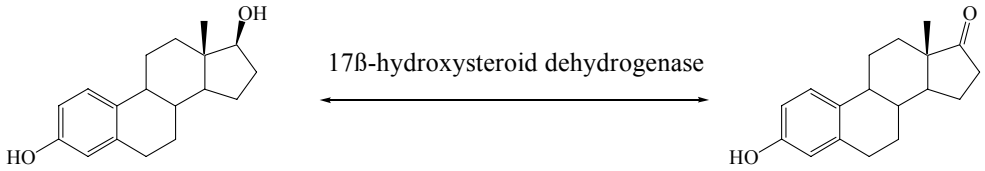
1 Introduction

1.1 Breast Cancer and Estrogen

Breast cancer is the most frequently diagnosed cancer in women. In Germany and other industrialized countries breast cancer is ascertained with approximately every tenth woman in the course of her life. In Germany breast cancer is responsible for 24% of all new cancer cases among women. It is estimated that there are 47,500 new breast cancer cases each year [Giersiepen, 1]. Also men can be suffer from breast cancer, even if very seldom. Breast cancer is frequently "hormone dependent". That is: hormones stimulate the cancer cells to grow. Vice versa this means that the growth of the cancer cells can be downregulated by the oppositely active hormones or so-called "anti-hormones". Therefore, a hormone therapy is possible as an adjuvant treatment on breast cancer as well as with metastases. Today operation, radiotherapy, chemotherapy and hormonal therapy form a common combination coordinated on each other of treatments.

The “dependent” natural female sex hormone means primarily the estrogen, 17β-estradiol (E2), which plays a prominent role in mediating the maturation, proliferation, differentiation, apoptosis, inflammation, metabolism, homeostasis and brain function and influences the growth and development of breast cancer [Edwards, 2; Song, 3; Yager, 4]. The biological activity of estradiol in hormone sensitive tissues is actively regulated by the interconversion between estradiol and the less active hormone, estrone, by 17β-hydroxysteroid dehydrogenase [Dubey, 5; see Scheme 1].



Estradiol (estrogen) Estrone (estrogen)

Scheme 1

Because of its special effects, the deficiency of E2 in healthy women might be associated with an increased risk of various diseases and, in contrary, the normal existence of E2 in women with hormone-positive breast cancer might worsen disease. In all these cases, hormone therapy may be sensible, effective and practical. For the deficiency of E2, the

hormone replacement therapy means an extra supplementation with exogenous natural estrogens which include estradiol, estrone and estriol as well as synthetic estrogens that have been used for years as imitations with similar characteristics as natural estrogens but are not the same biological chemicals that exist in our bodies from birth. In theory, treating hormonal deficiencies with natural estrogens should have many benefits over synthetic estrogens and, in fact, currently many patients do use natural estrogens for hormonal supplementation. For instance, E2 is used especially in postmenopausal women with reduced ovarian hormonal concentrations to prevent and treat cardiovascular diseases, to reduce lipoprotein cholesterol levels and lower blood pressure, to prevent spinal bone loss, to inhibit skin aging and improve glycemic control in patients with noninsulin-dependent diabetes mellitus [Glasnapp, 6]. For breast cancer, however, the hormone therapy means that synthetic estrogens and, in particular, antiestrogens are used to inhibit the physiological activities of E2 that stimulates the growth and development of breast cancer, so as to control and treat the disease.

1.2 Estrogen Agonists and Antagonists

In clinical settings, exogenous estrogens and antiestrogens are used for hormone replacement therapy and as anticancer agents [Cosman, 7]. They can be categorized into three pharmacological classes: estrogen agonists, mixed agonist-antagonists, and pure antagonists. The mixed agonist-antagonists are also referred to as selective estrogen receptor modulators (SERMs), because of their tissue-selective agonist or antagonist activities.

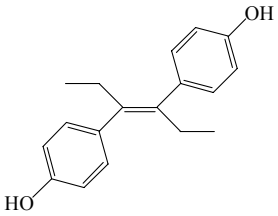
1.2.1 Agonists

Besides the above-mentioned natural estrogens 17β-estradiol and estrone, there are many other estrogens, e. g. diethylstilbestrol (DES, see Scheme 2). Diethylstilbestrol, a synthetic estrogen, was to revolutionize therapeutics with estrogen and was once used to treat breast and prostate cancer with high-dose of drugs as standard endocrine therapy before the discovery of antiestrogens (Haddow, 8).

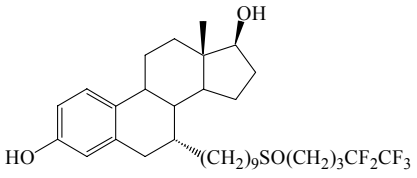
1.2.2 Selective estrogen receptor modulators

Since estrogens are known to play a role in the growth and development of many breast cancers, a logical approach for the treatment of estrogen-sensitive breast cancer is the use of

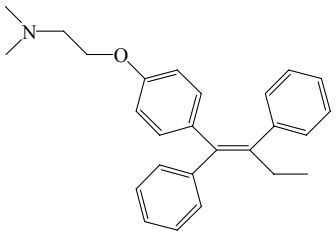
antiestrogens that inhibit the estrogen function in breast cancer cells. The first “classic antiestrogen” tamoxifen (see Scheme 2) was therefore developed. However, tamoxifen is now reclassified as a typical selective estrogen receptor modulator. Tamoxifen is largely inhibitory and functions as estrogen antagonist in breast cancer cells, but it also functions as an agonist in some tissues including the bone, uterus, liver and the cardiovascular system. These estrogen-like activities of tamoxifen are significant for woman taking antiestrogen against breast cancer. Its stimulatory effects on uterus and liver may underlie the increased incidence of endometrial hyperplasia that may lead to cancer, as well as alterations in liver function. The agonistic effects of tamoxifen in bone cells and in the cardiovascular system enhance bone maintenance, preserve a favourable blood lipid profile, and reduce risk of coronary problems [Katzenellenbogen, 9 and refs. therein]. Because of this function selectivity, tamoxifen has been used up to now as standard therapy by adjuvant hormone treatment on breast cancer.



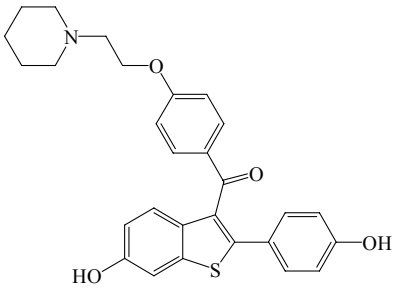
Diethylstilbestrol



Fulvestrant (ICI 182 780)



Tamoxifen



Raloxifen

Scheme 2

Another typical SERM is raloxifene, which has been shown to function as an antagonist in the breast and uterus, while functioning as an estrogen in the bone and cardiovascular system. Raloxifene was developed first as an antiestrogen for breast cancer in the late 1980s, but because it was found to maintain bone density, to prevent rodent breast cancer, and to inhibit tamoxifen-stimulated endometrial cancer growth, it was developed for osteoporosis, for which it is now an approved drug [Osborne, 10]. Raloxifene is an inhibitor of cultured breast cancer

cells and in vivo, it possesses antitumor activity. Like tamoxifen, raloxifene reduces total cholesterol but does not increase high-density lipoprotein cholesterol, a feature that may lessen any cardioprotective effects.[Osborne, 10; Balfour, 11].

1.2.3 Antagonists

Several classes of pure antiestrogens, which possess no known estrogen agonist effects, have been developed for the treatment of breast cancer. Pure antiestrogens, such as ICI 164 384, ICI 182 780 (fulvestrant, Scheme 2), and RU 54 876, may perhaps prove to be more effective than tamoxifen in treating hormone-responsive breast cancer, but are not effective in preventing bone loss and may have detrimental effects on the cardiovascular system [Katzenellenbogen, 9; and refs. cited therein]. Therefore, a pure antiestrogen, e. g. fulvestrant is recommended for treatment of breast cancer after failure of a first-line therapy with tamoxifen [Mckeage, 12].

