Structural Insights into Estrogen Receptors and Antiestrogen Therapies

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Abstract The differential impact of distinct antiestrogens (AEs) is the result of varying structural perturbations they confer to estrogen receptors (ERs) when these small-molecule synthetic compounds compete with endogenous hormones, such as 17β-estradiol. These structural changes translate to altered ability of ERs to conscript cofactors and consequently alter the transcription of their target genes. AEs, depending on the mechanism of action, are classified as either selective estrogen receptor modulators (SERMs), which display tamoxifen-like partial agonism, or as selective estrogen receptor downregulators (SERDs) that confer structurally induced posttranslational modifications (PTMs) that destine these receptors for proteosomal degradation. The conformational plasticity of the ER helix 12 (H12) and how its dynamics and conformational sampling is altered by different AEs are crucial to cofactor recruitment and selectivity, translating to varying degrees of receptor modulation and downstream functional effects. Dissecting these conformational state fluctuations within the context of variable cofactor profiles in different tissues, PTM induction, and emergence of hormonal treatment-related resistance mutations in ERs could lead to improved design of novel therapeutic molecules for breast cancer.

Keywords Nuclear receptor · Estrogen · Tamoxifen · Breast cancer · SERM · Raloxifene

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X. Zhang (ed.), *Estrogen Receptor and Breast Cancer*, Cancer Drug Discovery and Development, https://doi.org/10.1007/978-3-319-99350-8_10

1 Introduction

Estrogen receptors (ERs) are ligand-dependent transcription factors regulated by the main circulating estrogen hormone, 17β-estradiol (E2), which is normally produced by the ovaries or via alternative metabolic pathway starting with precursor hormones, such as testosterone [1]. Associated metabolites of E2, estriol and estrone, are also estrogen agonists but generally weaker than E2, and some have been found to have tissue-specific roles (Fig. 1a) [2]. These receptors regulate the function of the female reproductive system, control bone density maintenance, and have protective roles on the central nervous and cardiovascular systems. The effect of E2 on target tissues and organs is mediated by two distinct receptors, ERα (NR3A1) and ERβ (NR3A2), which are encoded by distinct genes [3, 4]. ERs have been implicated in pathological conditions ranging from breast and uterine cancers to cardiovascular and bone disease [5, 6]. Small synthetic molecules, such as

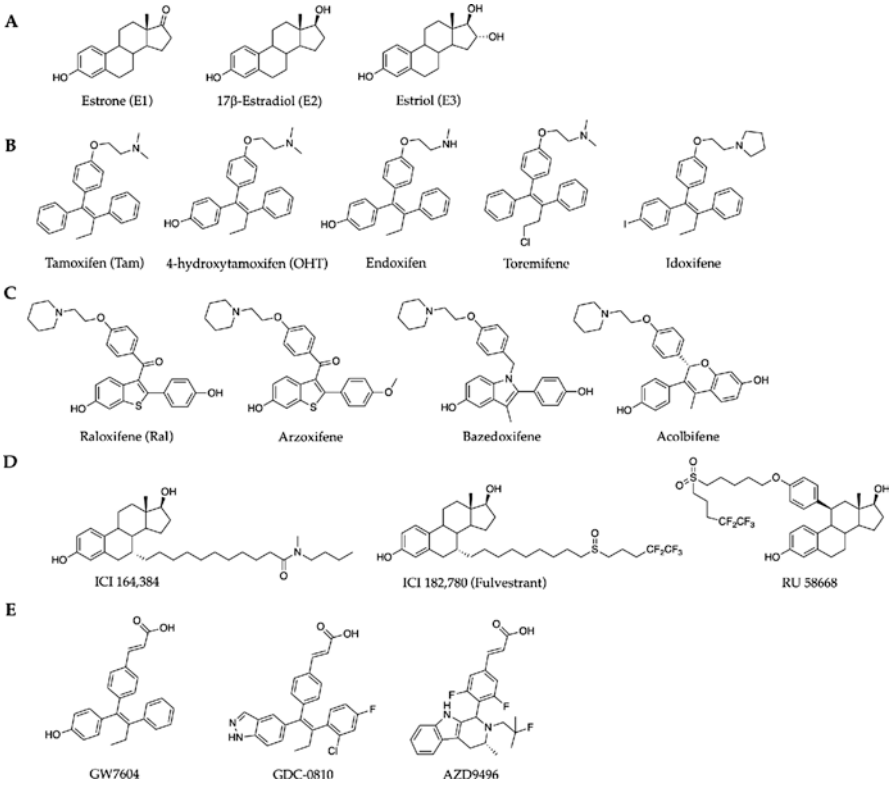

Fig. 1 Estrogen receptor agonists and antagonists. (**a**) Most abundant circulating estrogens, estrone, 17β-estradiol, and estriol. (**b**) Tamoxifen, its active metabolite, 4-hydroxytamoxifen, and SERMs derived from tamoxifen. (**c**) SERM antiestrogens with a steroid-like backbone and a tertiary amine side chain. (**d**) Pure antiestrogens with long side chains attached to a steroid-like scaffold. (**e**) SERDs with steroid-like scaffolds and an acrylic acid functional group

antiestrogen steroid or steroid mimics that are designed to block ERs, are used to treat breast cancer (Fig. 1b–e). Several antiestrogens demonstrate tissue-specific activity, such as selective estrogen receptor modulators (SERMs), tamoxifen and raloxifene (Fig. 1b, c).

