp351, but replacement with tyr351 extends the charge beyond the influence of the antiestrogenic side chain. The complex becomes estrogen-like with the D351Y mutation because of an inability to bind corepressors.[308]

can up-regulate ERα mRNA in T47D breast cancer cells.[312] Tamoxifen or pure antiestrogens do not affect the mRNA of ERα.

SERMs produce different actions on the level of ER protein. Tamoxifen (**2**) causes an increase in ER levels,[312] whereas the SERMs raloxifene, EM652 (**29**),[313]



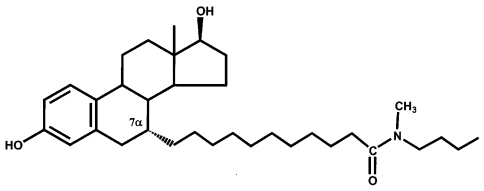
(29) EM652

and GW7604 (**26**)[120,305] all decrease ER levels. The dynamics of ER turnover are clearly multifaceted, and several mechanisms to stabilize or destabilize the ER complex are being investigated.

Changes in intracellular free Ca^{2+} concentrations can be translated into cellular events by calmodulin. The protein can change cell cycle progression, and inactiva-

tion of calmodulin can block the cell cycle. Incubation of ER-positive cells with tamoxifen causes a 5- to 10-fold increase in the association of ER with calmodulin,[314] whereas estradiol has no effect. Interestingly enough, the calmodulin antagonist trifluoperazine attenuates the interaction between calmodulin and the ER and causes a reduction in subcellular ER levels. Subcellular studies demonstrate that trifluoperazine does not affect ER mRNA, so calmodulin potentially stabilizes the protein by direct binding.[314]

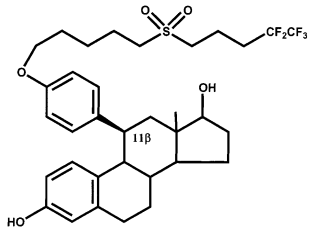
The pure antiestrogens bind to the ER and the long hydrophobic side chain significantly disrupts the protein structure, thereby resulting in cytoplasmic paralysis and rapid destruction.[315,316] The recent resolution of the binding site of rat ERβ with a pure antiestrogen ICI164,384 (**30**) illustrates the novel binding configu-



(30) ICI 164,384

ration of the complex[317] (see Pure Antiestrogens in part 2).

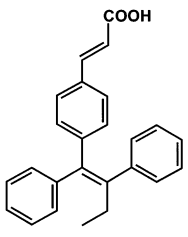
The pure antiestrogen RU58668 (**31**) causes a protein synthesis dependent paralysis of ER in the particulate



(31) RU 58,668

fraction of the cytoplasm that depends entirely on an intact ligand binding domain.[318] Indeed, the authors[318] suggest that antiestrogens that block ER nuclear localization will behave as pure antiestrogens in vivo.

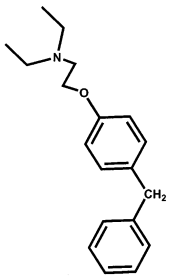
The destruction of the ER has been studied in detail by Wijayaratne and McDonnell.[319] The ER is a ubiquitinated protein within the cell, but the proportion of ubiquitination changes depending on the ligand. The tamoxifen (**2**) ER complex is hypoubiquitinated and the most stable, whereas the ICI 182,780 (**27**) ER complex is hyperubiquitinated and the least stable. It was also noted that GW5638 (**32**) caused a hyperubiquitinated



(32) GW 5638

ER complex,[319] thereby explaining the reduction observed in the ER levels when tamoxifen was compared to GW5638.[120,305] Obviously, the level of receptor can potentially play an important role in the ability of a SERM or antiestrogen to produce an agonist response through an alternative signal transduction pathway. High levels of a SERM ER complex present more opportunities for subcellular interactions, which may cause inappropriate cellular responses. In contrast, a low level of a SERM ER complex may have only limited opportunities to become promiscuous.