The biological effects of estrogens and antiestrogens mostly are mediated through the estrogen receptor (ER), which acts as hormone-activated transcription factor.

1.3 Estrogen Receptor

1.3.1 Discovery of the estrogen receptor

The estrogen receptor is a ligand-regulated transcription factor that belongs to the nuclear receptor superfamily and acts as a dimeric species. In the early 1960s, Jensen and Jacobsen first demonstrated that a specific protein was responsible for the concentration of physiological levels of estradiol in target tissues [Jensen, 13]. This protein is now known as the estrogen receptor (ER). Jensen and colleagues translated the basic science into clinical utility by proposing a predictive test, the ER assay, to determine which patients would respond to endocrine ablation. It was then established that patients with ER-rich tumors respond to endocrine therapy, whereas patients with ER negative tumors are unlikely to respond [Jensen, 14; Macgregor, 15]. There are two subtypes of ER, ERα and ERβ. The human ERα (hERα) was cloned and sequenced in 1986 from MCF-7 human breast cancer cells [Green, 16, Greene, 17]. The ERβ was first cloned from a rat prostate complementary DNA (cDNA) library [Kuiper, 18] and then the human ERβ (hERβ) was also identified and characterized in 1996 [Mosselman, 19].

The hERα and hERβ are expressed next to one another in many tissues, including the central nervous system, the cardiovascular system, the urogenital tract, the breast and the bone (Figure 1). In the uterus and mammary gland, ERα is an important estrogen receptor and much more present than ERβ. In addition, ERα is also expressed in liver, while in the gastrointestinal tract is only ERβ [Gustafsson, 20].

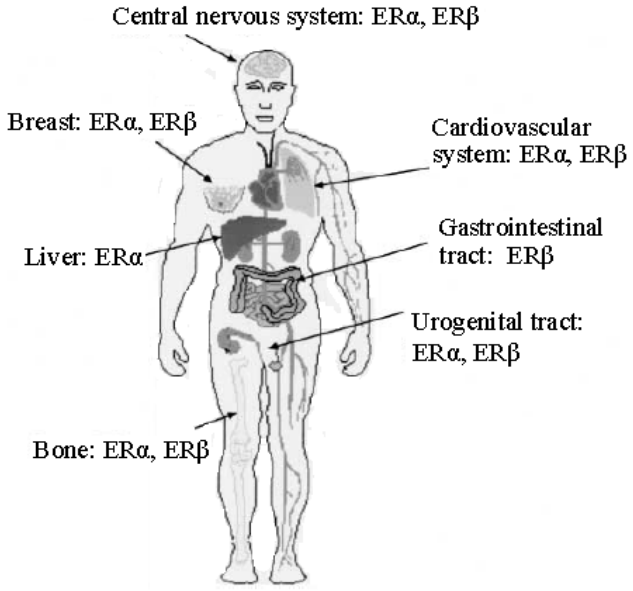


Figure 1 Overall distribution of ERα and ERβ in different tissues [Gustafsson, 20].

Both ERα and ERβ are mainly regulated by the endogenous estrogen E2. ER modulation is involved in the development and regulation of reproductive, cardiovascular, and bone health, in addition to controlling various aspects of cognitive function [McDonnell, 21]. Besides, an excessive activity of ER has been correlated with the development and proliferation of certain breast and uterine carcinomas [Persson, 22].

1.3.2 Structure of the estrogen receptors

ERα and ERβ represent two separate gene products. The hERα protein consists of 596 amino acids with a molecular weight of 66 kDa [Kong, 23; Green, 16] and is located on chromosome 6 [Menasce, 24], while the hERβ sequence encodes a protein of 530 amino acid residues with a molecular weight of 59 kDa [Ogawa, 25] and is positioned on chromosome 14 [Mosselman, 19].

Like other nuclear receptors, the ER has a multidomain structure consisting of six functional regions, from the N-terminal A/B domain to the C-terminal F domain, which show various degrees of sequence conservation (Figure 2) [Kong, 23].

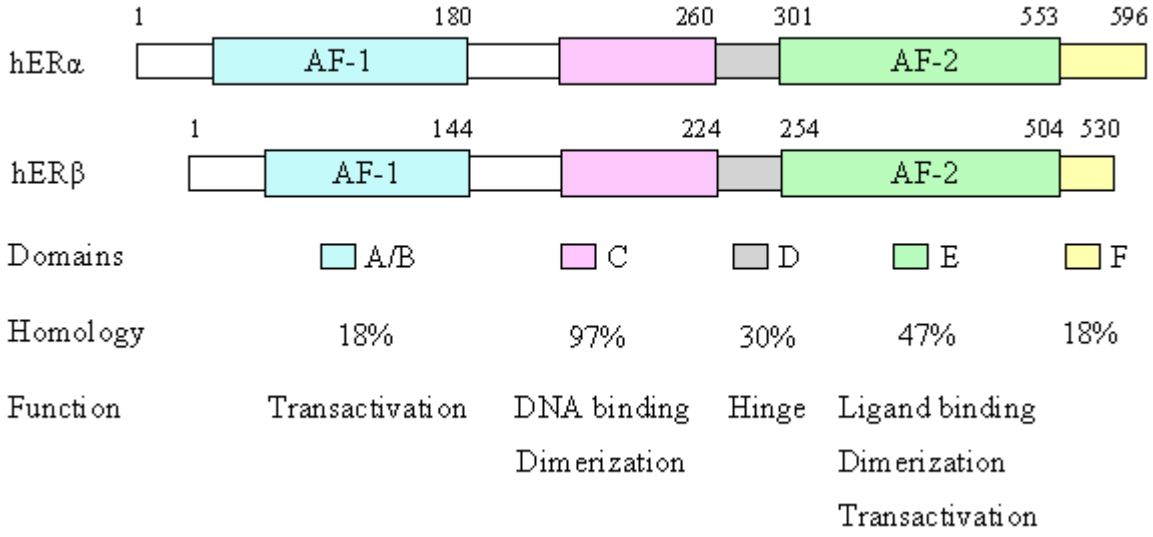


Figure 2. Domain structure representation of human ERα and ERβ isoforms [Kong, 23].

The poorly conserved combined A/B region contains the autonomous transactivation function AF-1. In this region, no clear secondary structure can be identified and no structural data have been obtained until now [Ruff, 26]. The better characterized parts, for which functional and structural data are available, are the highly conserved C region harboring the DNA-binding domain (DBD) and the conserved E region containing the ligand-binding domain (LBD) as well as the transactivation function AF-2. The D domain can be considered as a linker peptide between the DBD and the LBD, whereas F domain, a C-terminal extension region of the LBD, is not conserved [Ruff, 26]. Both ERα and ERβ share a modest overall sequence identity (47%) [Muramatsu, 27]. The DNA-binding domains of ERα and ERβ show a high degree 97% of homology (only three amino acids differ), but the ligand-binding domain shows only 47% homology [Gustafsson, 20; Kong, 23].

