Insights from the Structure of Estrogen Receptor into the Evolution of Estrogens: Implications for Endocrine Disruption

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Abstract In the last decade, there has been important progress in understanding the origins and evolution of receptors for adrenal steroids (aldosterone, cortisol) and sex steroids (estradiol, progesterone, testosterone) due to the sequencing of genomes from animals that are at key sites in vertebrate evolution. Although the estrogen receptor [ER] appears to be the ancestral vertebrate steroid receptor and estradiol [E2] is the physiological ligand for vertebrate ERs, the identity of the ancestral ligand(s) for the ER remains unknown. Here, using an analysis of crystal structures of human ERα with E2 and other chemicals and 3D models of human ERα with 27-hydroxycholesterol and 5-androsten-3β,17β-diol, we propose that one or more Δ5 steroids were the ancestral ligands for the ER, with E2 evolving later as the canonical estrogen. The evidence that chemicals with a β-hydroxy at C3 in a saturated A ring can act as estrogens and the conformational flexibility of the vertebrate ER can explain the diversity of synthetic chemicals that disrupt estrogen responses by binding to vertebrate ERs.

1. Introduction

The adrenal steroids, aldosterone and cortisol, and sex steroids, estradiol (E2), progesterone and testosterone, regulate a wide range of physiological processes including reproduction, development and homeostasis [Figure 1]. The physiological actions of these vertebrate steroids are mediated by nuclear receptors, a large and diverse group of transcription factors that arose in multicellular animals [1-4]. Nuclear receptors with transcriptional

responses to adrenal and sex steroids appear to have evolved in deuterostomes [5-7]; the estrogen receptor [ER] is the ancestral vertebrate steroid receptor [6-11].
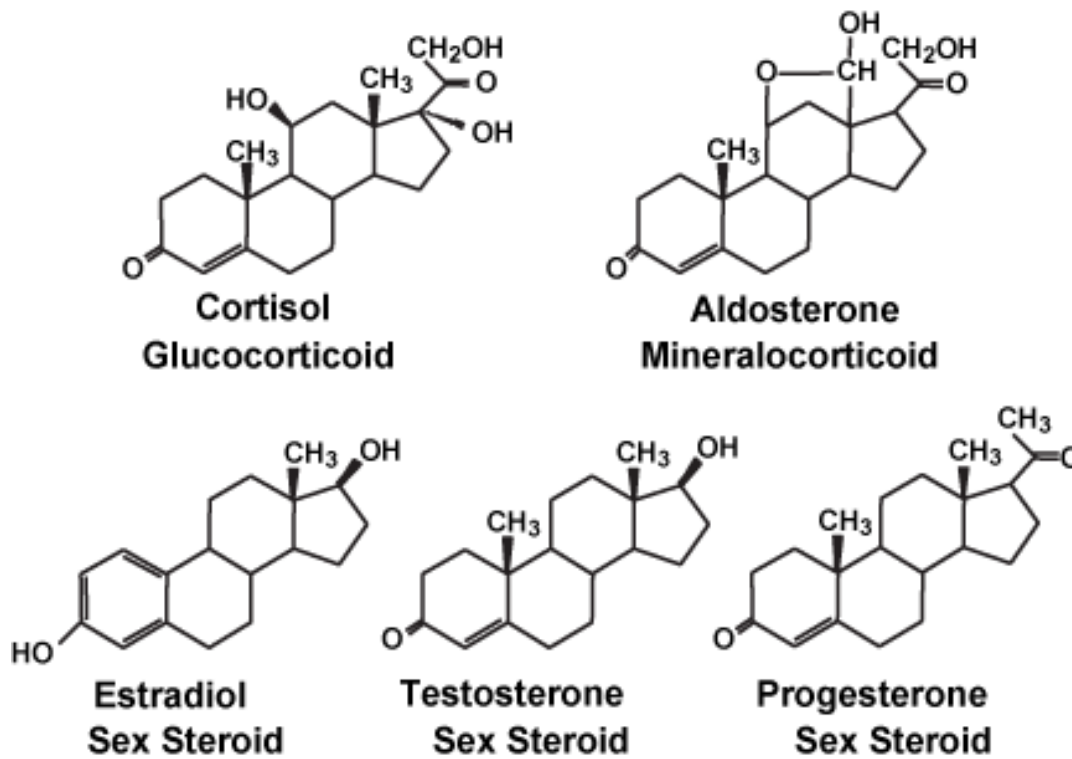

Figure 1. Structures of adrenal and sex steroids. The A ring of Estradiol has a C3 phenolic group.   The A ring in the other steroids has a C3-ketone.

In addition to an ER, the chordate amphioxus contains a steroid receptor [SR], which diversified in vertebrates into the androgen receptor [AR], glucocorticoid receptor [GR], mineralocorticoid receptor [MR] and progesterone receptor [PR] [8-9, 12-14].   Although mollusks and annelids contain receptors with sequence similarity to the human ER [15-17], there is disagreement as to whether these protostome receptors diverged from a common ancestor of chordate ER [17], or if the similarity between protostome proteins and the chordate ER is an example of convergent evolution [18-20].   The pros and cons of this controversy are discussed in [17-20] and are not the focus of this paper.

1.1 What was the ancestral ligand for the ER?

Here, we investigate another unresolved question about the ER: the identity of the ligand(s) for the ancestral ER [3, 7, 18, 21].   Phylogenetic analyses of lamprey and other

