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Advances in Brief

Molecular Classification of Estrogens[1]

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Abstract

Estrogens are involved in a multiplicity of programmed events in target tissues *e.g.*: uterus, breast, and pituitary gland, and hormone-responsive tumors occur at these target sites. We have addressed the possibility that all of the estrogens do not produce the same conformation of estrogen receptor α (ER). A novel assay *in vitro* was used to activate the *transforming growth factor α (TGF-α)* gene *in situ* in MDA-MB-231 cells stably transfected with cDNA for D351 ER or D351G ER. Three estrogen types were used: estradiol, diethylstilbestrol, and a triphenylethylene (TPE) derivative of tamoxifen without the antiestrogenic side chain. Computer molecular modeling was used to interpret data. A flat estrogen such as estradiol or diethylstilbestrol can induce *TGF-α* through a correctly positioned activating function 2 (AF2) and bind SRC-1. The TPE did not activate AF2 but activated the *TGF-α* gene through AF2b. This was demonstrated because D351 but not D351G ER activated the *TGF-α* gene with the TPE. We propose two classes of estrogens with different ER complexes that may incorporate different coactivators to function. Phytoestrogens and environmental xenoestrogens will fall into different classes based on structure and may exhibit selective actions and carcinogenic potential based on different ER conformations.

Introduction

Natural and synthetic estrogens are known to encourage the growth of breast and endometrial cancer (1). Estrogens have traditionally been defined as compounds that provoke vaginal cornification in ovariectomized rodents (2), and the assay was used successfully to identify synthetic estrogens based on stilbene (3) and TPE[3] (Ref. 4; Fig. 1). Extensive structure activity relationship studies resulted in the development of the stilbene DES (Ref. 5; Fig. 1) for the treatment of breast and prostate cancer and the substituted TPE trianisyl-chloroethylene (6) as a hormone replacement therapy. The initiation of estrogen action by all of the estrogens is considered to be the same in each target tissue. Estrogens first bind to the nuclear ER. An estrogenic ligand then causes a conformational change that encourages dimerization and interaction with either specific DNA sequences or a protein-protein interaction with AP-1 or SP1 sites in the promoter region of estrogen-responsive genes. These events herald the biological effects of estrogen in the specific target tissue or tumor. By way of example, estrogen may cause prolactin synthesis in cells of the anterior pituitary gland, vaginal cornification, uterine growth, or cell replication in hormone-responsive breast or endometrial cancers.

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[3] The abbreviations used are: TPE, triphenylethylene; DES, diethylstilbestrol; ER, estrogen receptor α; LBD, ligand-binding domain; 4OHT, 4-hydroxytamoxifen; AF, activating function; SERM, selective estrogen receptor modulator; E_2, estradiol; GST, glutathione *S*-transferase; 4OHTPP, fixed ring 4 hydroxy triphenylpentene.

Recent studies of the X-ray crystallography of the LBD of the ER have identified consistent conformational differences in estrogen (E_2 and DES) and antiestrogen (raloxifene and 4OHT) ER complexes (7, 8). In the estrogen-ER complex, the ligand is completely enveloped within the hydrophobic binding pocket with helix 12 sealing the pocket to allow coactivators to bind on the exposed AF2 site (8). In contrast, when a nonsteroidal antiestrogen occupies the LBD, helix 12 is displaced to block coactivator binding in the AF2 region. Silencing AF2 is a primary mechanism of action for nonsteroidal antiestrogens.

The antiestrogen tamoxifen (Fig. 1) is the *trans* isomer of a substituted TPE that is known to possess significant estrogen-like actions in select estrogen-target tissues (9). We have shown recently that the surface amino acid D351 in the LBD is critically important for the estrogen-like actions of SERMs. We hypothesized that the basic antiestrogenic side chain of 4OHT is unable to shield the charge at 351, which leads to full estrogen-like actions for the 4OHT-ER complex in MDA-MB-231 context at the *TGF-α* gene *in situ* (10). In contrast, the antiestrogenic side chain of raloxifene adequately shields the D351, thereby completely silencing all of the estrogen-like properties of the ER complex at the *TGF-α* gene (11).