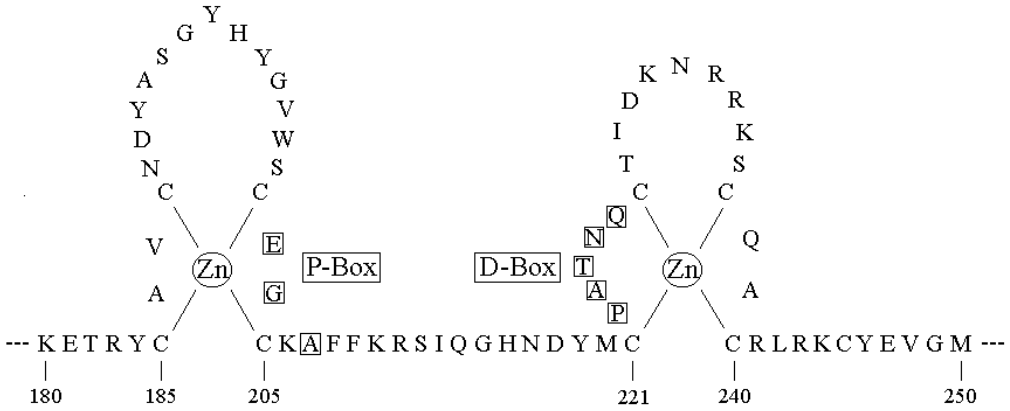


Figure 3. The DBD of hERα comprises two zinc finger motifs according to Ruff [26], Pettersson [28], Chen [29], Tsai [30].

The DBDs of the two ER isoforms share the same response elements. DBD structures are available only for ERα [Ruff, 26]. The topology of ER DBDs is characterized by two zinc finger motifs with eight cysteines that constitute the tetrahedral coordination of two zinc ions [Ruff, 26; Pettersson, 28; Chen, 29; Tsai, 30] (see Figure 3). These zinc fingers are essential components of the ER due to their non-fungible DNA binding function [Kumar, 31 and 32]. The first zinc finger sequence is neutral to slightly acidic, which determines the binding specificity to the so called estrogen response elements (ERE), whereas the second zinc finger structure harbors a positive net charge and governs unspecific DNA contacts as well as dimerization of the two DBD molecules [Wingender, 33]. The helical structure of the "P-box" (E, G, A) and downstream amino acids provides important deoxynucleotide contacts and fits into the major groove of the DNA helix. The amino acids in the "P-box" are responsible for base recognition and discrimination, whereas the residues participating in the "D-box" (P, A, T, N and Q) have been shown to be involved in the dimerization interface [Tsai, 30; Ruff, 26].

The LBD is a globular domain that harbors a hormone (ligand) binding site, a dimerization interface, and a coactivator and corepressor interaction function. Despite low sequence identity in LBDs of the nuclear receptor superfamily, the three-dimensional structures of the LBDs are similar [Ruff, 26]. The first reported crystal structure for a steroid receptor was that of ERα LBD in complex with estradiol and raloxifene [Brzozowski, 34]. Also the crystal structure of ERβ LBD was characterized in complex with genistein and raloxifene [Pike, 35]. ER LBDs are arranged in an antiparallel α-helical "sandwich" fold that was first described for the human RXRα apolipoprotein LBD [Bourguet, 36]. The liganded ER LBD (Figure 4)

contains 11 α-helices (H1, H3-H12) organized in a three-layered sandwich structure with H4, H5, H6, H8 and H9 flanked on one side by H1 and H3, and on the other side by H7, H10, and H11 [Ruff, 26]. The ligand pocket is closed on one side by an antiparallel β-sheet and on the other by H12, which is known to be directly involved in the transactivation function AF-2 by mutagenesis studies [Danielian, 37], and for which several "agonist" or "antagonist" conformations have been evidenced [Moras, 38].

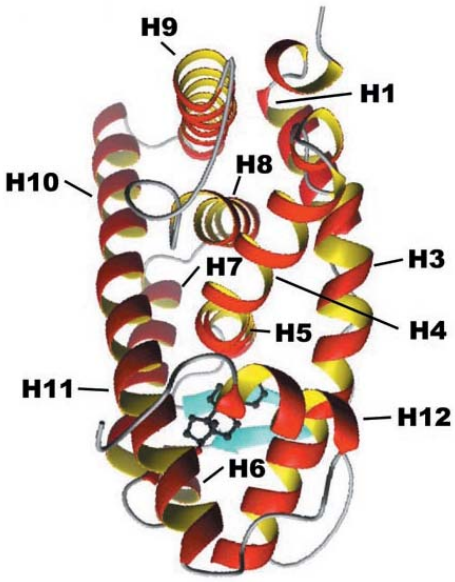


Figure 4. Three-dimensional structure of the wild-type ER LBD monomer, with the β-sheet colored in blue [Brzozowski, 34; Ruff, 26].

AF-1 located in the A/B regions mediates a constitutive activation potential and is responsible for the promoter-specific transcriptional activation independent of the presence of ligand. In addition, the AF-1 is thought to be responsible for the partial agonist activity of tamoxifen in cells that express ERα (McInerney, 39). AF-2 contained in the E domain provides ligand-specific activation. AF-1 and AF-2 are autonomous separately in their regions and also synergistic with each other in most case [Tzukerman, 40; Berry, 41].

1.3.3 Estrogen receptor transcription

The ER is a ligand-activated transcription factor. Both ERα and ERβ stimulate transcription of an ER responsive gene containing an estrogen responsive element (ERE) in an E2 dependent manner [Kuiper, 42]. Ligand-binding experiments revealed high affinity and

specific binding of E2 to both ER isotypes, and no obvious differences between the two isotypes alone or combined were observed in ERE transcriptional assays in the presence of E2 [Kuiper, 42]. ER-mediated transcription is a highly complex process involving a multitude of coregulatory factors and "cross-talk" between distinct signaling pathways [Shao, 43; Hall, 44], which could be depicted in a mode including ligand-dependent and ligand-independent ER transcriptional activations (Figure 5 and 6) [Katzenellenbogen, 9; Shao, 43; Edwards, 2].

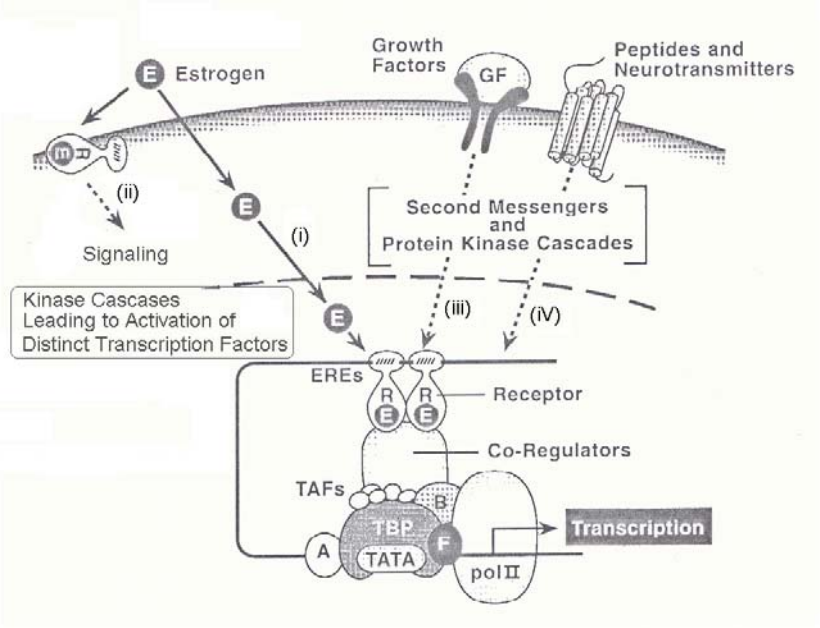


Figure 5. A model for estrogen receptor transcription [Katzenellenbogen, 9]. The abbreviations used are E, estrogen; R, receptor; ERE, estrogen response element; GF, growth factor; TBP, TATA binding protein; TAFs, TBP-associated factors; pol II, RNA polymerase II.

The basic pathway follows an E2-regulated ER transcription line (i): Upon binding E2, ER become activated through a process that involves dissociation from protein chaperones, conformational change, dimerization, and binding to EREs of target genes. ERE-bound ER recruits coregulators that stimulate gene transcription.