vertebrate steroid receptors established that the ER was the ancestral steroid receptor [7]. Duplication of this ER followed by sequence divergence led to the ancestral 3-keto-steroid receptor, which through further duplications and divergence led to the AR, GR, MR and PR [1, 6-7]. A problem with having the ER as the ancestral receptor is that estradiol [E2], the physiological ligand for vertebrate ERs, is at the end of the pathway for the synthesis of steroids from cholesterol [7, 22] [Figure 2].
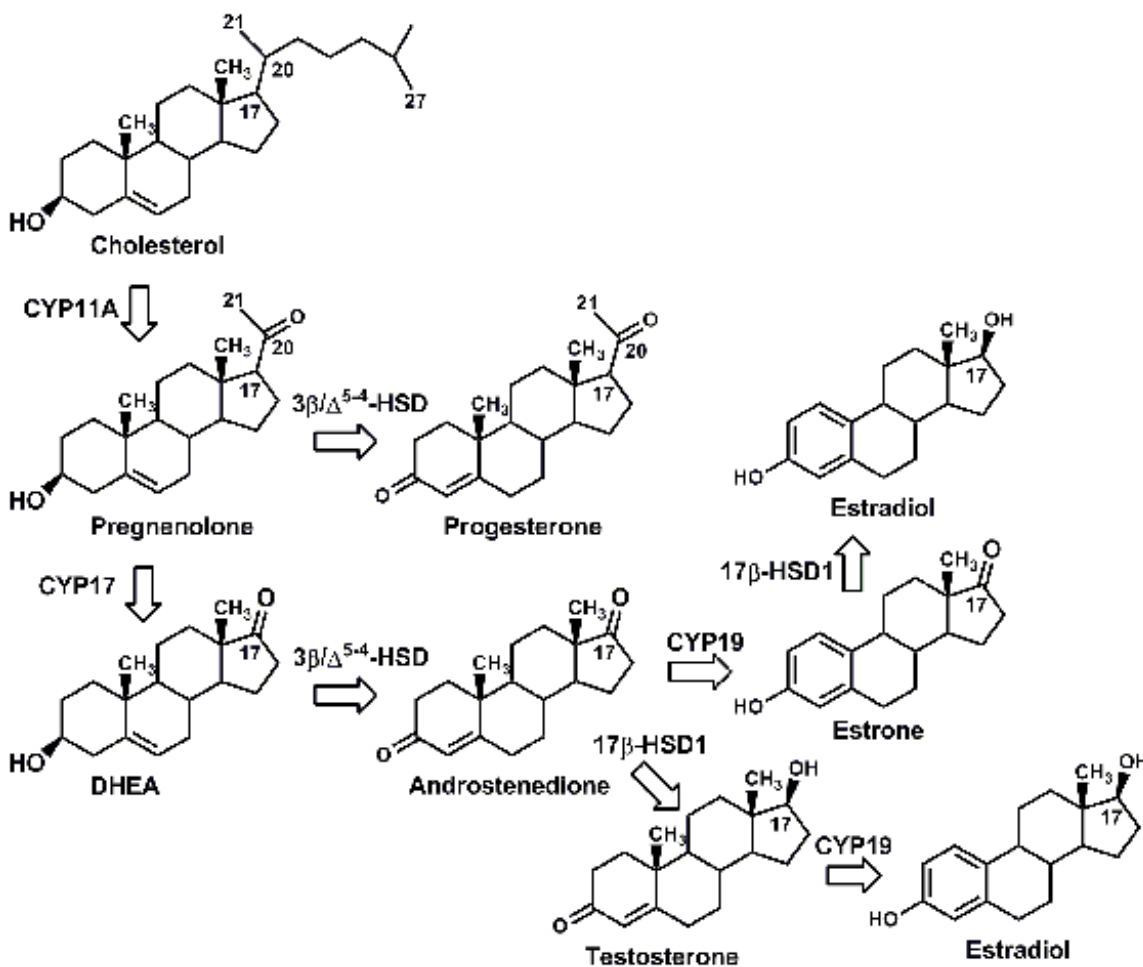

Figure 2. Enzymes involved in the synthesis of vertebrate steroids from cholesterol CYP450s, $3\beta/\Delta^{5-4}$-HSD and 17β-HSD-type 2 catalyze the formation of vertebrate steroids from cholesterol [18, 51-52].

E2 is synthesized from either testosterone, or from estrone [E1], which is synthesized from androstenedione. Thus, the AR would have been expected to have evolved before the ER. Moreover, $3\beta/\Delta^{5-4}$-hydroxysteroid dehydrogenase [$3\beta/\Delta^{5-4}$-HSD], which catalyzes the synthesis

of androstenedione from DHEA, also catalyzes the synthesis of progesterone from pregnenolone. Thus, the PR also would have been expected to have evolved before the ER. To solve this conundrum, Thornton [7] proposed the ligand exploitation model, in which vertebrate steroids that are upstream of E2, were present before their receptors evolved. In this model, progesterone and testosterone were present before the evolution of the PR and AR respectively. Progesterone and testosterone assumed novel signaling functions after the evolution of the PR and AR.

In an alternative model, 5-androsten-3β,17β-diol [Δ^5-androstenediol], which is upstream of E2, has been proposed as a ligand for the ancestral ER [22-23] [Figure 3].
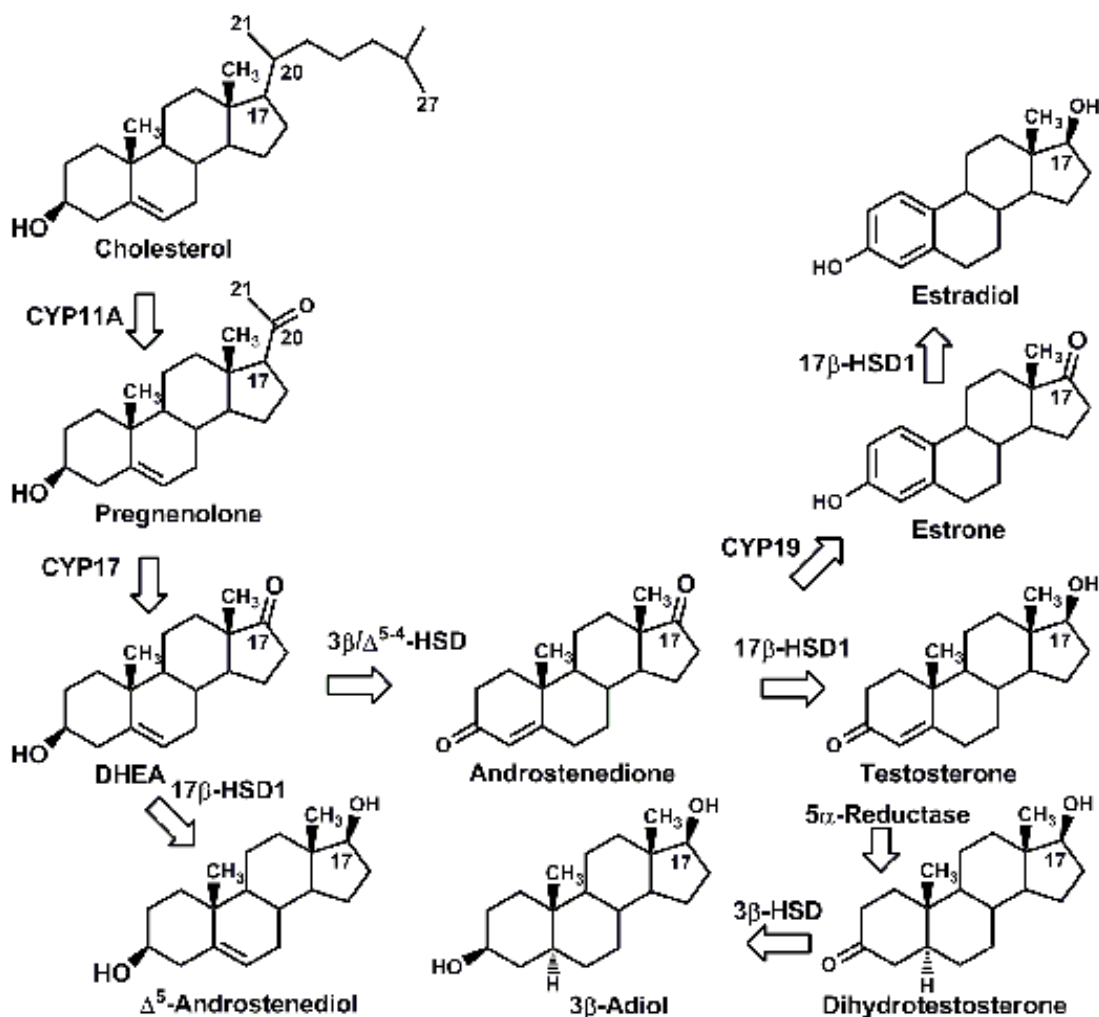