Structural studies have illuminated ligand-binding induced conformational reorganization of ER ligand-binding domain (LBD) that leads to stabilization of the ER dimer, thereby promoting interaction with coregulator proteins [7, 8]. Since coregulator proteins have cell-specific expression, estrogens have distinct cellular effects. In general, ERα is the principal receptor mediating E2 signaling in the mammary gland, skeletal muscle, uterus, adipose tissue, and pituitary gland, while ERβ plays a less dominant role in these tissues. In contrast, ERβ is found to be dominant in the central nervous and cardiovascular systems, as well as in the lung, ovary, and prostate gland [9–11]. Understanding the structural properties of ER and the molecular mechanisms underlying ligand-dependent conformational changes has led to the development of more selective ER ligands for more effective antiestrogen therapy. Antiestrogens, in a simplistic binary conformational description (i.e., active vs. inactive receptor), are thought to occupy the ligand-binding pocket, thereby blocking E2 access and locking ER into inactive conformations not conducive to coactivator recruitment. However, current evidence suggests an antagonist-specific continuum of conformational states in ERs that allow exposure of unique surfaces for coregulator recruitment [12, 13]. Furthermore the cell type-specific profiles of coregulators also dictate the transcriptional activities of ERs bound to antagonists [14].

Detailed ER domain structural analysis has proven critical to our understanding of receptor function [15]. X-ray crystallography studies provide structural snapshots that have revealed mechanisms of E2-ER interaction thereby providing invaluable clues to future drug design targeting the estrogen receptor. This chapter will focus on how the structural perturbations in estrogen receptors induced as a result of interaction with agonists and antagonists, posttranslational modifications (PTMs), and endocrine treatment-induced resistance mutations affect cofactor recruitment, transcription of target genes, and antiestrogenicity.

2 Structural Organization of ERs

2.1 Architecture and Sequence Homology of ER Subtypes

Similar to other transcription factors in the nuclear hormone receptor (NHR) family, ERs have distinct domains with structural and functional roles (Fig. 2) [16]. ERα and ERβ are encoded by distinct genes with varying expression levels in different tissues. Full-length ERα is a 66-kDa protein containing 595 amino acids [17], whereas ERβ is slightly smaller at 60 kDa, spanning 530 amino acids [18].

Typically, there are six functional regions [A–E (and in some receptors such as ERα, an F region as well)] in NHRs with significant level of sequence homology.

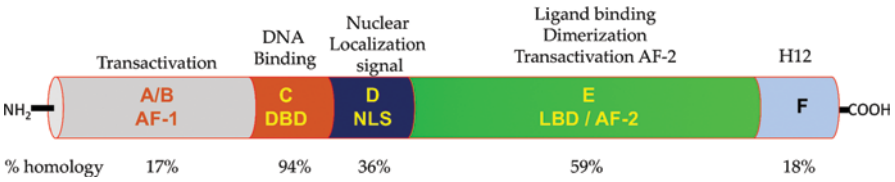

Fig. 2 Schematic of the ER architecture and details of the structural regions. A/B domain at the N-terminus contains activation function-1 (AF-1), the central C region is the DNA-binding domain (DBD), the hinge D contains the nuclear localization signal (NLS), region E is the ligand-binding domain and overlaps with the AF-2, and finally, domain F is the C-terminus of the AF-2 and contains the conformationally flexible helix 12 (H12). Percent homology of ERβ with respect to ERα are reported

Transcriptional activation is facilitated by two activation functions (AF) within the ERs, namely, the constitutively active AF-1 at the NH_2 terminus (A/B region, the least conserved between the ERs) and the ligand-dependent AF-2 at the COOH-terminal region (E region) that overlaps with the ligand-binding domain (LBD). The two ER subtypes share an overall sequence identity of 47%, which is primarily driven by high sequence identity in the central DNA-binding domain (DBD; C domain) and ligand-binding domain (LBD; E domain) with 94% and 59% identity between the receptor isotypes, respectively. Region D is considered a flexible hinge that also contains the nuclear localization signal (36% identity) [19]. The highly conserved DBD is responsible for DNA binding and recognition, while the LBD located at the COOH-terminal region is the site for small-molecule ligand binding. Both subtypes show high affinity for E2, which consequently stimulate transcription of an ER responsive gene containing an estrogen-responsive element (ERE) [20].