To demonstrate the critical importance of D351 for the estrogen-like properties of the 4OHT-ER complex, we removed the exposed surface charge resulting in the mutant ER D351G, which causes 4OHT to act as a complete antiestrogen at the *TGF-α* gene (12). Additionally, the analogue of 4OHT, GW7604, with a carboxylic acid side chain instead of a tertiary amine, dramatically repels rather than attracts D351 (13). The shift in surface charge with GW7604 converts the 4OHT-ER complex from an estrogen in MDA-MB-231 cells to an exclusively antiestrogenic complex (13). Thus, the exposed surface charge at D351 in an ER complex plays a pivotal role in the estrogen-like action of a SERM ER complex when helix 12 silences AF2.

We have devised an assay system that can discriminate between tamoxifen-like antiestrogens and true estrogens based on the structures of the ER complexes (12). The assay compares and contrasts the activation of the *TGF-α* gene *in situ* in MDA-MB-231 cells stably transfected with cDNA wild-type or D351G ER. Simply stated, it is classically believed that a true estrogen will activate AF2 and synergize with AF1 to produce estrogen action. In contrast, we suggest that an ER complex with a tamoxifen-like shape will produce an estrogen-like effect by activating the novel site AF2b (12). This site includes an exposed D351 and a repositioned, but intact, helix 12, which then interacts with AF1 to bind coactivators.

The dimethyaminoethoxy side chain of tamoxifen (Fig. 1) is essential to the antiestrogenic action of 4OHT. Removal or moving the position of the side chain on the TPE skeleton results in increased estrogenic activity. The derivatives will induce prolactin gene expression in the cells of the pituitary gland (14, 15), cause cell replication of T47D and MCF7 cells but not ER-negative MDA-MB-231 cells (16, 17), and induce increases in rat uterine weight.

The derivative of 4OHT, 4OHTPP, illustrated in Fig. 1, is a stable, nonisomerizable example of the TPE class (17). In this preliminary

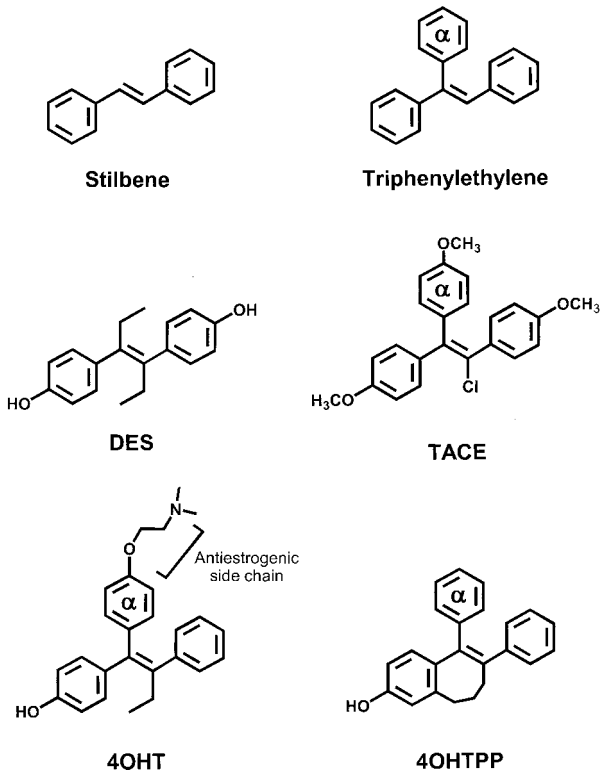

Fig. 1. The structural formulae of compounds referred to in the text.

study, we have evaluated the estrogenic TPE derivative in the TGF-α assay (12) and, based on our experimental finding, we propose a new classification of established estrogens into two different groups. The classification is based on the structure of their respective receptor complexes: (*a*) the DES type (class 1), which produces an ER complex with an activated AF2 to initiate estrogen action; and (*b*) the TPE type (class 2), which produces an ER complex with an activated AF2b site.