Figure 3. Synthesis of Δ^5-androstenediol and 3β-Adiol. Synthesis of Δ^5-androstenediol and 3β-Adiol does not require aromatase [CYP19]. Moreover, synthesis of Δ^5-androstenediol does not require 3β/Δ^{5-4}-HSD. Δ^5-androstenediol and 3β-Adiol have high affinity for the ER [30].

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Here, we provide support for this latter hypothesis, using data from crystal structures of human ERα with E2 and other steroids and 3D models of human ERα with Δ5 steroids. As discussed later, our analysis of crystal structures of human ER with novel steroids and 3D models of human ER with oxy-sterols also has implications for the binding to the ER of diverse synthetic chemicals, which can disrupt estrogen physiology in vertebrates [24-28].

2. Methods

The Insight II software and the Biopolymer and Discover 3 options were used to construct 3D models of human ERα [PDB:1G50] with Δ^5-androstenediol, 3β-Adiol, 27-OH-C and 24-OH-C, as described previously for constructing 3D models of 15α-hydroxy-estradiol complexed with human ERα and lamprey ER [29]. The crystal structure of human ERα with E2 was opened with Insight II. Then the Biopolymer option in Insight II was used to modify E2 to Δ^5-androstenediol, 3β-Adiol, 24-OH-C and 27-OH-C. Then the 3D model of ERα with each of these ligands was refined through energy minimization with Discover 3 for 10,000 iterations using the CVFF force field, with a distant dependent dielectric constant of 2. The final 3D models of ERα with Δ^5-androstenediol, 3β-Adiol, 24-OH-C and 27-OH-C are shown in Figures 6A, 6B, 8 and 9, respectively.

The crystal structure of human ERα with trifluoromethyl-phenylvinyl-E2 [PDB: 2P15] was downloaded for analysis with Insight II.

3. Structural Determinants for Binding of Estradiol to the ER

Estrogens such as E2 and diethylstilbestrol [DES], which activate ERα and ERβ [30], contain a C3-phenolic group [Figure 4], which also is required for high affinity binding to the ER by anti-estrogens, such as 4-hydroxy-tamoxifen, because tamoxifen is inactive [30] [Figure 4]. The crystal structures of human ERα with E2 [31-32], DES [33] and 4-hydroxy-tamoxifen [33] provide a structural explanation for importance of the C3-phenolic group on E2 and other estrogens.
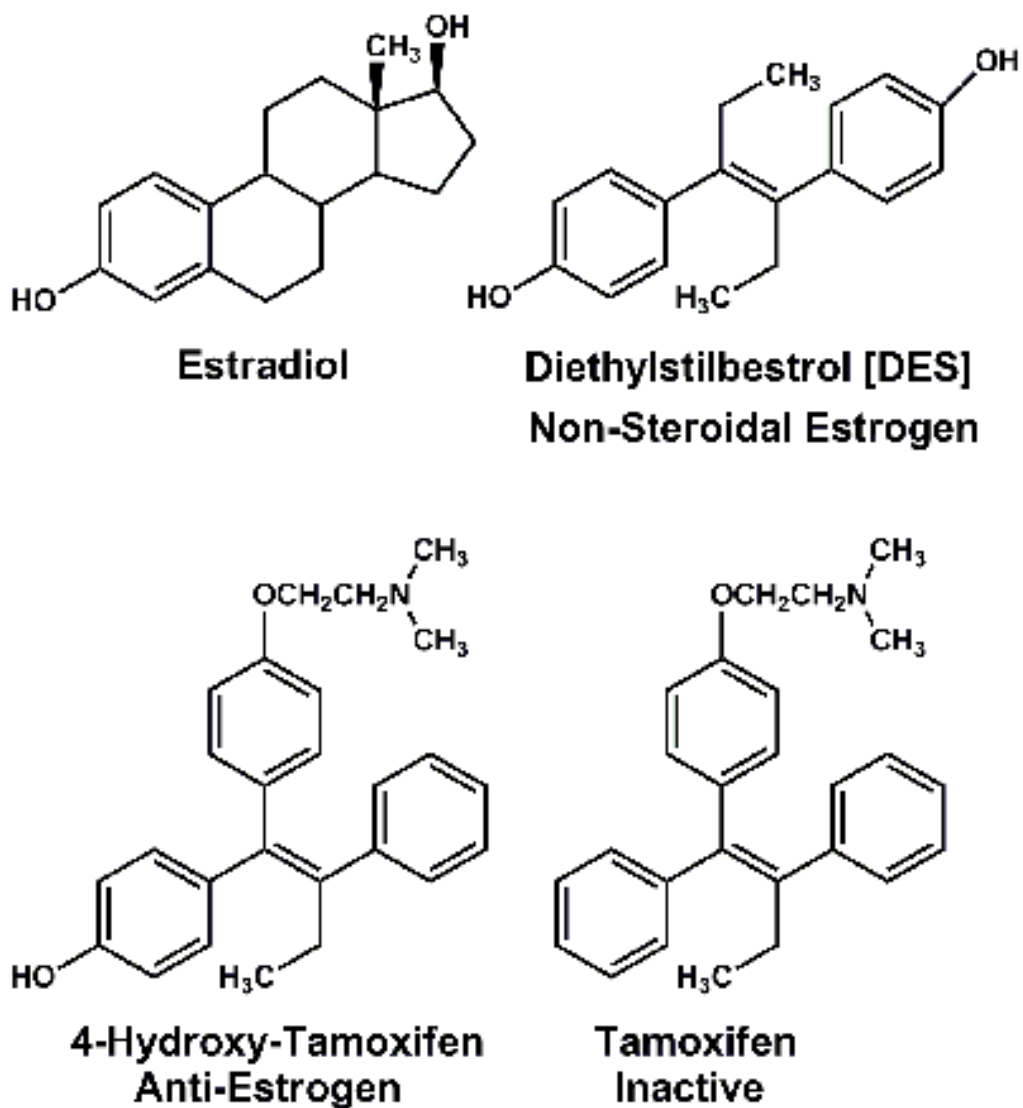
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Figure 4. Structures of estrogens and an anti-estrogen   E2 and DES, two estrogens, and 4-hydroxy-tamoxifen, an anti-estrogen have a phenolic A ring, which is important in their high affinity for the human ER.