In the non-active state, ER exists as a heterocomplex consisting of the heat shock protein 90 (Hsp90) and immunophilin FK binding protein 52 (FKBP52). Hsp90 binds directly to the ER LBD to form a less stable complex, which is stabilized by FKBP52 through a direct binding to Hsp90 and an electrostatical interaction with the NLS contained at the C-terminal end of the ER DBD [Pratt, 45]. The role of Hsp90 and other chaperons may be to maintain the receptors folded in an appropriate conformation to respond rapidly to hormonal signals [Pratt, 45; White, 46]. This inactive ER complex continually shuttles between the nucleus and cytoplasm with nuclear localization and nuclear export sequences. E2 diffuses through the

plasma membrane and cytoplasm of cells into the nucleus where it binds to the ER LBD. Once E2 binds to the ER, heat shock protein 90 and immunophillin dissociate and the receptor undergoes a conformational change transforming the receptors to the active form [Pratt, 45]. It has been demonstrated that different ligands induce different changes in receptor (ERα) conformation (see Section 1.4.1) and target cells can distinguish between these ERα-ligand complexes. These conformational changes have been shown to influence ERα-cofactor binding and, therefore, have a profound impact on ERα pharmacology [Paige, 47; McDonnell, 48; Wijayaratne, 49 and 50]. In addition, the nature of the bound ligand also influences the stability of ERα, and the rate of ERα degradation in the presence of E2 directly correlates with transcriptional activity [Wijayaratne, 50]. It was even concluded that acute degradation of ERα followed by an E2-dependent transcriptional activation of ERα mRNA is a general estradiol response [Pinzone, 51]. With these conformational changes, the receptors dimerize as homodimers (ERα/ERα and ERβ/ERβ) or heterodimers (ERα/ERβ) [Cowley, 52; Pace, 53]. The dimer complex is translocated to the nucleus of the target cells by nuclear localization sites. As mentioned in section 1.3.2, ER contains two dimerization domains, one in the DBD and one in the LBD. Dimerization by the LBD is ligand dependent, whereas dimerization by the DBD is ligand independent and mediated by sequences in the DNA, therein serine 236 located in the second zinc finger of ERα DBD plays an important role [Chen, 29]. However, hERα is phosphorylated by protein kinase A (PKA) on serine 236 and phosphorylation at this site can inhibit dimerization in the absence of estrogen and, therefore, inhibit DNA binding. Binding of estrogen to ER can overcome this inhibition [Chen, 29]. Even though this inhibition of dimerization was found in ERα, phosphorylation on multiple sites of ERα as a phosphoprotein, by ligand binding and other events, increases transcriptional activation of the receptor [Chen, 29]. The dimer complex either directly binds to EREs in target genes or indirectly interacts with DNA through tethering to other DNA-bound transcription factors, e.g. AP-1 [Paech, 54; Webb, 55] or Sp1 [Safe, 56], in a way that stabilizes the DNA binding of that transcription factor in the absence of direct ER-DNA binding, to alter the rate of transcription. These EREs may be consensus or nonconsensus and may exist as single or multiple full or half sites; they may also be composite sites, consisting of EREs flanked by response elements for other transcription factors (such as Sp1, Sp1 may play two roles, either in direct binding as “half site” or in indirect interaction as “tethering” [Safe, 56]), which themselves may or may not be occupied by their respective transactivating factors [Katzenellenbogen, 57]. The ERE sequence is an allosteric effector of ER action. Binding of ER to natural and synthetic EREs with different nucleotide sequences alters ER binding

affinity, conformation, and transcriptional activity and, therefore, impacts physical and functional interaction of ERα and ERβ with coregulators [Klinge, 58]. Both direct and indirect interaction between ER and EREs result in recruitment of coregulators and components of the RNA polymerase II transcription initiation complex that enhances target gene transcription [Klinge, 59].

Coregulators can be broadly divided into coactivators, which augment the activity of receptors, and corepressors, which mediate the repressive effects of receptors [Rosenfeld, 60]. In recent years, at least 28 different ERα coactivator proteins have been identified [Klinge, 58]. Many coactivators required for ER activity are histone acetyltransferases (HATs), e.g. CBP/p300 [Ogryzko, 61]. Transcriptional activation involves alterations in chromatin structure mediated by ATP-dependent chromatin-remodeling enzymes in conjunction with factors that contain histone acetyltransferase activity [Kingston, 62], and transcriptional competence correlates with the acetylation of chromosomal histone proteins at their N-termini, which results in destabilization of protein-DNA contacts and chromatin decompaction [Orphanides, 63]. Briefly, coactivators facilitate ER transcription through their functions of (1) acetylating the N-terminal tails of lysine residues in histones H3 and H4 leading to “relaxed” chromatin structure, (2) acetylating other transcription factors and coactivators, (3) recruiting secondary coactivators including coactivator associated arginine methyltransferase 1 (CARM 1) and protein arginine methyltransferase 1 (PRMT 1) that methylate histones, (4) interaction with components of various ATP-dependent chromatin-remodeling complexes, and (5) direct interaction with and stabilize basal transcription factor binding [Klinge, 58 and refs. cited therein]. Most of coactivators, e.g. p160 family proteins, interact with the AF-2 domain of agonist-bound ERs through multiple LxxLL (L = leucine, x = any amino acid) amino acid motifs [Heery, 64], whereas some coactivators, such as the steroid receptor RNA activator SRA and the RNA helicases p68/p72, interact with and regulate the AF-1 domain of ER [Lanz, 65; Endoh, 66; Watanabe, 67].

Opposing the coactivators, corepressors negatively regulate transcription, namely promote transcriptional repression, via their recruitment of histone deacetylases (HDACs). The best characterized corepressors are the structurally related proteins NCoR and SMRT, which are recruited by ER to the promoter of target genes in the presence of antagonists such as tamoxifen [Shang, 68; Huang, 69]. But NCoR and SMRT differentially impact E2-induced transcriptional activity in an ER subtyp- and ERE sequence-dependent manner [Klinge, 58]. Other proteins act to repress ER-mediated transcription by distinct mechanisms. For instance, the ER-specific corepressor REA, as well as the orphan receptors SHP and DAX-1 act by

competing with the p160 coactivators for binding agonist-bound ER [Moggs, 70 and refs. therein].

Upon binding to ERE and in cooperation with coregulators, ER binds to a promoter and forms a transcription preinitiation complex. Upon further interaction with coregulators, components of the core transcriptional machinery “TATA-box” and RNA polymerase II (pol II), a transcription initiation complex is complete. RNA polymerase II is recruited to the transcription start site and begins transcription [Cosma, 71; Tsai, 30].

Besides the E2-dependent basic pathway (i), there are other E2-dependent or -independent pathways, that play an important role for gene transcription of the ER. These include signaling pathway (ii) regulated by membrane ER, and modulation of ER activity by growth factors (including epidermal growth factor, insulin-like growth factor-1, insulin, and transforming growth factor-β), neurotransmitters such as dopamine, and second messengers such as cAMP and others that affect protein kinase cascades including the MAP kinase signaling pathway (iii) and (iV) (Figure 6)[Edwards, 2; Shao, 43; Katzenellenbogen, 9].