Materials and Methods

Compounds. E_2 and DES were obtained from Sigma Chemical Co. (St. Louis, MO). 4OHT was a generous gift from AstraZeneca (Macclesfield, England, United Kingdom) and 4OHTPP (referred to previously as compound 3; Ref. 18) was synthesized and characterized as reported previously (17).

Cell Lines and Northern Blot Analysis. The characterization and growth conditions used for the MDA-MB-231 breast cancer cell lines stably transfected with wild-type ER (S30) and D351G (JM6) have been reported previously (12). Cells were treated for 24 h with compounds as indicated in the figure legends, and Northern blot analysis was completed as described previously (12). A human TGF-α cDNA probe derived by *Eco*RI digestion of a *TGF-α*-containing plasmid was a generous gift from Dr. Rick Derynck (Genentech, San Francisco, CA). Bands were quantified densitometrically using ImageQuaNT analysis (Sunnyvale, CA). Experiments were repeated at least three times. ANOVA test was used to analyze differences between treatments using two-tailed *t* test StatMost 2.5 (Datamost Corp., Salt Lake City, UT).

***In Vitro* Protein-Protein Interaction.** A GST pull down assay was performed as described previously (19) using ^{35}S-labeled wild-type ER, which was made from pSG5 HEGO using an *in vitro* transcription-coupled translation system (Promega Corp., Madison, WI).

Computer Graphics Docking and Lowest Energy Calculations. The structural model of dimeric human ER bound to DES, 4OHT, or 4OHTPP was constructed from 3ERT.pdb (8) using crystallographic symmetry operations. The shape of the OHTPP was deduced from the known X-ray crystallography of fixed-ring tamoxifen (20). After removing all of the water molecules except the ordered water-forming hydrogen bonds with DES or 4OHT, the model was minimized into the consistent valence force field using Discover (Molecular Simulations, Inc. San Diego, CA).