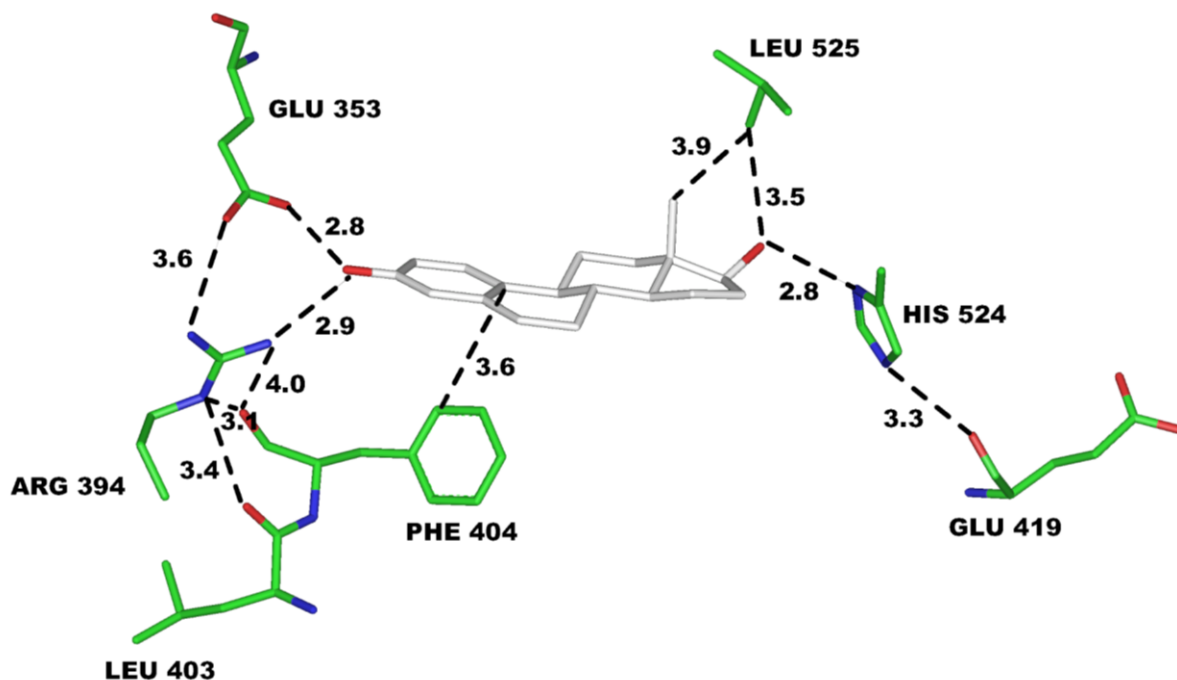As shown in Figure 5, the C3 phenolic group has a hydrogen bond with the γ-carboxylate of Glu-353 in human ERα.

Figure 5. Key amino acids in human ERα that bind E2. The crystal structure of human ERα with E2 reveals that the A ring of E2 has hydrogen bond with γ-carboxylate of Glu-353 [4, 31-32]. This interaction is characteristic of the ERα and ERβ. In receptors for 3-keto-steroids, Glu is replaced by Gln, in which the amido NH2 group donates a hydrogen bond to the 3-keto group.

The specificity of this interaction for the ER is seen in the replacement of Glu-353 by a corresponding Gln in the PR [32, 34] and AR, MR and GR [4], which are activated by steroids with 3-keto-groups [Figure 1]. The amido NH2 group on this Gln donates a hydrogen bond to the 3-keto-group on progesterone, testosterone, cortisol and aldosterone [4].

3.1 Human ERa binds Δ^5-androstenediol and 5α-androstane-3β,17β-diol with high affinity

The evidence that the major physiological estrogens, E2 and E1, and anti-estrogens, such as 4-hydroxy-tamoxifen and raloxifene [31], have an aromatic A ring supports the notion that aromatase [CYP19] was necessary for the evolution of the steroid that activated the ancestral ER [17]. However, steroids that lack an aromatic A ring and contain a 3β-hydroxyl group, such as Δ^5-androstenediol and 5α-androstane-3β,17β-diol [3β-Adiol] also have high affinity for the mammalian ER [30] and could have served as ligands for the ancestral ER [22-23]. Indeed, 3β-Adiol is an active estrogen in the prostate [35], as well as in the brain, under conditions in

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which E2 is not present [36].   As shown in Figure 3, synthesis of Δ^5-androstenediol and 3β-Adiol does not require aromatase [CYP19].   Thus, either steroid could have been an active estrogen before the evolution of CYP19.   Moreover, Δ^5-androstenediol could have been an active estrogen before the evolution of $3\beta/\Delta^{5-4}$-HSD [22-23].