Pathway (ii) is about membrane ER action. ER is one of nuclear receptor super families. The majority of them in the cell reside in the nucleus in the presence of estrogen. But a small fraction of total receptors are also localized at or near the cell membrane in either the presence or absence of estrogen. Several studies suggest that ER localization to the cell membrane is facilitated by association with other proteins that themselves translocate to the cell membrane. Candidate proteins reported to fulfill this role are caveolin-1, the adaptor molecule Shc, and IGF1 receptor [Edwards, 2; and refs. therein]. The adaptor molecule, Shc, through a coupling of ER with IGF1 receptor, has also been suggested to mediate ER translocation to the cell membrane in an estrogen dependent manner. The signaling pathway (iii) also is mediated by the interaction between growth factor receptor and Shc as well as membrane ER. The ER LBD alone is sufficient for estrogen-dependent translocation to the cell membrane and also sufficient for mediating many of the described effects of estrogen on signal transduction pathways in different cell types [Edwards, 2; and refs. therein]. These extranuclear signaling pathways converge upon and activate nuclear transcription factors by phosphorylation and thus may ultimately affect gene expression patterns by integration with nuclear signaling by ER in the cell.

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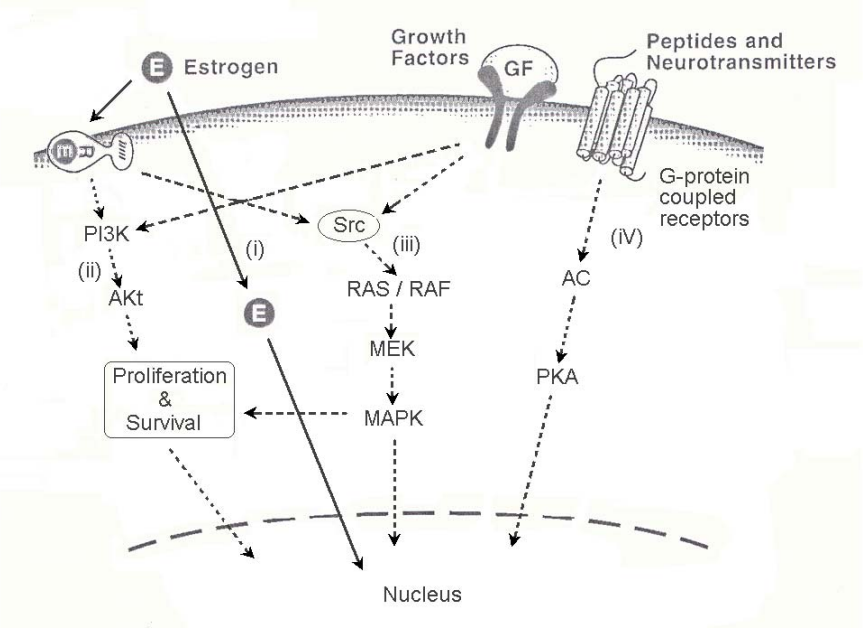


Figure 6 (see Figure 5). Extranuclear signaling pathways in ER transcription. Membrane ER, and / or growth Factor receptors, can interact directly with components of the cytosolic signaling molecules, including the regulatory subunit of PI3K, leading to the activation of the serine/threonine kinase Akt pathway (ii); Growth factors such as epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), insulin, and transforming growth factor-β (TGF-β) bind to and activate their receptors, which, and / or membrane ER, in turn activate the Src-RAS–RAF–MEK-MAPK and the phosphoinositide 3-kinase (PI3K) pathways (iii); Other extracellular stimuli such as dopamine and cyclic AMP bind G-protein-coupled receptors and activate adenylyl cyclase (AC) and protein kinase A (PKA) pathway (iV). The activated kinases subsequently phosphorylate and activate ER and other transcription factors in the nucleus [Shao, 43; Edwards, 2].

In present time, more understanding of molecular mechanisms of steroid receptors is in terms of transcriptional signaling in the nucleus, whereas the clear mechanisms by which conventional steroid receptors interact with and modulate the activities of extranuclear cell signaling pathways still remain to be uncovered [Edwards, 2]. The mechanisms with those pathways depicted in figure 6 is only a model established on present studies. Nevertheless, it may be a great help to understanding more about ER transcriptional action.

As mentioned above, both ER subtypes recognize similar target DNA sequences and bind and respond similarly to E2, but there are differences in DNA-binding affinity and specificity for pharmacological ligands. In addition, ERα is a more potent transcriptional activator than ERβ, and in tissues where both ERs are expressed, ERβ has been suggested to have a role as an attenuator of ERα [Edwards, 2; and refs. cited therein).

1.4 Estrogen and Antiestrogen Action through Estrogen Receptor

The estrogen receptor transcription described in section 1.3.3 is based on ER action regulated by the natural ligand E2 and the ability of cells to distinguish and response. In clinical settings, the bound ligands to ER are not only estrogens but also antiestrogens including SERMs, and the ER can adopt multiple conformations upon binding different ligands [Beekman, 72; McDonnell, 48]. The impact of such conformational changes was further revealed when steroid receptor coactivator-l (SRC-l), and subsequently other cofactor proteins, coactivators and corepressors, were isolated [McKenna, 73 and 74]. Furthermore, analysis of the crystal structure of the ER LBD-ligand complexes provided the molecular basis of the interaction of the estrogen receptor with its ligands [Brzozowski, 34; Shiau, 75 and 76; Pike, 77 and 78] and so that a better understanding of estrogen and antiestrogen actions through the estrogen receptor was established.

1.4.1 The X-ray crystal structure of ER LBD-ligand complexes

The first crystal structure of an ERα LBD was reported in complexes with 17β-estradiol and the nonsteroidal selective estrogen antagonist raloxifene [Brzozowski, 34].

In the E2-ERα LBD complex (Figure 7a), the E2 cavity is completely shielded from the external environment involving parts of H3, H6, H8, H11 and H12 as well as a small two-strand antiparallel β-sheet (see Figure 4 shown in Section 1.3.2). E2 binds diagonally across the cavity between H11, H3 and H6 and adopts a low-energy conformation. H12 sits over the ligand-binding cavity, without direct contact with E2 and is packed against H3, H5/6 and H11, with its inner hydrophobic surface toward the bound hormone [Brzozowski, 34].

Hormone recognition is achieved through a combination of specific hydrogen bonds and van der Waals contacts of the binding cavity to E_2's non-polar character (Figure 7b). They are involved in the anchoring of the E2 hydroxyl moiety at positions 3 and 17. The phenolic hydroxy group of the A-ring (3-OH) is hydrogen bonded to Glu353 from H3, and to Arg394 from H6 and a water molecule. The hydroxy group of the D-ring (17β-OH) forms a single hydrogen bond with His524 in H11. The remainder of the molecule participates in van der Waals contacts that are concentrated over the A, A/B interface and D-rings [Brzozowski, 34].

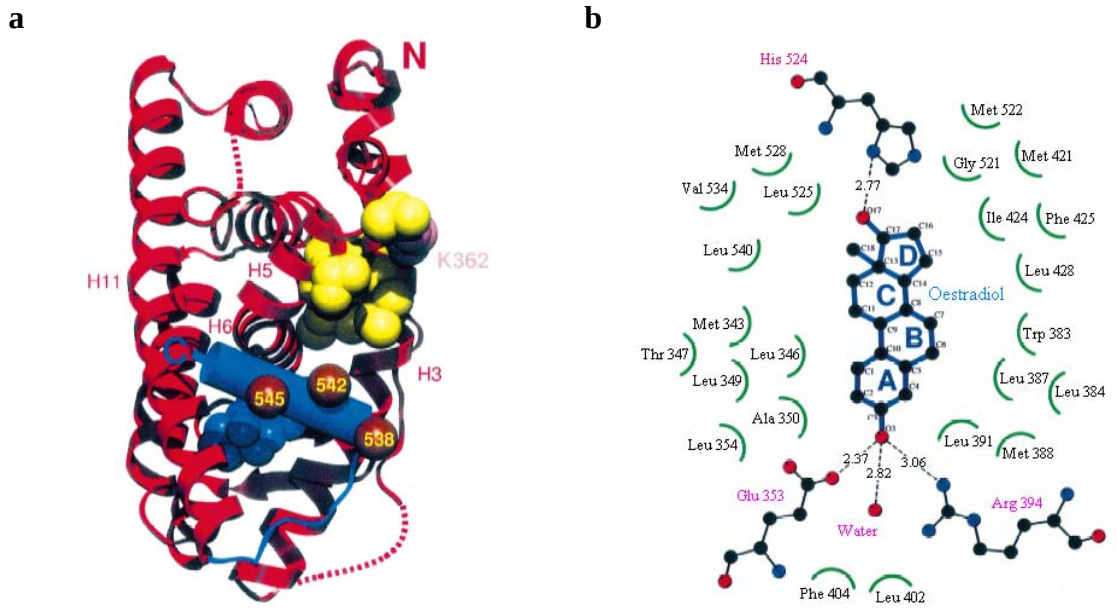


Figure 7. a, The three-dimensional protein structure of the E2-ERα LBD complex including H12 (blue cylinder) and hydrophobic residues (yellow); Dotted lines indicate unmodelled regions of the structures.

b, The interaction of E2 with critical amino acids in the ERα LBD [Brzozowski, 34].