(d) Non-ER Interactions. Tamoxifen binds with high affinity ($K_D = 10^{-9}$ M) to an "antiestrogen binding site" (AEBS)[320] in most tissues around the body. The highest concentrations of AEBS are the liver microsomal fraction.[321,322] Specific ligands that bind to AEBS and not to ER show cytoxic actions in tumor cells.[323,324] Indeed, a compound originally designed to be a blocking drug for a novel histamine receptor, *N*,*N*-diethyl-2-ethanamine·HCl(DPPE) (**33**),[325] potentiates cytotoxic



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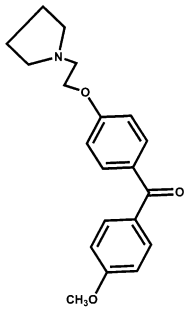
drugs in vitro[326,327] and in clinical trial.[328,329] Alternatively, labeled compounds have been used to photoaffinity-label protein targets for tamoxifen-like compounds. An azido photoaffinity derivative of DPPE was used to identify microsomal epoxide hydrolase as a tamoxifen target,[330] which was subsequently shown to be a predictor of tamoxifen response in primary breast cancer.[331] Current studies with new probes of the AEBS based on intestinal metabolic products of triphenylethylene antiestrogens (**34**)[332] may prove to be valuable in



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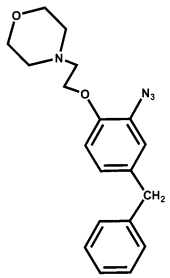

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deciphering the actions of tamoxifen on lipid metabolism.[333] One azide derivative[334] (**35**) has recently been applied to photoaffinity labeling of target enzymes.

It is unclear what role, if any, the AEBS plays in the actions of triphenylethylene-type antiestrogens, but it could be responsible for inadvertently extending the duration of action of SERMs or may turn out to be involved in the actions of SERMs on cholesterol metabolism.

Mechanisms of Action of SERMs: Problems

Although much progress has been made with our understanding of estrogen and antiestrogen action, there is no unifying theory that has explained the target site-specific actions of SERMs. Despite this deficit, there are opportunities to imagine multiple mechanism. In other words, there may be different mechanisms at different sites or groups of targets. By way of example, it is intriguing that raloxifene expresses less estrogen-like activity than 4-hydroxytamoxifen in breast and uterine cells. The shapes induced by the ligands in the ligand binding domain are similar enough that one could conclude that both raloxifene and 4-hydroxytamoxifen silenced AF-2. However, until the whole ligand−receptor complex has been crystallized, it is not possible to visualize the relationship between AF-1 and AF-2. If 4-hydroxytamoxifen is known to silence only AF-2 but AF-1 is easily activated in the correct environment,[56] then on the basis of the results at the TGFα gene, where the raloxifene receptor complex is without activity,[303] one would have to conclude that raloxifene is less estrogenic overall because it silences AF-1 and AF-2. This conclusion would be a reasonable explanation, based on the shape of ERα for the reduced estrogenicity in the rodent[237] and human uterus.[335] However, if only the shape of the raloxifene and 4-hydroxytamoxifen ERα complexes dictates the silencing of AFs selectively, then ERα shape alone cannot explain the similarity of the estrogen-like properties of raloxifene and tamoxifen on bone[35,247] and circulating cholesterol.[336,337] Additional target site-specific mechanisms need to be devised.

Another way of approaching the problem is that the shape of the ER complex is similar everywhere but the signal transduction pathways are controlled indirectly by the proportion of coregulators in a particular target tissue. The hypothesis would also apply to an understanding of drug resistance to tamoxifen. One could imagine that tamoxifen-stimulated (-resistant) breast cancer cells, with wild-type receptor, will be cloned out to grow if an excess of estrogenic coactivators could facilitate gene transcription through the tamoxifen ER complex.[296] This is the basis for McDonnell's[122] observation that the coactivators for tamoxifen-stimulated gene expression bind at sites different from those on the E2 ER complex. Less estrogenic antiestrogens would therefore be valuable second-line agents for treating tamoxifen-resistant breast cancer[338] because the new shape of the complex or destruction of the receptor would prevent gene transcription.[120] Furthermore, the hypothesis suggests, based on AF-1 and AF-2 silencing,[303] that raloxifene-like compounds may not be completely cross-resistant with tamoxifen. Unfortunately, this seems to be untrue in standardized models of tamoxifen resistance.[339] However, this may be true initially during the development of resistance; i.e., tamoxifen is overall more promiscuous as an estrogen with appropriate coactivators than raloxifene. In other words, drug resistance may develop more rapidly with tamoxifen than with other SERMs that are less able to exploit coactivator pools. The challenge is to identify the coactivator molecules and produce a target site-specific map around the body.