2.2 *ER Ligand-Binding Domain Structure and the Helix 12 Conformational Switch*

Like the LBDs of all NHRs, the ER LBDs form three-layered antiparallel α-helical folds [21]. ERα LBD has 12 helices, where the central core is formed by helices H5/H6, H9, and H10 sandwiched between helical layers, L1 (H1–4 and H7) and L2 (H8 and H11), which creates a ligand-binding pocket associated with helices H3, H6, H8, H11, H12, and the hairpin S1/S2 [22]. The dynamically mobile H12 and small two-stranded antiparallel β-sheet flank the major triple layer scaffold [21, 22]. ERβ closely resembles ERα as both have an unstructured F region. However, the ERβ COOH terminus has only an extremely short extended F region. While the F domain of ERα appears to have a role on transcriptional activity modulation, dimerization, receptor stabilization, and coactivator recruitment, the analogous role in ERβ remains unclear [23–25].

The LBDs of ERα and ERβ display considerable structural similarities, with the ligand-binding pocket of ERβ differing only at two residue positions with respect to ERα. Amino acid residues outside the binding cleft influence the size and shape of the

respective ER pockets, which explains the subtype-selective binding of certain ligands, as exemplified by the ERβ-specific agonist, diarylpropionitrile [26]. Subtype-selective agonists and antagonists are invaluable for dissecting the biological effects specific to ERα and ERβ, which could corroborate findings from ER-knockout animal models.

Dimerization is crucial to ER function as amino acid substitutions that interfere with dimer formation abrogate receptor transcriptional activity [27]. The dimerization domain of ERα is created predominantly by helix 11, with some contribution from the DBD, amino terminal ends, and residues from H8 and loop H9/H10 of each monomer [28]. The ERα dimer binds ligands via hydrogen bond interactions and hydrophobic contacts with nonpolar ligands in a hydrophobic groove formed by helices, H3, H4, H5, and H12 [29]. Charged residues, namely, E353, R394, H524 and E260, R301, H430 in human ERα and Rat ERβ, respectively, stabilize the binding of agonist and antagonist by interacting with the hydroxyl groups of the estrogenic steroidal backbone.

The ligand-dependent transcriptional activation function-2 (AF-2) of ER is a conformationally dynamic region of the LBD that contains the conformational switch, helix 12 (H12). Depending on the class of ligand bound to ER, H12 is oriented differently with respect to the rest of the LBD (Fig. 3). Binding of an agonist, 17β-estradiol (E2), positions H12 over the ligand-binding cavity to generate a competent AF-2 interaction surface for coactivator docking, which is essential for transcriptional activation (Fig. 3a, b). Specifically, this opens up a new surface

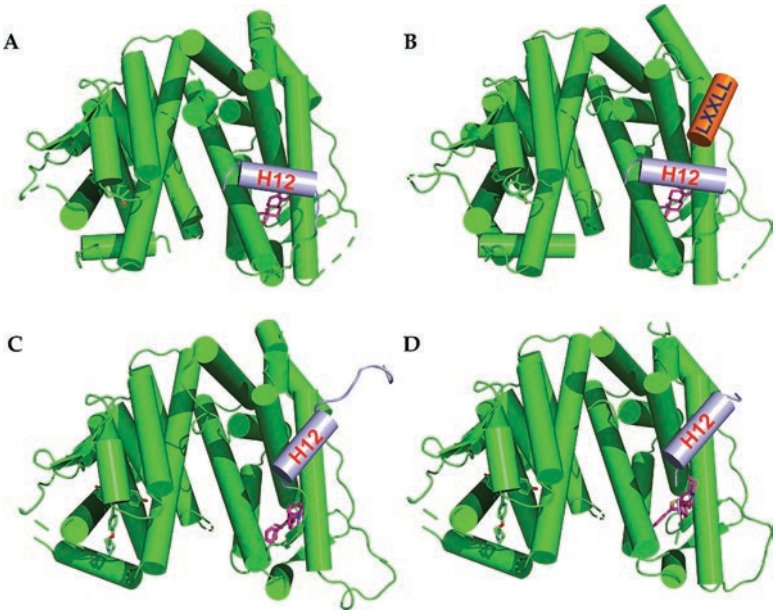

Fig. 3 ERα bound to agonists or antagonists with or without coactivator peptide. (**a**) ERα LBD bound to estradiol (E2) (PDB ID 1ERE). (**b**) ERα LBD bound to E2 and SRC-2 NR box 3 (PDB ID 1GWR). (**c**) ERα LBD bound to 4-hydroxytamoxifen (4-OHT) antagonist (PDB ID 3ERT). (**d**) ERα LBD bound to SERM antagonist, raloxifene (PDB ID 1ERR)

consisting of D538, L539, E542, and M543 to facilitate the interaction with a coactivator [22, 30, 31]. In this H12 conformation, the AF2 surface is conducive to recruitment of helical segments with LXXLL motif, where L and X are leucine and any residue, respectively—a motif found in many coactivators including the p160 steroid receptor coactivators (SRC) [30, 32]. In addition, the 17-OH group in E2 interacts with H524, which is forced to form an H-bond with the peptidic carbonyl group of E419 in loop 6–7. This facilitates the salt bridge formation between E339 from H3 and E419 from H7 to form a salt bridge network with K531 from H11 that favors the agonist orientation of H12 (Fig. 4a) [22].