Our 3D models of human ERα with Δ^5-androstenediol and 3β-Adiol reveal that stabilizing contacts are retained between the 3β-hydroxyl and Glu-353 and Arg-394 and between the 17β-hydroxyl and His-524 [Figure 6], which is consistent with the evidence that both steroids have nM affinity for ERα and ERβ [30] . This expands the ligands that can bind to the ER to include steroids with a 3β-hydroxyl on a saturated A ring.
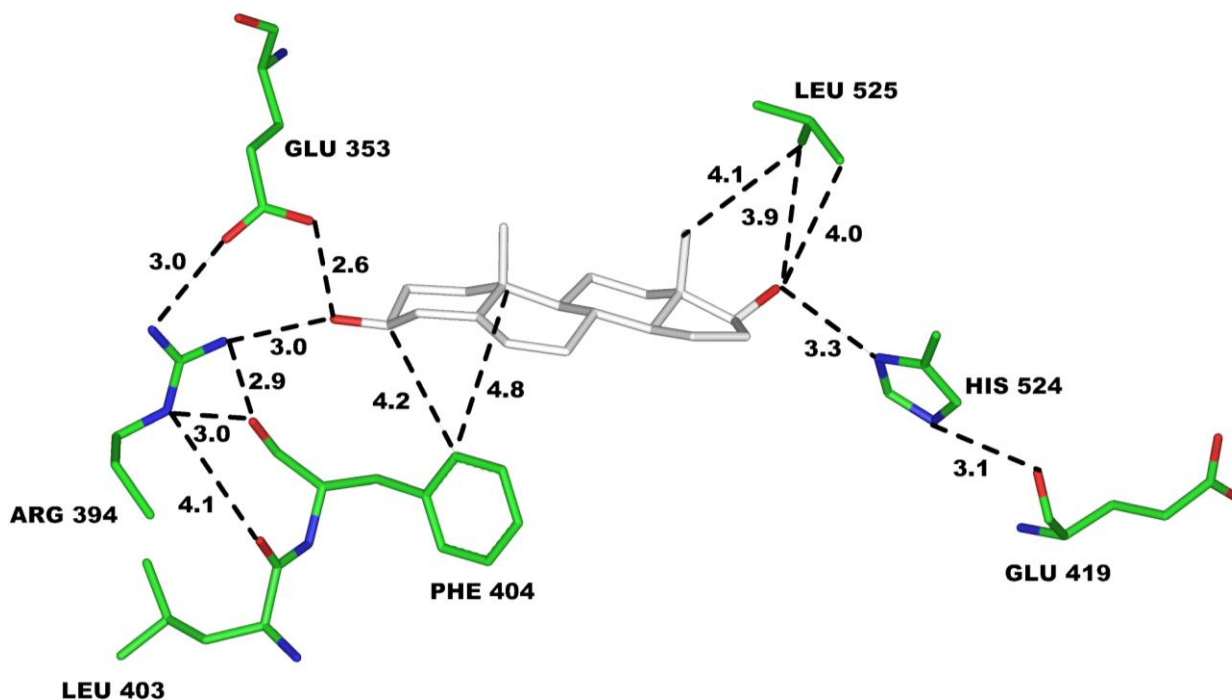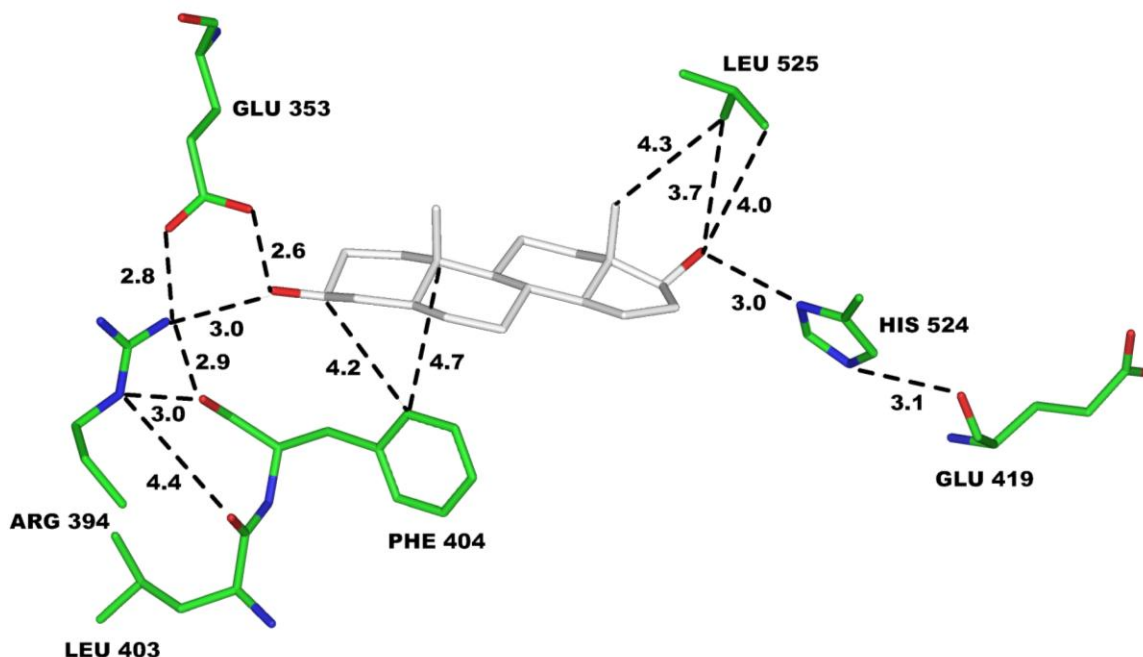
Figure 6. Interaction of human ERα with Δ^5-androstenediol and 3β-Adiol

A. 3D model of human ERα with Δ^5-androstenediol

B. 3D model of human ERα with 3β-Adiol.

In both 3D models, the 3β-hydroxyl group on Δ^5-androstenediol and 3β-Adiol has favorable contacts with Glu-353 and Arg-394.   The 17β-hydroxyl on Δ^5-androstenediol and 3β-Adiol has a favorable contact with His-524.

3.2 Human ERα can accommodate steroids with large substituents in the D ring

E2 has the smallest van der Waals volume of the vertebrate steroids [37] and the ER has the smallest ligand-binding cavity of vertebrate steroid receptors [38].   Crystal structures of the ER place the estrogen-binding site in a hydrophobic cavity, with select polar residues stabilizing the A and D rings.   These polar residues are Glu-353 and Arg-394, which stabilize the A ring, and His-524, which caps the D ring with a hydrogen bond to the 17β-hydroxyl that is characteristic of the ER [Figure 5].   Conversion of the 17β-hydroxyl to a ketone, as found in E1, reduces the affinity for the ER [30].   The lack of a C17 side chain in E2, in contrast to aldosterone, cortisol and progesterone [Figure 1], and the compact ligand-binding cavity in the ER indicated that compact ligands bind to the ER.