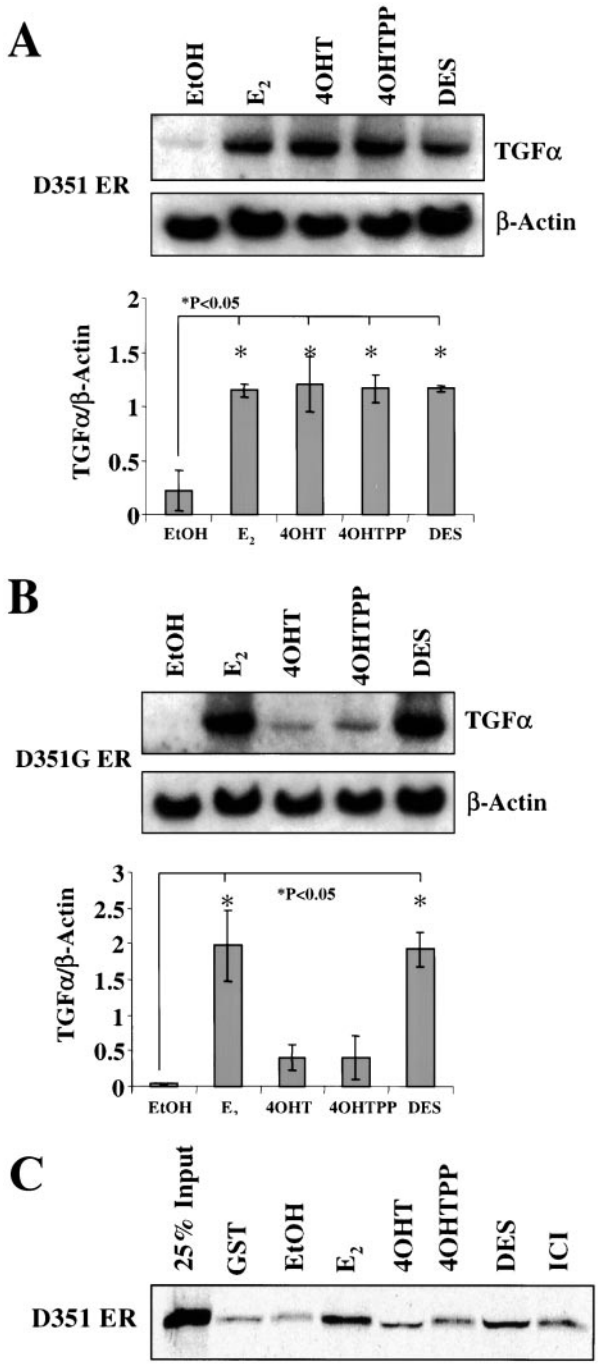

Fig. 2. The biological activities of test compounds in the *TGF-α* gene activation assays. Northern blot analysis of TGF-α mRNA in MDA-MB-231 cells stably transfected with cDNA D351 (*A*) or D351G ER (*B*). Cells were grown in E_2-free medium and treated for 24 h with vehicle (ethanol), 10^{-9} M E_2, 10^{-9} M DES, 10^{-7} M 4OHT, or 10^{-7} M 4OHTPP. β-Actin mRNA was measured to ensure even loading. Densitometric analyses of Northern blots were analyzed by ANOVA. Results were confirmed in at least three independent experiments and represented as mean; *bars*, ± SD. All compounds in (*A*) produce a significant ($P < 0.05$) increase in *TGF-α*, but only E_2 and DES produced a significant increase ($P < 0.05$) in *TGF-α* in experiments with D351G ER (*B*) The recruitment of SRC-1 to the LBD of D351 (*C*). The pulldown assay was conducted as described in "Materials and Methods." [^{35}S]D351 ER (5 μl) was incubated with GST or GST-ΔSRC-1 with vehicle or different ligands (10^{-9} M E_2, 10^{-9} M DES, 10^{-6} M 4OHT, or 10^{-6} M 4OHTPP). The bound [^{35}S]D351 ER was resolved by 7.5% SDS-PAGE. The pull-down assay is representative of three independent experiments. Only DES and E_2 bound SRC-1 indicating AF2 activation.