In contrast, binding of an antagonist, such as 4-hydroxytamoxifen (4-OHT), disrupts the formation of the salt bridge network (Fig. 4b), thereby conferring increased conformational plasticity to H12 and ability to adopt a conformation that occludes the AF-2 groove, which physically blocks coactivator binding (Fig. 3c, d). This physical occlusion is made possible by an internal sequence in H12 that mimics an LXXLL motif, which enables a part of H12 to bind the coactivator groove [30]. A more compelling alternative explanation is that H12 contains an extended corepressor box sequence that binds the AF-1 surface, thereby preventing or hampering corepressor interaction [33, 34]. The latter explains why deletion of H12 confers strong enhancement of ER interaction with corepressors, such as NCoR and SMRT [35, 36]. Even though the importance of NR corepressors to ER signaling remains unclear, studies show that both agonist- and antagonist-bound ERs are capable of recruiting other proteins that repress ER activity [37]. Molecular dynamics (MD) simulation of 4-hydroxytamoxifen (4-OHT)-bound ERα demonstrates structural flexibility of H12, which fluctuates from an initial antagonist position to structurally distinct continuum of H12 positions between an agonist and antagonist conformation, explaining the mixed agonist-antagonist effects of 4-OHT [38].

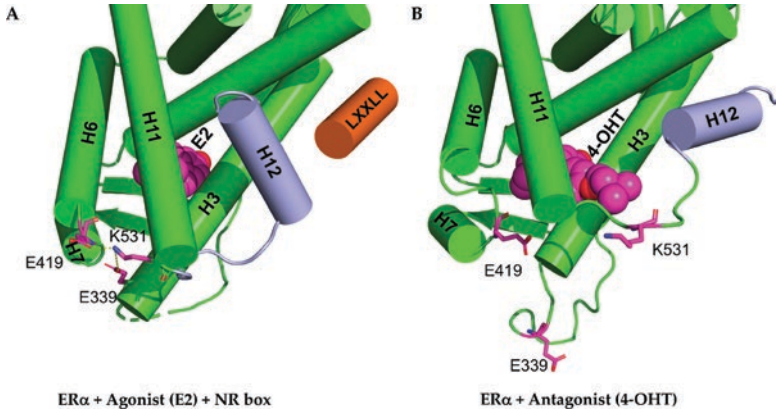

Fig. 4 (**a**) ERα LBD bound to E2 and SRC-2 NR box 3 reveals two salt bridges: K531-E419 and K531-E339 (PDB ID 1GWR). (**b**) When bound to the 4-OHT antagonist (PDB ID 3ERT), the salt bridges are disrupted

2.3 *ER DNA-Binding Domain Structure and Response Element Recognition*

The centrally positioned DNA-binding domains of the ERs are highly conserved and interact with identical DNA sequences. Crystal structures of ERα DBD with or without DNA reveal a topology characterized by two zinc finger-like motifs consisting of four cysteine residues that each coordinate with Zn^{2+} in a tetrahedral geometry (Fig. 5) [39–42]. Amino acid residues in the D box contribute to ER dimerization and discriminate half site spacing, while residues in the P box are involved in estrogen response element (ERE) recognition. Specifically, P box residues E203, G204, and A207 determine DNA-binding specificity and sequence discrimination and are critical to ERE binding. EREs, which are located at various positions from the transcription start site and/or within a gene locus, are variations of the palindromic sequence, 5′-GGTCAnnnTGACC-3′, where n is any nucleotide acting as a spacer [43, 44].

Gene expression modulated by the binding of E2-complexed ER (E2-ER) to EREs relies on a signaling pathway described as "ERE-dependent" [45–49]. Meanwhile, regulation of target gene expression that is mediated by transcription factor interaction with E2-ER, such as activation protein (AP) 1 and stimulatory

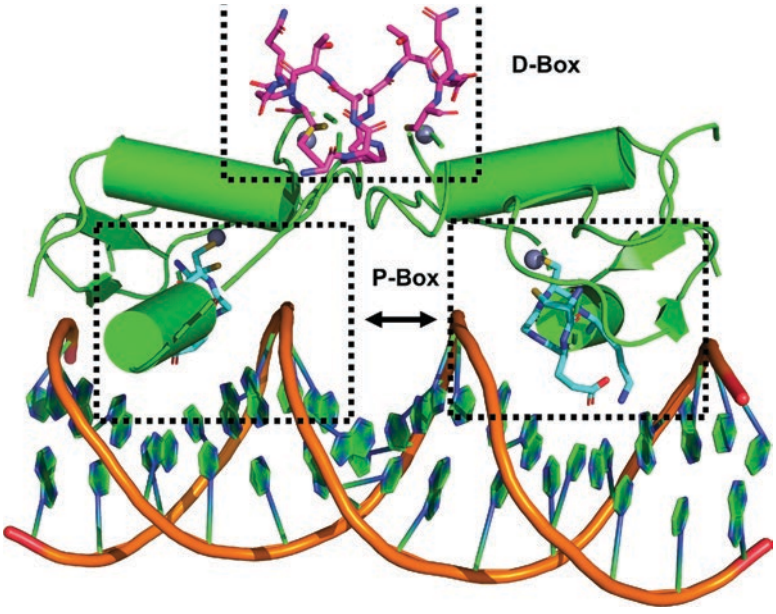

Fig. 5 The DNA-binding domain (DBD) of estrogen receptor α (ERα) dimerbound to the consensus sequence of the estrogen response element (ERE), GGTCAnnnTGACC, where n are nonspecific bases acting as spacer (PDB ID 1HCQ). The first zinc-finger module called P box determine DNA-binding specificity, while the second zinc module termed D box is involved in half-site spacing discrimination