However, in the last decade it has become clear that there is substantial conformational flexibility in the ER, which allows high affinity binding of E2 analogs with large substituents at

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C17 to E2.   An important example is trifluoromethyl-phenylvinyl-E2 (TFMPV-E2), which has a bulky 17α-substituent and a Kd of about 50 pM for human ERα [39].   TFMPV-E2 is an agonist for human ERα.   To understand the molecular interaction of TFMPV-E2 with human ERα, they were cocrystallized (PDB 2P15) by Nettles et al.   Analysis of this crystal structure [Figure 7] revealed that despite the bulky substituent at C17 on TFMPV-E2, it has favorable contacts with key residues in the ligand-binding pocket [39].
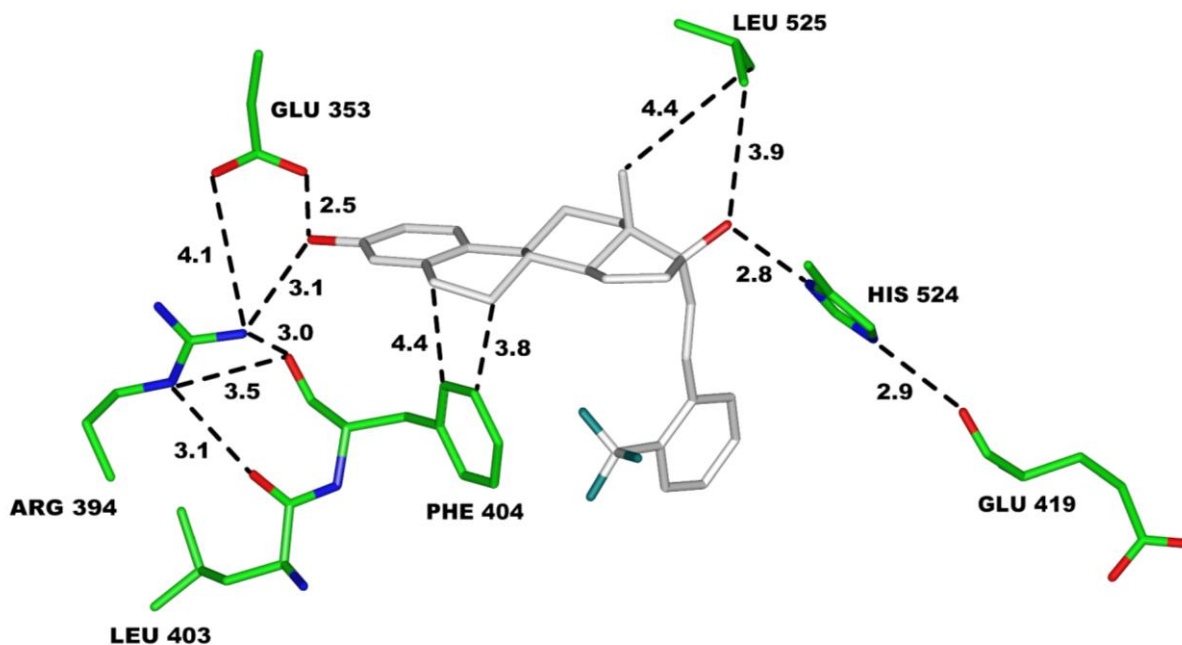

Figure 7. Human ERα complexed with TFMPV-E2   The crystal structure of human ERα with TFMPV-E2 [PDB: 2P15] [39] was downloaded for analysis with Insight II.   Despite the large 17α-substituent, TFMPV-E2 has favorable contacts with His-524 and no steric clashes with ERα.   In fact, TFMPV-E2 is a potent estrogen with a Kd of about 50 pM for human ERα [39].

4. Was the ancestral estrogen a cholesterol analog?

The finding that transcription by the ER can be activated by an estradiol analog with a bulky substituent at C17 indicates that a steroid with a large substituent at C17 could have been the ligand that activated the ancestral ER, which would be expected to have a different sequence from mammalian ERs, as well as a different cellular milleu.   Recently, Umetani et al [40] reported that several hydroxylated cholesterols including 27-hydroxycholesterol [27-OH-C], 22R-OH-C, 24S-OH-C and 25-OH-C bound human ERα and human ERβ.   27-OH-C was the

most potent, and in some mammalian cells 27-OH-C functions as a partial agonist for human

ERα and ERβ [40-43].   The Kd of 27-OH-C for ERα and ERβ is about 1.3 μM and 0.4 μM,

respectively [40-42], which is over 10^{3} times higher than the Kd of E2 for human ERα and ERβ

[30, 40].   Nevertheless, a Kd of 1 μM is physiologically relevant because the circulating

concentration of 27-OH-C is from 0.15 to 0.73 μM, [40-42].

To understand the molecular interaction of 27-OH-C with human ERα, we constructed a

3D model of human ERα with 27-OH-C [Figure 8].   The 3D model reveals that binding of the

A ring to Glu-353 and Arg-394 on human ERα is favorable, but His-524 does not have a

stabilizing hydrogen bond with 27-OH-C [Figure 8], which may explain the low affinity of
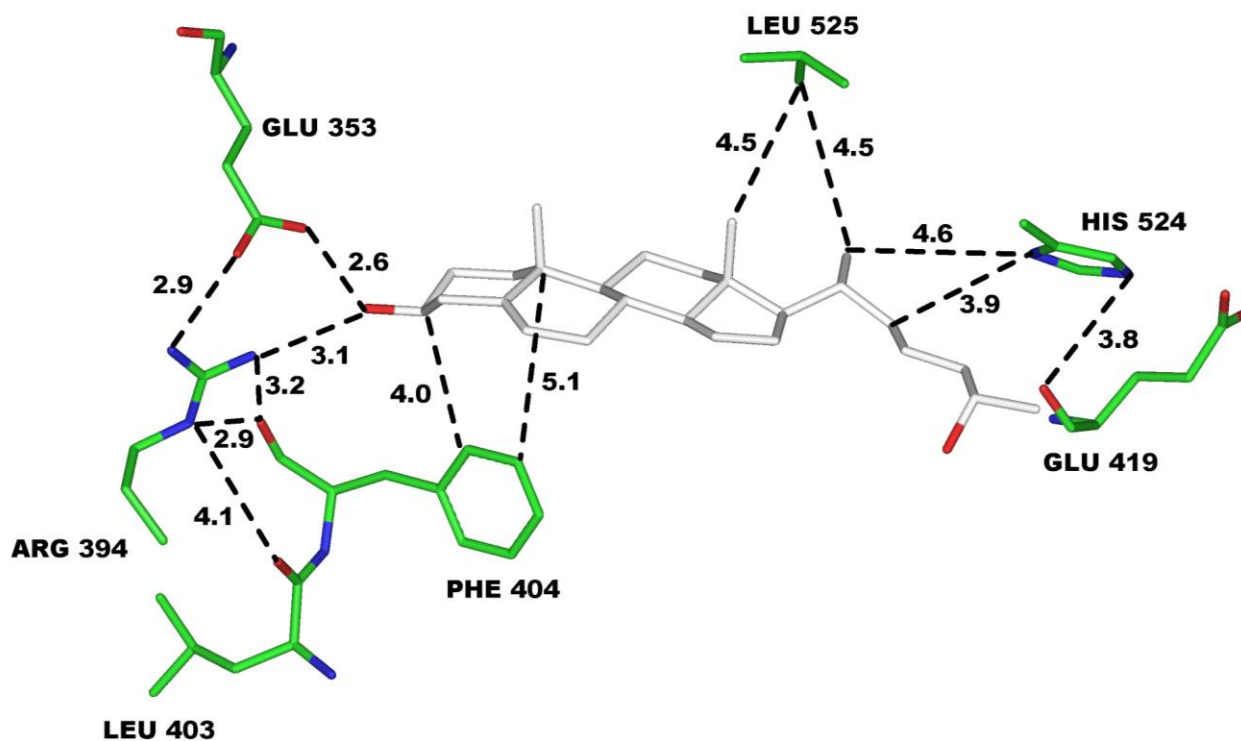
human ER for 27-OH-C.