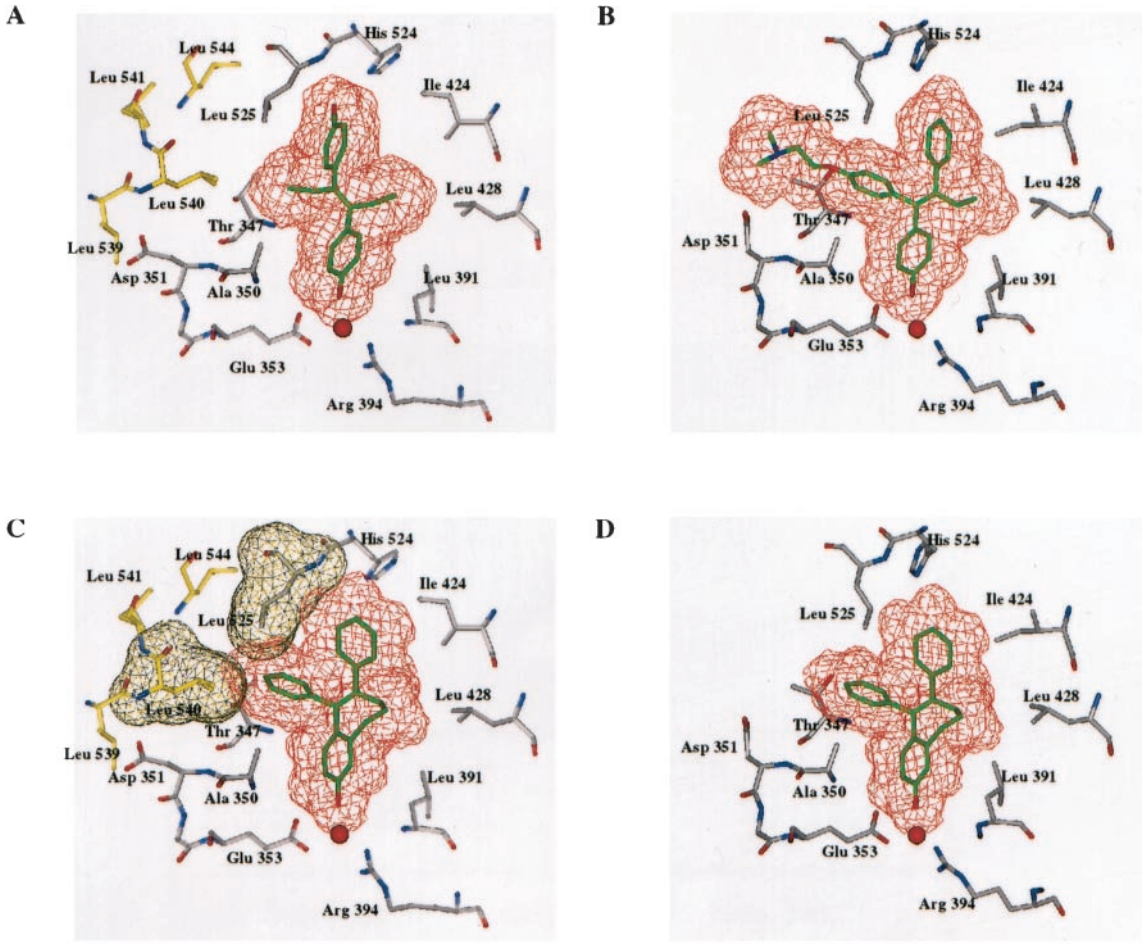

Fig. 3. Cross-sectional views of the ER ligand-binding sites. Van der Waals surfaces of ligands are represented as a *red grid*. Helix 12 carbon atoms are shown in *yellow*, the remaining protein carbon atoms in *gray*, and ligand carbon atoms in *green*. Oxygen and nitrogen atoms are show in *red* and *blue*, respectively. An ordered water molecule position between E353 and R394 and ligands is shown as a *red ball*. *A*, DES bound form; *B*, 4OHT bound form; *C*, 4OHTPP is positioned in DES bound form by superimposing the compound docked receptor onto the DES bound receptor. Van der Waals surfaces of L540 and L525 are shown as a *yellow grid*. Helix 12 residues, L540 and L525, sterically clash with the compound. *D*, 4OHTPP is docked to 4OHT bound form (3ERT.pdb). The conformation of the benzocycloheptene ring of 4OHTPP was derived from the small molecule X-ray structure (20).

Results and Discussion

We have addressed the hypothesis that two classes of synthetic estrogens exist, which function through distinctly different molecular mechanisms. In our first series of experiments we tested several representative compounds that were anticipated to produce estrogen-like effects with D351 ER at the *TGF-α* gene. As noted previously in extensive concentration response studies (10, 12, 13), single concentrations of the compounds E_2, DES, and 4OHT selected in the present study were complete estrogens (Fig. 2*A*). Concentration response studies have been reported with 4OHTPP (17) using the growth of MCF7 and T47D breast cancer cells as the end point of estrogenic action. On the basis of this evidence, one would conclude that each compound bound to the ER and caused full estrogen action. As predicted (17), 4OHTPP was also an estrogen in the TGF-α assay with wild-type ER. In contrast, 4OHTPP and 4OHT did not induce TGF-α mRNA in cells stably transfected with cDNA for D351G ER (Fig. 2B). The TPE derivatives were distinctly different in their responsiveness from DES and E_2. Concentration response studies with 4OHTPP reported previously also did not induce TGF-α mRNA between 10^{-9}-10^{-6} M using D351G ER (18).