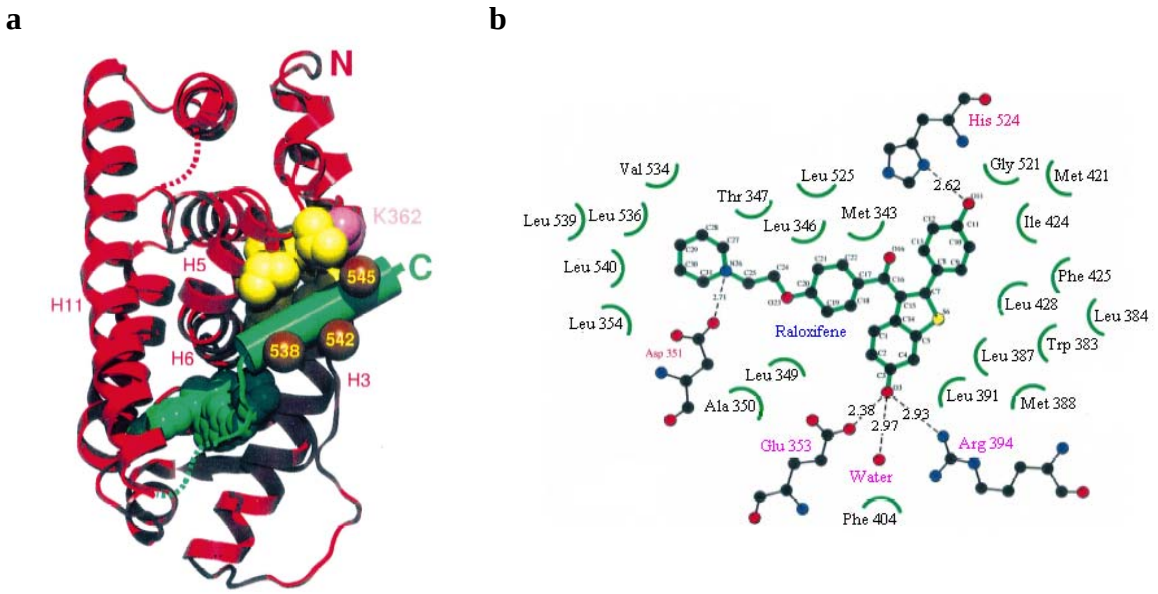


Figure 8. a, The three-dimensional protein structure of the RAL-ERα LBD complex including H12 (green cylinder) and hydrophobic residues (yellow);

b, The interaction of RAL with critical amino acids in the ERα LBD [Brzozowski, 34].

In the RAL-ERα LBD complex (Figure 8a), RAL binds at the same site as E2 within the LBD. The side chain of RAL makes extensive hydrophobic contacts with H3 and H5/6, H11 and the loop between H11 and H12. In addition, the long side chain displaces H12 and protrudes from the pocket between H3 and H11. Instead of the alignment of H12 over the cavity in E2-ERα LBD complex, the helix H12 lies in a groove formed by H5 and the carboxy-terminal end of H3, with a rotation of 130° combined with a 10-Å rigid-body shift toward the amino terminus of the LBD compared with the E2-induced conformation [Brzozowski, 34].

Hydrogen bonds between the hydroxyl group of the benzothiophene moiety and H3 (Glu353), H6 (Arg394) and a water molecule (Figure 8b) are similar to those of the A-ring phenolic hydroxy of E2. In the binding mode of RAL at the "D-ring end" of the cavity, the phenolic hydroxy hydrogen bonds with His 524 whose imidazole ring makes a rotation. The remainder of the core is involved in non-polar contacts similar to those seen for E2. The side chain of RAL is anchored to the protein by a direct hydrogen bond between Asp351 and the piperazine ring nitrogen [Brzozowski, 34].

The crystal structures of the complexes of synthetic agonist, diethylstilbestrol (DES), and the selective antagonist, 4-hydroxytamoxifen (OHT), respectively bound to the ERα LBD [Shiau, 5] are similar to those of E2 and RAL, namely, the conformation and interactions of ERα LBD with DES are similar to those of ERα LBD with E2, and the conformation and interactions of ERα LBD with OHT resemble that of ERα LBD with RAL (Figure 9 and 10). Remarkable is that the DES-LBD complex binds to the NR box II peptide, while the OHT-LBD cannot [Shiau, 75].

In DES-LBD-NR box II peptide complex, the ligand is completely encased in a predominantly hydrophobic cavity with two of the phenolic hydrogen binding to the corresponding amino acid residues and a water molecule. Besides, DES contacts two regions of the ligand binding pocket not occupied by E2, located at the 7-α and 11-β positions of E2, and filled by the two ethyl groups of DES. H12 makes a similar conformation as that in E2-LBD complex. In OHT-LBD complex, OHT is bound within the same pocket that recognizes DES, E2, and RAL. Besides the hydrogen bonds of its hydroxyl group with Glu353, Arg394 and water, OHT stretches its side chain between H3 and H6, and the positioning of the flexible dimethylaminoethyl region of the side chain is stabilized by van der Waals contacts with Thr-347, Ala-350, and Trp-383 and by a salt bridge between the dimethylamino group of the side chain and the β-carboxylate of Asp-351. The orientation of H12 mimic that in RAL-

LBD complex. The remainder of the molecule of DES as well as OHT participates in van der Waals contacts with the corresponding residues [Shiau, 75].

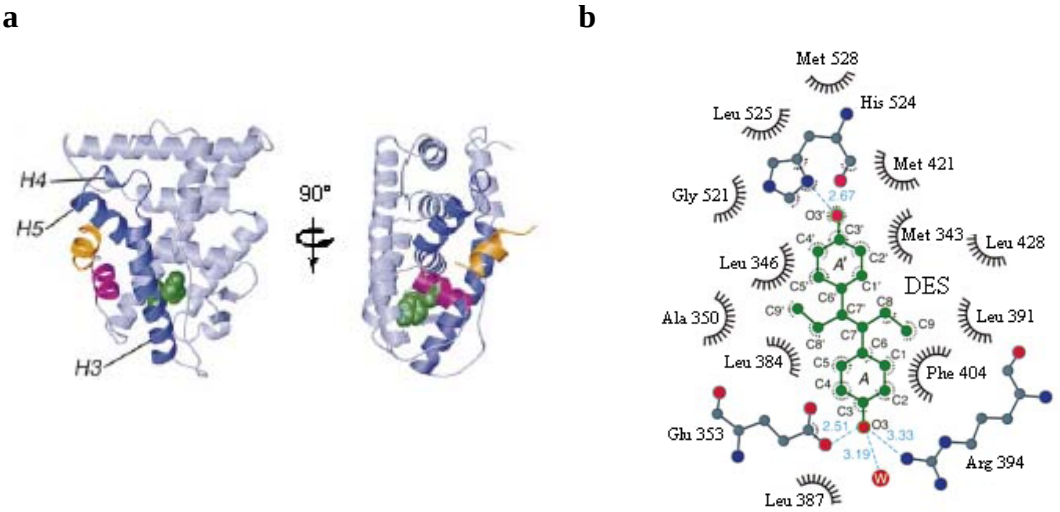


Figure 9. a, The three-dimensional protein structure of the DES-ERα LBD-GRIP1 NR box II peptide complex; Two orthogonal views of the complex including the coactivator peptide (gold), helix 12 (magenta), H3, H4, and H5 (blue) and DES (green) shown in space-filling representation.

b, The interaction of DES with critical amino acids in the ERα LBD [Shiau, 75].