Figure 8. 3D model of human ERα with 27-hydroxycholesterol   The 3D model of human
ERα with 27-OH-C shows that Glu-353 and Arg-394 are 2.6A and 3.1A, respectively, from the
C3-hydroxyl.   His-524 on ERα does not contact the 27-hydroxyl, which may explain the
substantially the lower affinity of 27-OH-C for ERα and ERβ [40-42].

The 3D model in Figure 8 indicates that a C24-hydroxyl on cholesterol could have a stabilizing contact with His-524.   In Figure 9, we show a 3D model of human ERα complexed with 24-OH-C, which shows that the C24 hydroxyl is 3.3A from His-524.   Bioassays are needed to determine if 24-OH-C has estrogen agonist or antagonist activity.
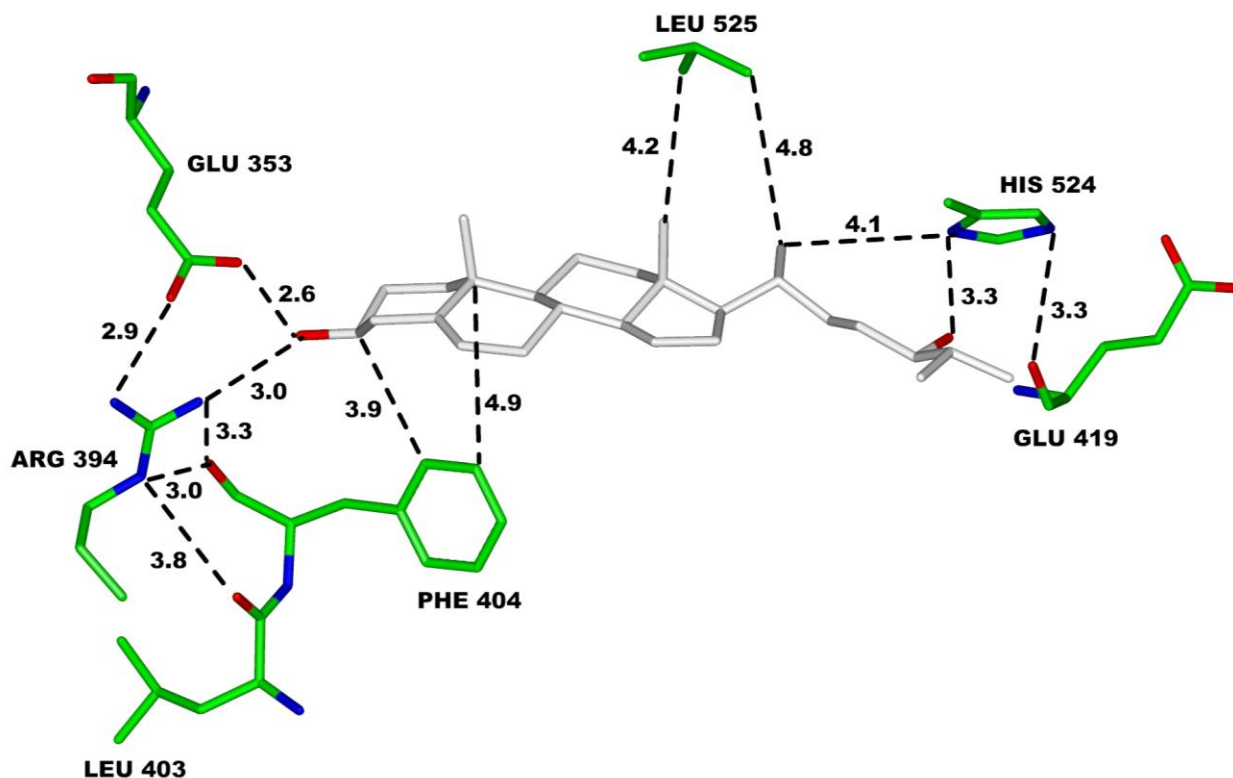

Figure 9. 3D model of human ERα with 24-hydroxycholesterol   The 3D model of human ERα with 24-OH-C shows that Glu-353 and Arg-394 are 2.6A and 3.0A, respectively, from the C3-hydroxyl.   His-524 on ERα is 3.3A from the 24-hydroxyl.   There are no steric clashes between 24-OH-C and amino acids in the 3D model of human ERα.

The partial agonist activity of 27-OH-C for human ERα is relevant for the identity of ligand that activated transcription by ancestral ER.   The Δ^5-ligand that regulated estrogen physiology through binding to the ancestral ER may have been 27-OH-C, 24-OH-C or another hydroxy-cholesterol derivative.   Regarding the ancestral ligand, an important consideration is that the ancestral ER would be expected to have a different sequence from mammalian ERs, and,

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thus, may have a higher affinity for a cholesterol analog or another ligand than has a mammalian ER. Also, the cellular milleu for the ancestral ER is likely to differ from that in mammalian cells. For example, the ancestral ER-ligand complex may have had increased transcriptional activity due to binding to ancestral co-activators, which differ from their mammalian orthologs. If a cholesterol analog is the ancestral estrogen then it would support the model of Markov and Laudet that the estrogen response evolved before the cholesterol side-chain cleavage enzyme CYP11A [20]. It also means that a cholesterol analog preceded Δ^5-androstenediol or another Δ^5-derivative of DHEA as the physiological estrogen for the ancestral ER.

The different physiological concentrations of steroids and cholesterol may have been important in the evolution of steroids as ligands for vertebrate nuclear receptors. In contrast to the high concentration of cholesterol in blood, vertebrate steroid concentrations are at nM or lower levels, consistent with the nM affinities of vertebrate steroids for their cognate receptors. Thus, the evolution of CYP11A and CYP17 would provide a transition to C21 or C19 ligands that would function at nM concentrations to regulate transcription by the ER and other vertebrate steroid receptors.