To determine whether the coactivator SRC-1 would bind to an activated AF2 site in the LBD of all of the estrogens with D351 ER, we performed GST pull down assay with a D351 construct. The estrogens E_2 and DES induced SRC-1 binding with D351, but neither 4OHT nor 4OHTPP increased the binding of SRC-1 with D351 (Fig. 2*C*) despite the fact that the compounds produced full estrogen-like actions at the *TGF-α* gene (Fig. 2*A*). To interpret our experimental observations (Fig. 2, *A–C*), we have drawn on both the X-ray crystallography data for E_2, DES, and 4OHT (7, 8) and the idea (21) that coactivators bind at different sites on either E_2 or 4OHT ER complexes to initiate gene transcription. Recently, we suggested that the novel site for coactivator binding on the 4OHT ER complex is called AF2b (12). Our suggestion for the novel site is based on experimental evidence that AF2b includes an intact but repositioned helix 12 and an exposed negatively charged amino acid (aspartate, tyrosine) at position 351 on the LBD that synergizes with AF1. Although is has been proposed that a D351Y ER mutant could reactivate weak AF2 activity with 4OHT (22) that synergizes with AF-1 to produce an estrogenic effect, we believe that silencing and reactivation of the ER liganded with a SERM can be modulated entirely through the interaction of a negatively charged amino acid at 351 without relocation of helix 12 (23).

Using the available X-ray crystallographic data, we have docked the 4OHTPP derivative into either the DES (Fig. 3*A*) or 4OHT (Fig. 3*B*) structure and completed the lowest energy calculations for ligand fit. The 4OHTPP estrogen is strictly hindered when fitting the DES-ER structure (Fig. 3*C*) with the α ring (identified in Fig. 1) bumping L540 on the underside of helix 12 (Fig. 3*C*). It is most