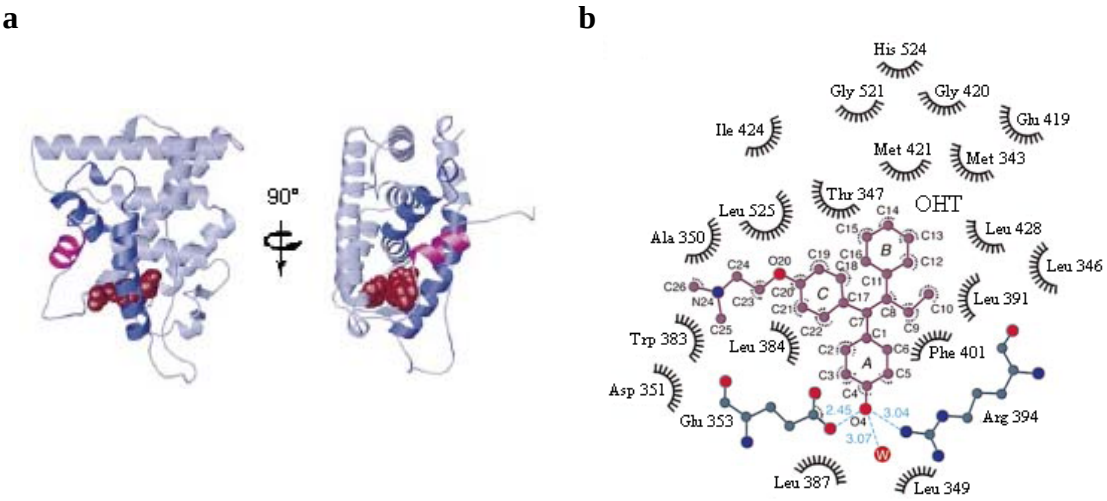


Figure 10. a, The three-dimensional protein structure of the OHT-ERα LBD complex; Two orthogonal views of the complex including helix 12 (magenta), H3, H4, and H5 (blue) and OHT (red) shown in space-filling

b, The interaction of OHT with critical amino acids in the ERα LBD [Shiau, 75].

Based on these analyses of the crystal structures of the ligand-ERα LBD complexes, the relative positioning of H12 is compared in figure 11 [Ruff, 26]. These analyses revealed that the activation function 2 (AF-2) packet, when bound by an agonist, undergoes a conformational change that forms a hydrophobic groove on the surface of an agonist-bound LBD formed by residues from H3, H4, H5 and H12 and allows the docking of a conserved leucine-rich NR box LxxLL motif present in all p160 and most of other coactivator proteins. Conversely, binding of an antagonist alters the AF-2 structure, especially, H12 with an NR box-like sequence (LxxML versus LxxLL) functions as an intramolecular mimic of the coactivator helix interacts with the hydrophobic groove, so that it is incompatible with coactivator docking [Shiau, 75].

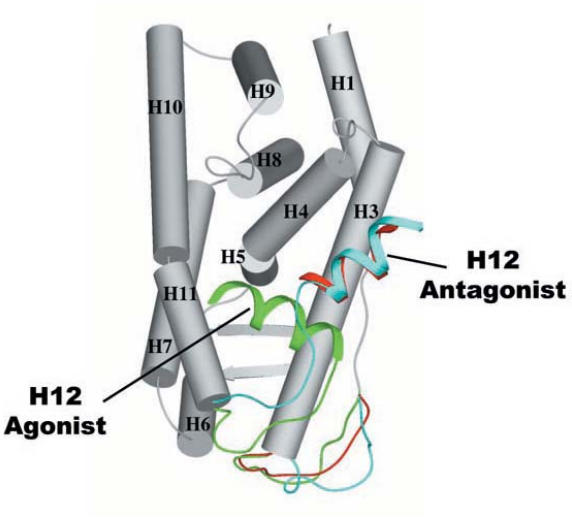
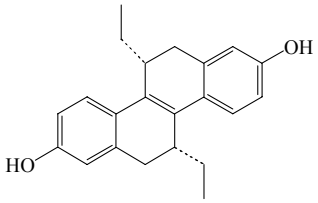


Figure 11. Superposition of the three-dimensional structure of ERα LBD complexed with estrogens (green conformation), antiestrogens (red and blue conformations). [Ruff, 26]



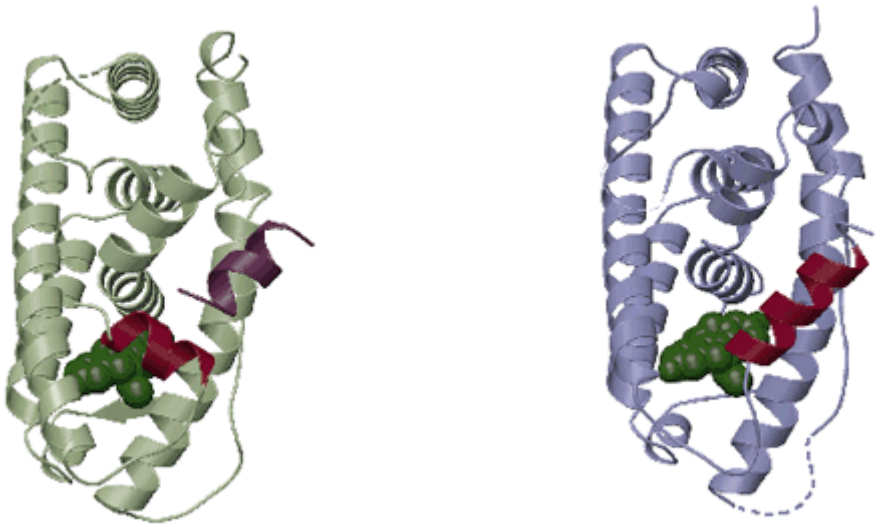
Scheme 3. 5,11-*cis*-Diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC)

These different conformation changes of LBD with binding to agonist and antagonist also were revealed by analyses of the crystal structure of ERβ LBD complexed with genistein (GEN), RAL [Pike, 77] and ICI-ERβ [Pike, 78]. In the GEN-ERβ LBD complex, H12 is bound over the ligand-binding pocket in a position such that it occludes the coactivator recognition surface only partially, being consistent with that genistein acts as an ERβ partial agonist [Pike, 77]. The position of H12 in the RAL-ERβ complex is similar to that in RAL-ERα LBD, also with a tethering interaction between H12 and the hydrophobic groove [Pike,

77]. In the ICI-ERβ LBD complex, the pure antagonist ICI side chain binds directly to the coactivator-binding site of ERβ LBD, causing H12 to be completely disordered [Pike, 78].

