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Structure–activity relationships of bisphenol A analogs at estrogen receptors (ERs): Discovery of an ERα-selective antagonist

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ABSTRACT

Our multi-template approach for drug discovery, focusing on protein targets with similar fold structures, has yielded lead compounds for various targets. We have also shown that a diphenylmethane skeleton can serve as a surrogate for a steroid skeleton. Here, on the basis of those ideas, we hypothesized that the diphenylmethane derivative bisphenol A (BPA) would bind to the ligand-binding domain of estrogen receptors (ERs) in a similar manner to estradiol and act as a steroid surrogate. To test this idea, we synthesized a series of BPA analogs and evaluated their structure-activity relationships, focusing on agonistic/antagonistic activities at ERs and ERα/ERβ subtype selectivity. Among the compounds examined, **18** was found to be a potent ERα-antagonist with high selectivity over ERβ and androgen receptor under our assay conditions. A computational docking study suggested that **18** would bind to the antagonistic conformation of ERα. ERα-selective antagonists, such as **18**, are candidate agents for treatment of breast cancer.

The multi-template approach to drug design is based on the fact that the number of three-dimensional spatial structures (fold structures) of human proteins is much smaller (more than 50 times smaller) than the number of human proteins (50,000–70,000).[1–4] Therefore, ignoring physical/chemical interactions, a template/scaffold structure which is spatially complementary to one fold structure might serve as a multi-template for structural development of ligands that would interact specifically with more than 50 human proteins. In other words, the structures of ligands that bind to one member of a particular group of fold structures may be used for the development of novel lead compounds for other members of the same fold structure group. Lead compounds obtained in this way may possess polypharmacologic character, and so structural modification is then necessary to optimize target selectivity.

A skeleton that corresponds structurally to the steroid skeleton would be a potential multi-template for structural development of ligands for a number of important proteins, and we have focused on diphenylmethane. Indeed, non-secosteroidal vitamin D receptor (VDR) agonists with a diphenylmethane skeleton have been developed, such as LG190178[5,6] (Fig. 1), and they show improved stability and reduced calcium-increasing effects. We have extended this approach to obtain novel ligands of nuclear receptors (NRs) and steroid-related enzymes, such as VDR/androgen receptor (AR) dual ligands (VDR agonists/AR antagonists),[7,8] AR-selective antagonists,[9] farnesoid X receptor (FXR) agonists[10] and inhibitors of steroid metabolism-related enzymes, including 5α-reductase[11] (Fig. 1). Those results indicated that the diphenylmethane skeleton is an effective steroid surrogate.[4,12]

Estrogen receptors (ERs) are activated by the endogenous hormone 17β-estradiol (Fig. 2). Following endogenous ligand binding, ERs undergo conformational changes and biochemical modifications that induce release of inhibitory proteins, receptor dimerization, and interaction with DNA. Estrogen action is mediated through two ER subtypes, ERα and ERβ, which have distinct target tissue distributions and functional activities.[13] Estrogens play an important role in the initiation and progression of breast cancer, and so ERs are regarded as important drug targets. For example, classical selective ER modulators (SERMs), tamoxifen[14] and raloxifene (Fig. 2), have been used for the treatment of breast cancer. However, acquired resistance to tamoxifen is a serious problem.[15–17] Although many breast cancer patients benefit from tamoxifen therapy, resistance to this drug develops in the majority of cases; moreover, if tamoxifen treatment is not discontinued after the onset of resistance, the drug can actually promote, rather than inhibit, tumor growth.

The presence of ERα appears to be associated with an increased risk of breast cancer.[18] In addition, this receptor subtype has been shown to be a prerequisite for growth stimulation of MCF-7 breast

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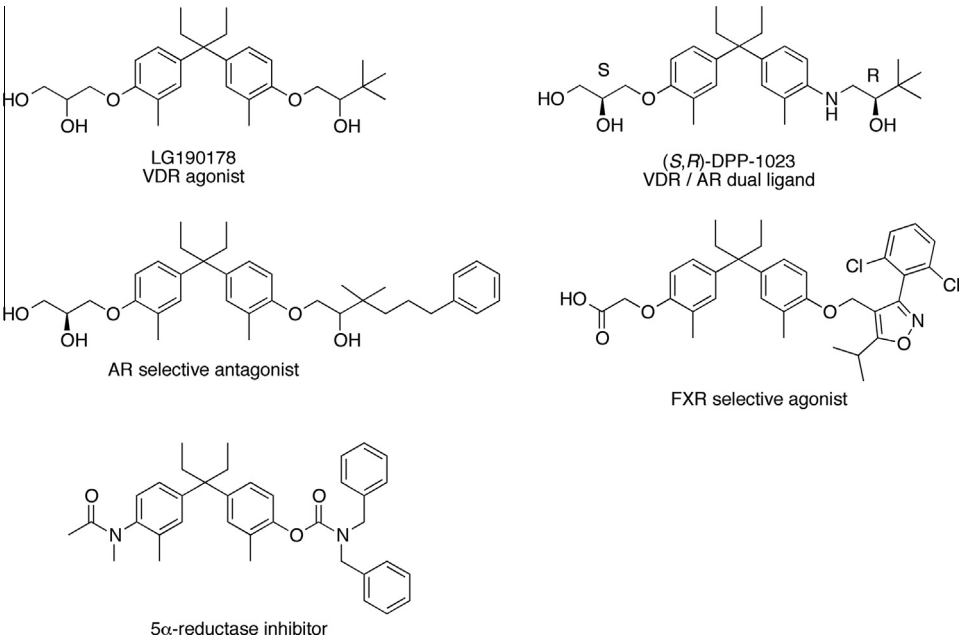


Figure 1. Chemical structures of steroid surrogates bearing a diphenylmethane skeleton.