5. Implications for disruption of estrogen physiology by xenobiotics

An unexpected outcome in preparing this commentary was the discovery that the ER can accommodate ligands such as TFMPV-E2 and 27-OH-C, which have larger substituents at C17 than does E2. The conformational elasticity in the ligand-binding site of the ER and the evidence that the ER can be activated by ligands with a 3β-hydroxyl instead of a C3-phenolic group substantially expands the suite of chemicals that need to be considered as possible disruptors of normal functioning of the ER in vertebrate physiology. Indeed, although there is evidence that the ER binds environmental chemicals with diverse structures [Figure 10] [25, 27-28], synthetic chemicals and phyotchemicals that lack key features of E2 should not be assumed to be inert towards the ER as either agonists or antagonists.
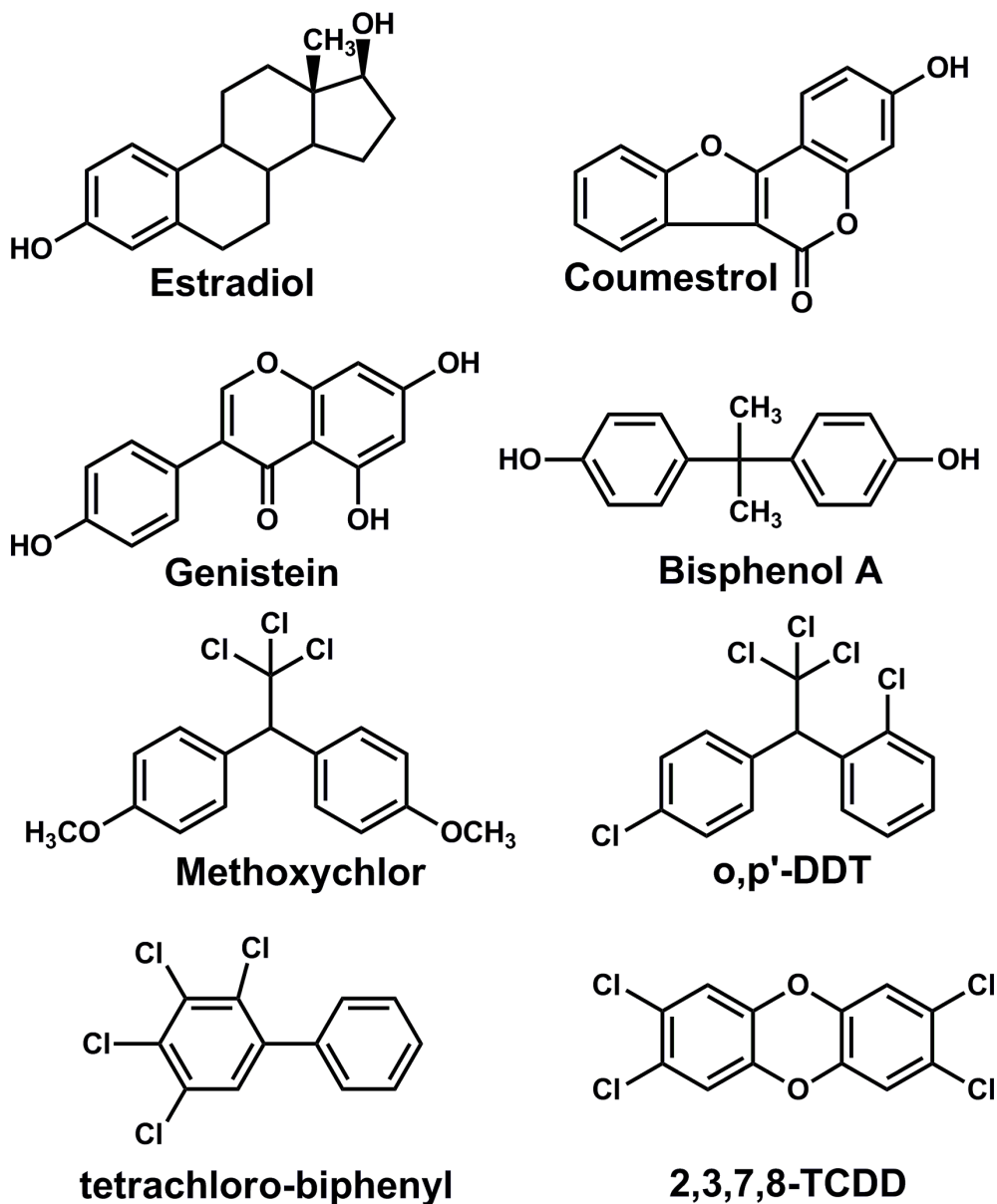
Figure 10. Chemicals with diverse structures bind to the ER.   Bisphenol A and genistein contain hydroxyls that can mimic some of the properties of the C3 and C17 hydorxyls on estradiol.   Coumestrol has only one hydroxyl.   Methoxychlor, o,p'-DDT, 2',3',4',5'-Tetrachloro-4-biphenyl and 2,3,7,8-Tetrachloro-dibenzodioxin (2,3,7,8-TCDD) lack hydroxyl substituents.

Before one assumes that these chemicals do not disrupt estrogen physiology, they should to be screened *in silico* for binding to vertebrate ERα and ERβ using various docking algorithms [44-45].   Chemicals with high docking scores can be tested for agonist or antagonist activity with high throughput screening of cells containing an ER and a reporter gene.

Interestingly, conformational flexibility in the ligand-binding pocket has been found in other nuclear receptors.   The ligand binding pocket on the GR can accommodate steroid analogs that are substantially larger than cortisol.   In particular, deacylcortivazol, which has a large substituent at the A ring and thus lacks a C3 ketone, has a 10-fold higher affinity for the GR than does dexamethasone [46].   The crystal structure of the GR-deacylcortivazol complex [PDB:3BQD] shows that the ligand binding pocket doubles in size.   Crystal structures of LXR [PDB:1PQ6, 1PQ9, 1PQC] [47], the ecdysone receptor [PDB 1R20, 1R1K] [48] and thyroid hormone receptor [PDB:1X7X, 1Y0X] [49-50] indicate that these receptors have a flexible ligand-binding pocket.   Thus, these receptors also may bind chemicals that are larger than their cognate ligands, with important implications for disruption of their physiological activity.

Lastly, conformational flexibility of the ligand-binding pocket in the ER and other nuclear receptors may have been important in the evolution of their response to oxysterols and vertebrate hormones from ancestral signals [3, 18, 20].

Acknowledgments

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