Nevertheless, the estrogenic properties of some ligands are selective for both subtypes ERα and ERβ. For instance, THC (see Scheme 3) acts as an ERα agonist and as an ERβ antagonist, which can be explained by analysis of the crystal structure of their complexes (see Figure 12), and therein a novel antagonistic mechanism was proposed [Shiau, 76].

a **b**



THC-ERα LBD-GRIP1 NR box II peptide THC-ERβ LBD

Figure 12. a, The three-dimensional protein structure of the THC-ERα LBD-GRIP1 NR box II peptide complex including the coactivator peptide (purple), helix 12 (red), and THC (green) shown in space-filling;
b, The three-dimensional protein structure of the THC-ERβ LBD complex including helix 12 (red), and THC (green) shown in space-filling [Shiau, 76];

The ERα LBD when bound to THC adopts the same conformation (Figure 12a) as it does when bound to the full agonists E2 and DES, Whereas the THC-ERβ complex shows an similar H12 orientation (Figure 12b) to that observed in the GEN-ERβ complex, without H12 binding to the region of the coactivator recognition groove. In contrast to that antagonism (OHT, RAL or ICI) with a bulky side chain that directly or "actively" precludes the agonist-bound conformation of H12 by steric hindrance, termed "active antagonism", the antagonism by THC-ERβ, without H12 precluding from adopting the agonist-bound conformation, was

termed “passive antagonism”. This “passive antagonism” lies in the difference of ligand-binding pocket residues of ERβ from that of ERα [Shiau, 76].

These analyses of crystal structures of ligand-ER LBD complexes reveal that the position and orientation of H12 are important indicators for understanding the conformation changes by ER binding to agonists and antagonists including SERMs, but not determining factors. The determining conformation changes lie in overall structure of ligand-ER complex, including AF2 and AF1 as well as degradation of ER.

1.4.2 Comparison of estrogen and antiestrogen actions

Due to that ligand actions mostly are mediated by the transcription factor ligand receptor, vice versa, ligand receptor actions mostly are regulated by ligands, estrogen and antiestrogen actions, in fact, are interconnected with ER transcriptions to form united physiological events. The ER transcription described in section 1.3.3 relates to the normal transcriptional process including estrogen action, but involving seldom distinction from antiestrogen action. This section focusses on a brief comparison between the normal estrogen action and clinical antiestrogen action.

Estrogen binding to the ER facilitates such a conformational change as to be favorable for ER binding to coactivators (see Section 1.4.1). This change brings AF-2 and AF-1 of ER in direct association with one another leading to synergy. After dimerization, ligand-ER dimer binds to ERE and promotor, with cooperation of coregulators and transcriptional machinery as well as growth factors, results in transcription (Figure 13. see also Section 1.3.3).

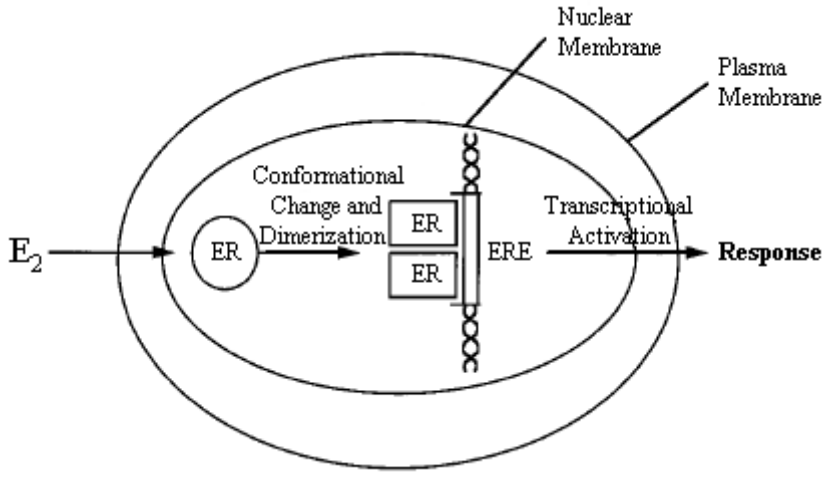


Figure 13. A simple mode of estrogen action through estrogen receptor [Macgregor, 15].

Antiestrogens including SERMs can be used to inhibit or prevent this estrogen action in breast, so as to treat estrogen-dependent breast cancer. Such an antiestrogen action mediated also by ER is depicted as a simple mode in figure 14.

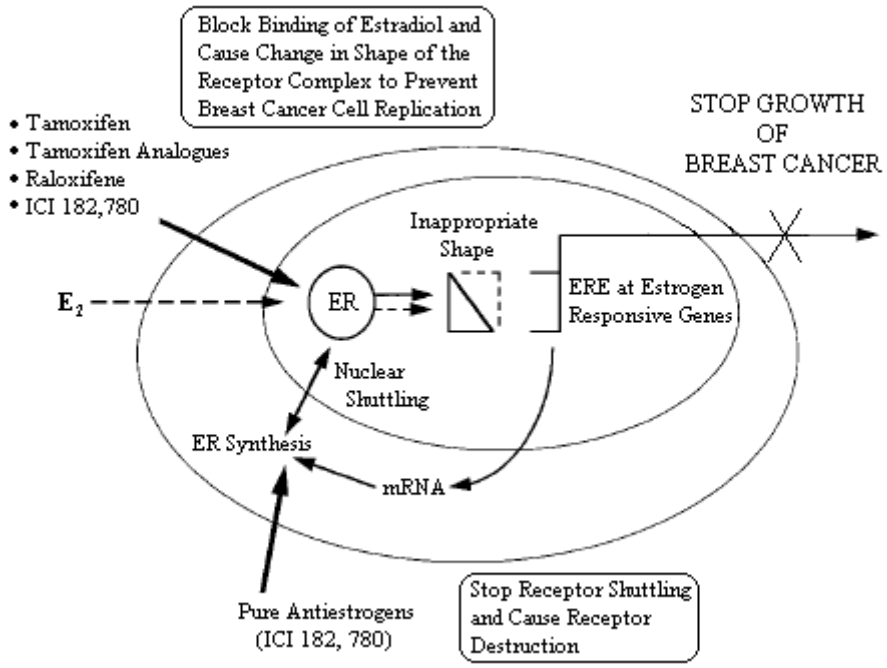


Figure 14. A simple mode of antiestrogen action through estrogen receptor [Macgregor, 15].

An antiestrogen competitively binds to ER and induces an ER conformational change, which blocks ER binding to coactivators (see Section 1.4.1) and / or is favorable for ER binding to corepressors [Huang, 69; Shang, 79] as well affects ER dimerization and interaction with ERE. Thus the genetic estrogen response is inhibited and the growth of breast cancer cells is stopped. In addition, the pure antiestrogens can destroy ER [Macgregor, 15]. The ER is synthesized in the cytoplasm and transported to the nucleus where it functions as a transcription factor. A pure antiestrogen such as ICI 182,780 binds to the newly synthesized receptor in the cytoplasm and prevents transport to the nucleus. The paralyzed receptor complex then is destroyed rapidly [Dauvois, 80]. The complete destruction of available ER will prevent any estrogen-regulated events from occurring.

Besides these antagonistic effects in breast tissue, SERMs such as tamoxifen and raloxifene also act as agonists in some tissues, e. g. bone and the cardiovascular system. These biological selectivities in different tissues may be explained, besides by the nature of ligands, by both the multitude of transcription factors and cofactors in the modes and the differences and particularities of tissues [Shao, 43; Dutertre, 81; Macgregor, 15]. For instance, 1) different

transcription factors (or subtypes) such as ERα and ERβ can regulate different even opposite biological events with a same ligand; 2) Different cofactors such as coactivators or corepressors possess different recognition to ER; 3) Specific EREs or a particular promoter that interacts with the altered ligand-ER complex result the corresponding response; 4) Different intracellular environments influence or even determine ligand biocharacter through different signaling pathways. However, an exact mechanism of SERM action in tissues is not presented up to now.