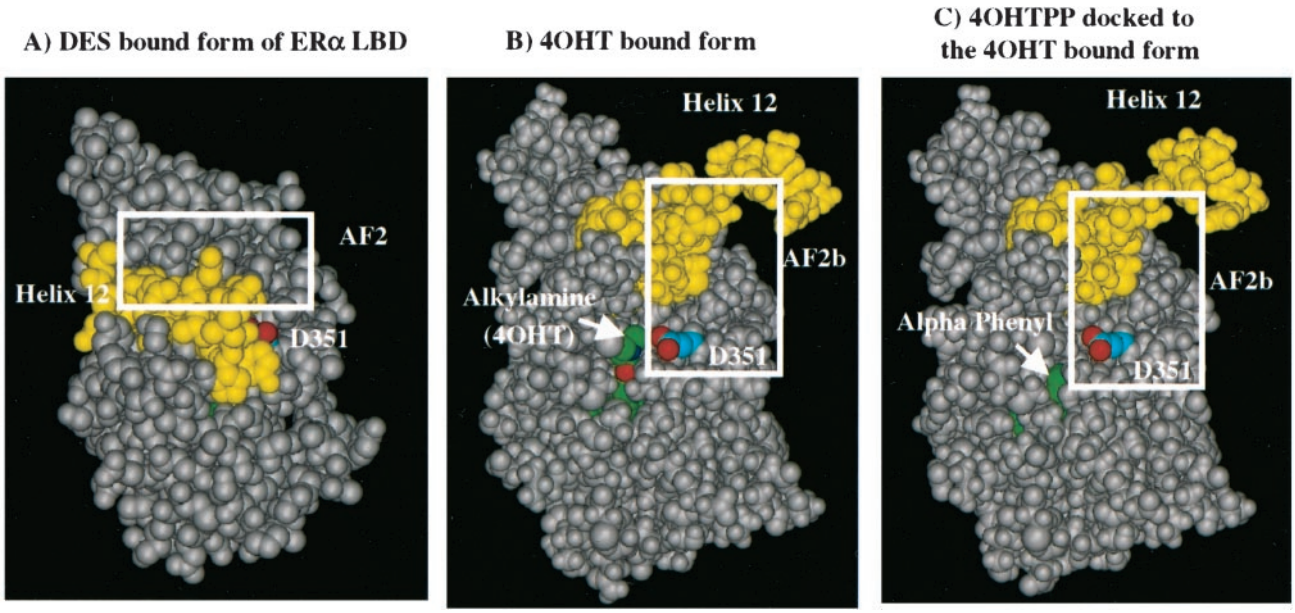

Fig. 4. Surface location of D351 and differing conformations of helix 12 in various ER complexes. *A*, DES bound form (3ERD.pdb); *B*, 4OHT bound form (3ERT.pdb); and *C*, 4OHTPP is docked to the 4OHT bound form. Monomeric ER structures are rendered in space-filled form. ER residues in helix 12 are shown in *yellow*; D351 side chain carbon and oxygen atoms in *teal* and *red*, respectively; the remaining ER residues are in *gray*. Compound carbon atoms are shown in *green*, oxygen in *red*, and nitrogen in *blue*.

interesting to note that L540Q (21) and L539A/L540A (24) mutations of ER reduce the estrogen-like actions of E_2 but retain the estrogen-like properties of 4OHT. In contrast, the TPE 4OHTPP is more likely to fit in the 4OHT ER structure thereby exposing the surface D351 once the helix 12 is repositioned in the AF2 silenced configuration (Fig. 4, *B* and *C*). This would indicate that both 4OHTPP and 4OHT would initiate estrogenic action by binding coactivators at the AF2b position and not the AF2 position as established for DES or E_2 (8). The idea that different estrogens force the ER into different shapes is best illustrated by examining the external surface of the complexes. Classical estrogen action occurs through the planar ligand being sealed within the LBD by helix 12. The external shape of the DES-ER complex is shown in Fig. 4*A* with D351 in close contact with helix 12. This conformation activates AF2. In contrast, the TPE-type estrogen causes helix 12 to silence AF2 (Fig. 4*C*) in much the same way as observed for 4OHT (Fig. 4*B*). This exposes the charge at D351 necessary for AF2b activation. Removal of the charge in D351G ER silences AF2b and prevents estrogen action (Fig. 2*B*) with 4OHTPP but does not affect the planar estrogens DES and E_2 (Fig. 2*A*) that employ an AF2 coactivator site.

However, it is important to stress that the compounds 4OHTPP and 4OHT have dissimilar actions using the complex estrogen assay techniques of breast cancer cell replication or rat uterine growth (17). 4OHT is classified as an antiestrogen, whereas unsubstituted TPE derivatives are estrogens. Antiestrogen action is clearly more sophisticated than a simple silencing of AF2 with helix 12. Coactivators and corepressors probably program different shaped complexes to enhance or suppress gene function (25). Nevertheless, the idea that a TPE-like ligand that repositions helix 12 but remains an estrogen may have important biological implications.

On the basis of our experimental results, we propose that synthetic estrogens can now be reclassified into two types: class I estrogens are planar and related to DES and E_2 so they can easily be sealed within the hydrophobic pocket by helix 12. Class I estrogens will use the AF2 coactivator binding site, which will synergize with AF1 to produce optimal estrogen-like actions. The angular TPE type of estrogens are class II. These compounds do not activate AF2 but use the AF2b site that includes acidic surface amino acids on a repositioned helix 12, an exposed aspartate at position 351 and AF1 (12). Each component of the novel AF2b site is mutually interdependent.

We suggest that in the future, endocrine disrupters in the environment or phytoestrogens should be evaluated to determine whether they can be subdivided in separate classes of estrogens based on their molecular mechanism of action. The novel subclassification of estrogens may provide new information about the carcinogenic potential of different compounds and whether estrogen with a novel shape, that diverges from the natural hormone, forms ER complexes that recruit different coactivators to initiate transcription. This knowledge may provide new targets for the prevention of hormonal carcinogenesis or provide rationalization of the carcinogenic potential of xenoestrogens.

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