protein (SP) 1 bound to associated regulatory elements on DNA, is classified as "ERE-independent" and employs a signaling pathway mechanism that remains unclear [45, 46, 49, 50]. ER could interact with transcription factors directly or indirectly through coregulatory proteins using interaction surfaces that include the DBD, while transcription control is conferred by combined effects transmitted through the NH_2 and COOH termini.

2.4 The Intrinsically Disordered ER NH_2 Terminus

The amino terminus (domain A/B in Fig. 2) encompasses the AF-1 region and is highly variable among members of the nuclear hormone receptor family [51]. In yeast and chicken cells, ERα AF-1 functions independently of the AF-2 in a ligand- and promoter-independent manner, but decoupling of AF-1 from AF-2 in mammalian cells resulted in a dysfunctional AF-1 in terms of influencing transcription [52–55]. Therefore, ERα AF-1 function depends on three factors, namely, cell type, ligand agonism, and structural integrity of the LBD. This is supported by further studies that demonstrate full activity of ER only when AF-1 and AF-2 are functionally integrated [54, 56–58].

Due to the intrinsically disordered nature of the ER AF-1, the underlying biochemical and structural mechanism of AF-1 action remains elusive. Interestingly, this disordered nature leads to the formation of a large ensemble of rapidly and reversibly interconverting conformational states [51, 59–61]. Inter-domain allosteric cooperativity, protein interaction, and posttranslational modifications (i.e., phosphorylation) control these conformational changes. For instance, interaction of the TATA box-binding protein upon interacting with the NH_2 terminus of ERα induces formation of an ordered structure [61]. Meanwhile, the S118 phosphorylation of ERα complexed with either E2 or tamoxifen via growth factor signaling led to Pin1 recruitment, which isomerizes the S118-P199 bond from cis to trans and promotes a conformational change that favors ligand-independent and agonist-inducible ERα activity [62]. Protein interactions with ERα are important for inducing conformational changes that stabilize interaction with coregulatory proteins, which translates to effective transcription [51]. In contrast, ERβ NH_2 terminus is devoid of AF-1 [48, 58, 63–65], does not interact with the COOH terminus [58], and impairs the ERβ-ERE interactions [66].

3 Structure-Activity Relationships in Antiestrogen Therapy

3.1 Selective ER Modulators and Selective ER Downregulators

Selective ER modulators (SERMs) such as tamoxifen, raloxifene, and analogues (Fig. 1b, c) are ER ligands that display gene- and/or tissue-specific agonist/antagonist activity. The first clinically approved SERM, tamoxifen, is the standard therapeutic

regimen for all stages of breast cancer and has benefitted 70% of women with ERα-positive breast cancer [67, 68]. Aside from the antagonist effects of tamoxifen that inhibit breast cancer proliferation, the drug has desirable agonist effects on bone and lipid profiles [69–72]. However, tamoxifen and its active metabolite, 4-hydroxytamoxifen (OHT) has an estrogenic effect in the uterus of mice and rat models, which translates to higher risk of endometrial cancer development during the course of treatment [73, 74]. Moreover, the emergence of tamoxifen resistance without loss of ERα expression has been observed in primary tumors in majority of metastatic cancer patients [75, 76], although remissions are observed after tamoxifen withdrawal or altered treatment regimen, suggesting ongoing ER signaling activity in some tamoxifen-resistant tumors [77, 78].

To hamper the unwanted side effects while concomitantly improving the efficacy of tamoxifen, synthetic analogues (Fig. 1b) were designed, such as the halogenic/pyrrolidino derivatives, toremifene and idoxifene, and the secondary amine variant of 4-OHT, endoxifen [67]. Unfortunately, these tamoxifen analogues did not demonstrate improved efficacy or prevent drug resistance [67, 77, 79]. The benzothiophene SERM derivative, raloxifene (Fig. 1c), retained 76% of tamoxifen efficacy while reducing endometrial cancer incidence but is ineffective against tamoxifen-resistant breast cancer cells [67, 77, 80]. Meanwhile, the raloxifene analogue, arzoxifene, failed to be on par with tamoxifen efficacy for metastatic breast cancer in a phase III clinical trial [81] despite being more potent than tamoxifen and 4-OHT on inhibiting human mammary carcinoma cell proliferation [82, 83]. Relative to tamoxifen and raloxifene, a structural analogue bazedoxifene is more effective than other SERMs at inhibiting gene expression in MCF-7 cells and hampering the growth of tamoxifen-resistant xenograft [84].