An alternative way of interpreting the different intrinsic activities of SERM ER complexes is that the differently shaped complexes can bind corepressor molecules that impair the attachment of coactivators or interfere with the assembly of a competent transcription complex. A number of corepressor molecules have been identified such as N-CoR and SMRT that interact with thyroid hormone or RARs,[340−343] but their role in antiestrogen action is not well defined. SMRT and N-CoR do, however, suppress the agonist activity of the tamoxifen ER complex, but they do not enhance the inhibitory effectiveness of antiestrogens nor do they block the activity of the estradiol ER complex.[344] Recently, Katzenellenbogen's group[345] described a new corepressor named "repressor of estrogen receptor activity" (REA). The 37 kDa protein is an ER-selective coregulator that competitively reverses SRC-1 activity. Norris and co-workers[309] also described RTA as a corepressor, but surprisingly high levels of the mRNA are noted in heart and nontarget tissues. The fact that RTA was discovered in a cDNA library from an ER-negative cervical cancer cell does not support the view that RTA is a specific inhibitor of the estrogenic actions of SERMS in estrogen target tissue. One goal that would enhance knowledge of SERM action at the target sites would be to document the family of corepressors that may be present in different *target* tissues.

An alternative idea is that the antiestrogen ERα complex interacts with another sequence of DNA that could be referred to as "an antiestrogen response element" in contrast to an ERE. Such an idea has been suggested by McDonnell and co-workers by screening cDNA libraries,[346] and a raloxifene response element has been proposed.[347] The latter authors subsequently suggested that there is no ER−DNA interaction but an ER−protein interaction that enhances the estrogen-like properties of raloxifene at a transforming growth factor β promoter.[348] This could explain the bone effects of raloxifene but 4-hydroxytamoxifen was not tested in the system, so a unifying mechanism cannot be proposed. The concept of gene activation by the tamoxifen ERα complex via a mechanism other than traditional EREs is illustrated recently by Shang and Brown.[310]

The idea of antiestrogen receptor complexes providing gene activation through a protein interaction (tethered) at the genome is appealing in light of the observation that 4-hydroxytamoxifen ERβ complexes can initiate gene transcription at AP-1 (fos and jun) sites.[74−76] One major concern is that the effect of raloxifene at ERβ is not related to the affinity of the ligand for the receptor. The raloxifene ERβ complex only activates an artificial reporter gene at extremely high concentrations of ligand, i.e., 1−10 μM.[74] Another strange finding is the observation that the pure antiestrogen ICI 164,384 (**30**)-can also activate AP-1 sites through ERβ, but estradiol and diethylstilbestrol block gene activation.[74,76] Clearly, these data are inconsistent with the reported effects of estrogen and pure antiestrogens on bone remodeling.[349]

Another idea for the modulation of SERM action is that the ratio of ERα to ERβ is important for expressing the estrogen-like effects of antiestrogens.[350−352] In simple terms, it could be imagined that with a higher proportion of ERβ there would be a greater likelihood of an estrogen-like effect with tamoxifen through nonclassical (AP-1) signal transduction pathways. At present, the hypothesis is being tested in breast tumors but available

antibodies for ERβ[353−356] have not resolved the issue. All current results on the distribution of ERβ are based on the use of RT-PCR and extrapolation of RNA levels to deduce the presence of a pharmacological target. The increase in ERβ in tumors is thought to be the reason for tamoxifen-induced drug resistance though the AP-1 pathway (see part 2). However, the fact that pure antiestrogens also activate AP-1 through ERβ[76] is inconsistent with clinical observation. The pure antiestrogen ICI 182,780 (fulvestrant) (**27**) is effective as a treatment for tamoxifen-resistant breast cancer.[338]

It is important to appreciate that progress in deciphering the multifaceted mechanism will come from a precise analysis of the actions of SERMs in the clinic. An action described for a SERM at a gene target in engineered cells must, as far as possible, be related to a biologic effect in humans. In other words, the recognition of the actual action of SERMs in the human must guide the interpretation of biochemical data from the laboratory. At present, there is a wealth of useful information about the target site-specific actions of tamoxifen and raloxifene as well as a range of analogues of tamoxifen that have been tested and rejected. This database is the foundation for improving drug targeting for SERMs and is considered in part 2.

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Biography

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