Steroidal compounds with long side chains, such as ICI 164,384, ICI 182,780 (fulvestrant), and RU 58668 (Fig. 1d), were developed to minimize partial agonist activity. These drugs were initially referred to as pure antiestrogens (AEs) due to their lack of partial agonist effects in breast and endometrial cell lines [85–88] but were later designated as selective ER downregulators (SERDs) as they promote ERα degradation via the ubiquitin-proteasome pathway in ERα-positive breast cancer cells [89–92]. The pure AE character of fulvestrant did not confer an advantage over tamoxifen against advanced or metastatic breast cancer [79, 93], which could be attributed to its poor pharmacokinetic properties. This limitation is circumvented by doubling the intramuscular injection dosage to 500 mg, which increased patient survival rate [94–96]. In comparison, certain SERM derivatives of tamoxifen, such as GW7604, GDC-0810, and AZD9496, demonstrate SERD ability to induce ERα degradation with similar efficacy as fulvestrant but with improved oral bioavailability [78, 97–99].

In aggregate, antiestrogens demonstrate varying SERD activity, ranging from drugs that lack ERα downregulating capacity, such as tamoxifen, to SERM analogues with disparate levels of ERα reduction ability (e.g., raloxifene, bazedoxifene, GDC-0810, and GW7604). The strongest SERD activity is associated with pure AEs with long side chains, such as ICI 164,384, fulvestrant, and RU 58668.

3.2 *Molecular Rationale of Antiestrogen Effects*

AEs bind to ERα LBD akin to estradiol—with bulky side chains attached at steroid core positions 7α or 11β conferring antiestrogenicity by positioning these substituents between H3 and H11 of the binding cavity. Presence of bulky functional groups of different length and size results in structural rearrangements that can cause varying levels of steric hindrance to the positioning of H12 over the ligand-binding pocket.

Tamoxifen, raloxifene, and synthetic SERM analogues (Fig. 1b, c) contain alkylaminoethoxy side chains with varying tertiary amine groups. The dimethylamino group of tamoxifen or the piperidyl group of raloxifene sterically hinders the positioning of H12 to the coactivator-binding site. In an unobstructed state, H12 will dock to the coactivator groove via its hydrophobic residues, L540, M543, and L544, in a manner similar to the LXXLL motif in coactivators [22, 30, 100]. Synthetic analogues and ER mutants were made to validate this structural observation. For instance, a raloxifene derivative with a nitrogen-to-carbon replacement of a crucial compound side chain abrogated the drug's antagonist activity [101]. Furthermore, substitution of the crucial D351 residue to glutamic acid altered the effect of raloxifene from a pure antagonist to a tamoxifen-like antagonist in HepG2 cells. A hydrogen bond binds the tertiary amine of raloxifene to D351 in ERα LBD; the mutation to E351 altered this crucial interaction [22, 102]. In transfected MDA-MB-231 cells, a similar mutation D351G abolished tamoxifen-induced expression of TGFA—an estrogen target gene [103]. In addition, a D351A substitution rendered ERα inactive on a reporter gene in tamoxifen-induced HepG2 cells [102], corroborating the importance of D351 in mediating the partial agonist activity of SERMs.

Relative to SERMs, pure AEs such as fulvestrant and ICI 164,384 (Fig. 1d) have longer side chains. A crystal structure of the latter with rat ERβ shows position 7α protruding out of the ligand-binding cleft reminiscent of SERM side chains but bends at carbon 5 by 90°, thereby positioning the rest of the chain onto the coactivator-binding surface [104]. Side chains L261, M264, I265, and L286 in the coactivator-binding surface of rat ERβ form hydrophobic contacts with the terminal n-butyl group of ICI 164,384, which displaces H12 from the same position in crystal structures of 4-OHT and raloxifene-bound ERα (Fig. 3c, d). The long side chain sterically clashes with H12 residues L540 and M543 in the agonist conformation and with L536 and L540 when H12 is positioned in the coactivator-binding surface. Substitution of the aforementioned residues to alanine increased the pure AE-induced ERα transcriptional activity [105–108].

In contrast to ICI 164,384, fulvestrant antiestrogenicity is not affected by D351 mutations, but introduction of tertiary amine functional group in analogues ZK-253 and ZK-703 improved growth inhibition ability toward mouse xenografts from tamoxifen-resistant and estrogen-sensitive breast cancer cell lines [109]. However, these studies did not address whether direct interaction with pure AEs is crucial for enhanced activity.

Aside from amine functional groups, the effect of chain length was also investigated. To reveal optimal chain length for pure antiestrogenicity, derivatives of ICI 164,384 with variable side chain lengths were synthesized. Side chain lengths consisting of 15–19 atoms display optimal antiestrogenicity, while shorter side chains (13–14 carbon atoms) show agonist or SERM-like activity in reporter assays in HepG2 cells transiently transfected with ERα. These results suggest that longer chains are necessary for pure AEs to reach the coactivator-binding surface. Moreover, hydrophobicity and presence of terminal pentafluoropentyl group are crucial factors for pure AE activity, as supported by a study that shows higher potency and efficacy of fulvestrant relative to ICI 164,384 for growth inhibition in *in cellula* and in vivo human breast cancer models [88].

Partial or full SERD activity has been observed upon changing the shorter side chains of SERM analogues. For instance, the derivative bazedoxifene differs from raloxifene by having a larger heterocyclic amine ring (Fig. 1c) conferring enhanced steric clash with H12. Furthermore, GW5638 (Etacstil), the prodrug of the active metabolite GW7604 (Fig. 1e), is a tamoxifen derivative where the dimethylaminoethyl group is substituted with an acrylic acid side chain. In its protonated state, the carboxylate group in GW5638 form hydrogen bonds with the peptidic backbone of H12 and E351, inducing a conformation of H12 where the side chain of hydrophobic residues L536, L539, L540, and M543 are pointing toward the aqueous environment, which effectively increases the hydrophobic surface area of H12 relative to 4-OHT-bound ERα while concomitantly maintaining interaction in the coactivator-binding surface [110]. Therefore, pure antiestrogenicity positively correlates with hydrophobic surface area of H12 and is independent of H12 positioning in crystal structures. However, the effect of these structural perturbations on protein-protein interactions and overall ERα stability is not yet clear.

3.3 Effect of AE Binding on Cofactor Recruitment and Gene Transcription

The AF1 and AF2 activation functions at the N- and C-termini, respectively, are utilized by ERs to conscript a large number of cofactors in the presence of agonists. Such cofactors include chromatin remodeling complexes, histone acetyltransferases (HAT), methyltransferases (HMTs) and deacetylases (HDACs), and transcriptional machinery components [111]. The altered recruitment of cofactors to ERα is conformationally induced by AEs that modify the protein surface available for interaction [84]. HATs such as SRC1–3 (NCOA1–3) and CMP/p300 and HMTs including CARM1 and PRMT1 are some of the coactivators that interact directly with the AF2 of E2-ERα or perturb it allosterically [112, 113]. Tamoxifen, but not raloxifene, selectively recruit SRC-1 to promoter genes in Ishikawa and ECC-1 cell lines. Repression of SRC-1 in Ishikawa cells abrogates the partial agonist activity of tamoxifen on target genes [14]. In contrast, when SRC-1 is overexpressed in MCF-7

cells, the behavior of tamoxifen switches from agonist to antagonist suggesting that difference of SRC-1 expression level in breast and uterine cells explains the tissue-specific tamoxifen effects on transcription [14]. Analogously, SRC-2 and p300 overexpression in HeLa cells transfected with ERα amplified the partial agonist activity of tamoxifen but only have moderate and negligible effects in the presence of raloxifene and fulvestrant, respectively [36]. These results suggest that presence of coactivators may contribute to cell- and gene-specific partial agonist activity of SERMs. In addition, 11% of breast tumors show increased SRC-3 expression and is associated with unfavorable prognosis and tumor phenotype, which is explained by the impact of SRC-3 on the cell cycle regulation of both $ER\alpha^{+}$ and $ER\alpha^{-}$ tumors [114].

The activity and ligand-independent AF-1 function of ERα is linked to the partial agonist activity of tamoxifen and, to some degree, of raloxifene in a cell- and promoter-specific manner [56, 115, 116]. Case in point is the agonist effect of tamoxifen in HEC1 cells that is dependent on the AF1 of ERα [65]. In addition, Zwart and coworkers swapped the AF1 domain of ERα with that of ERβ and consequently abolished tamoxifen-induced transcriptional activity in U2OS cells, showing that the AF1 region is crucial to the partial agonist activity of tamoxifen. This result is corroborated by studies that show the ability of ERα, but not of ERβ, to conscript SRC-1 via the AF1 region [117, 118].

ERα recruits corepressors NCOR1 and NCOR2 (SMRT) in the presence of tamoxifen in MCF-7 cells resulting in the repression of estrogen target genes. Increase of ER target gene expression is observed in the same cells in the presence of tamoxifen after siRNA knockdown of the aforementioned corepressors. Analogously, recruitment of corepressors is absent on genes upregulated by tamoxifen in Ishikawa cells [14, 119, 120]. Moreover, SMRT2 overexpression in HepG2 cells inhibits partial agonist activity of tamoxifen [121]. In comparison to SERMs, ERα bound to fulvestrant is more efficient than raloxifene or tamoxifen at recruiting NCOR1 C-terminal fragment in ChIP experiments in HeLa cells [36]. However, the difference in corepressor recruitment mechanism between SERMs and SERDs remains elusive. It only became possible for raloxifene-bound ERα to co-crystallize with a corepressor NR sequence after H12 deletion, where the peptide occupies the AF2 surface subtended by H3 and H5, and with the raloxifene side chain packed against the peptide N-terminus. Further studies are needed to confirm whether the differential H12 conformation between SERM- and SERD-bound ERα increases corepressor recruitment in the presence of SERDs.

3.4 Impact of ERα Posttranslational Modifications to Pure Antiestrogenicity

Cofactor recruitment is likely modulated by ER posttranslational modifications (PTMs). Mass spectrometry has become an invaluable tool for the identification of acetylation, methylation, and phosphorylation sites in ERα. A study shows that

phosphorylation of S104, S106, and S118 in the AF1 region and of S305 in the AF2 may be linked with tamoxifen resistance [122]. Presence of pure AEs induce the phosphorylation of the same serine residues on the AF-1 region, but the link of these PTMs to SERD transcriptional downregulation remains unclear [123, 124]. MCF-7 breast cancer cells with dephosphorylated Y537 displayed increased sensitization to SERMs and fulvestrant [125]. Sensitization of breast cancer cells to AEs may also be induced by other PTMs on ERα, such as acetylation, SUMOylation, ubiquitination, and methylation [122, 126].

SERDs induce the degradation of ERα in breast and uterine cancer cell lines. Presence of 4-OHT increases ERα expression [92, 106, 127], with some level of decrease in the presence of endoxifen, raloxifene, and bazedoxifene and substantial decrease in the presence of pure AE, such as fulvestrant [84]. Moreover, ERα ubiquitination is doubled in the presence of fulvestrant [92].

ERα turnover differs in the presence of AEs and E2. For instance, α-amanitin transcriptional inhibition prevents E2-induced degradation of ERα, but not by fulvestrant [128]. Similarly, partial inhibition of ERα degradation by E2 but not by pure AEs is afforded by cycloheximide or kinase inhibitor treatment [129, 130]. In spite of the aforementioned differences in degradation mechanisms between E2 and SERDs, the Neddylation pathway seems to be important for both E2- and pure AE-induced turnover [131].

Overexpression of ERα to saturate the degradation process has no effect on the capacity of SERDs to act as AEs in MCF-7 cells [132]. Moreover, the steady-state level of ERα unexpectedly increased in the presence of fulvestrant in HepG2 cells, but this ligand still functioned as an inverse agonist while tamoxifen has partial agonist activity [106, 127] suggesting an alternative mechanism to afford enhanced efficacy of pure AEs for inhibiting ERα activity in HepG2 cells. SUMOylation of ERα is strongly induced by pure AEs in MCF-7 breast cancer cells, HEK293 and HepG2 cells, and abolishing SUMOylation attenuates transcription in the presence of pure AEs, with no effect on the corresponding activity induced by E2 or tamoxifen, suggesting that SUMOylation contributes to pure antiestrogenicity [127]. Interestingly, SUMOylation activity peaked at 15–19 carbon atom chain length and decreased when chain length is >22, which correlates with inverse agonist activity in HepG2 cells and with the ability of the AE side chain to dock at the coactivator-binding cleft. Furthermore, the SERM raloxifene also induce SUMOylation to a lesser extent, which positively correlates with its ability to suppress basal transcription activity in HepG2 cells [127]. Possibly, differential SUMOylation could explain the varying SERM effects in different tissues.

3.5 Effect of ER Mutations on AE Action

Emergence of endocrine treatment resistance remains a challenging issue in treatment of patients with ER^+ breast cancers. After developing resistance, majority of tumors still express ERα, suggesting the role of ERα in tumor growth. Coactivator

overexpression inducing estrogen-dependent transcription is a potential mechanism of desensitization, as is the signaling pathway activation that controls the activity of ERα and/or its associated coactivators [133]. A recent review has highlighted the role of ERα mutations as an additional hormonal treatment resistance mechanism [134], as initially hinted by a constitutively active ERα Y537N mutant isolated from metastatic breast cancer cells [135]. Majority of hormone therapy-resistant tumors contain gain-of-function mutations, such as E380Q, L536Q/R, Y537S/C/N, and D538G that result in ERα activity that is ligand-independent [136–138]. These mutants show higher levels of S118 phosphorylation, enhanced recruitment of SRC1–3, increased ligand-independent tumor growth, and/or S118 phosphorylation [138–140].

Crystal structures of ERα mutants Y537S and D438G in the apo state adopt an agonist-like conformation [138–141]. As a result, affinity of binding of E2 and 4-OHT to said mutants is tenfold weaker relative to wild-type ERα, and higher doses of 4-OHT and fulvestrant are required to affect levels of activity inhibition in mutant ERα similar to wild type. This could lead to clinical resistance to AE therapy when required concentrations for activity suppression of ER mutants are not reached [136, 138, 140]. Furthermore, the structural changes relative to wild type in 4-OHT-bound ERα mutant LBDs may result in different effects on ER target genes at saturation [139].

4 Epilogue

ERα and ERβ have similar structures but display distinct as well as overlapping regulatory potentials in cells in a tissue-specific manner. Antiestrogens have diverse conformations and structures that modulate AF-1 and/or AF-2 activity that translate to varying levels of antiestrogenicity in breast cancer cells. The conformational dynamics of AE binding to ERs has several downstream consequences on posttranslational modifications and ER degradation mechanisms and needs to be explored further. Hormone therapy resistance is caused by the emergence of ER mutants that need to be characterized for their individual responses to various clinically available AEs, which will guide the design of future drugs for breast cancer.

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