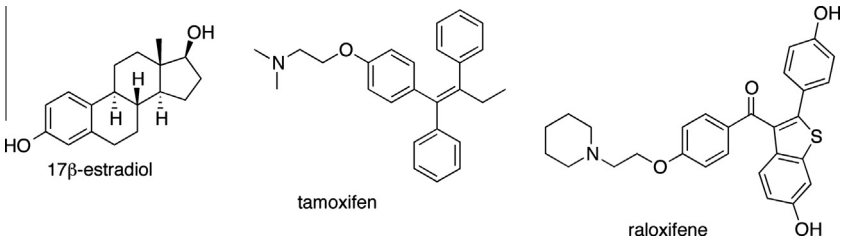


Figure 2. Chemical structures of ER ligands.

cancer cells by estradiol. An MCF-7 cell line without ERα did not proliferate in the presence of estradiol, but recovered its ability to proliferate when ERα was reintroduced.[19] However, classical SERMs have essentially no selectivity for either ER subtype. On the other hand, several ERα-selective antagonists[20–24] have been reported.

Bisphenol A (BPA, 4,4′-(propane-2,2-diyl)diphenol, **1**) is used primarily in the manufacture of polycarbonate plastic and epoxy resins, and is also used as a non-polymer additive to other plastics (Fig. 3). Several lines of evidence indicate that BPA has estrogenic activity. For example, BPA induced the expression of estrogen-responsive genes and promoted proliferation of MCF-7 cells.[25] However, it has also been reported that the activity of BPA depends on both the ER subtype and the cell type.[26] Although bisphenol AF[27] and HPTE[28] (Fig. 3) have been reported to be an agonist for ERα and an antagonist for ERβ, respectively, only a few structure–activity relationship (SAR) studies of BPA have been reported,[29–32] and most of them did not evaluate ER-subtype selectivity.

We hypothesized that BPA, which has a diphenylmethane skeleton, would bind to the ligand-binding domain (LBD) of ERs in a similar manner to 17β-estradiol, and act as a steroid surrogate. Herein, we report SAR studies of BPA analogs by means of ERα and ERβ reporter gene assays, focusing on agonist/antagonist functional switching and subtype selectivity, in order to evaluate our hypothesis. One of the BPA analogs examined proved to be a potent and selective ERα-antagonist.

If BPA binds to the LBD of ERs, as many known NR ligands do, it should be a good lead compound for development of ER antagonists, because it has a simple structure so that synthesis of analogs should be straightforward. Further, it might be possible to utilize canonical SAR information available for numerous NR ligands, including structural requirements for switching from agonistic to antagonistic activity. Specifically, classical SARs for NRs indicate that introduction of bulky substituents leads to a switch from agonist to antagonist, because bulky substituents interfere with the folding of helix 12, which is important for agonistic activity. In addition, classical SARs for NRs also indicate that hydrophobicity

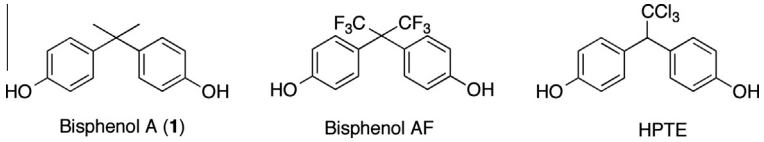
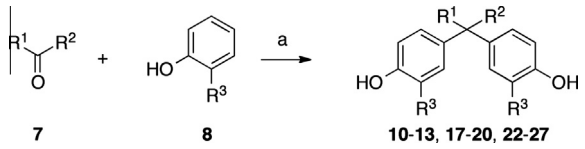


Figure 3. Chemical structures of bisphenols.



Scheme 1. Reagents and conditions: (a) MsOH, rt, 7–35%.

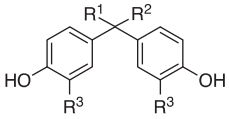
is important for strong affinity. Therefore, we designed a variety of BPA analogs in which these factors were modified.

Symmetrical BPA analogs bearing various alkyl chains were synthesized as shown in Scheme 1. Symmetrical ketones **7** were treated with phenols **8** in the presence of MsOH to generate BPA analogs **10**–**13**, **17**–**20** and **22**–**27**. Some BPA analogs, **1**, **9**, **14**–**16** and **21**, were commercially available. Tetramethyl analog **28** (Table 2) was synthesized from **7** and 2,6-dimethylphenol.

Synthesis of asymmetrical analogs is illustrated in Scheme 2. Reaction with methyl 4-hydroxybenzoate (**29**) and R^1MgBr yielded ternary alcohols **30**, which were reacted with *o*-cresol to give **31** and **32**. Aniline analog **33** was obtained from **17**.[7] Sila-analogs **36** and **37** were synthesized as shown in Scheme 3. 4-Bromo-2-methylphenol (**35**) was lithiated with *n*-BuLi, and then reacted with dialkyldichlorosilane to obtain **36** and **37**.

Agonistic and antagonistic activities of BPA analogs were evaluated by means of reporter gene assay using a Gal4-human ERα or ERβ reporter system.[33] Antagonistic activity was measured in the

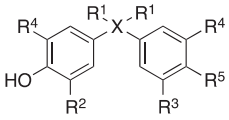
Table 1
Structure–activity relationships of bisphenol A analogs



Compound	R^1	R^2	R^3	ERα		ERβ	
				EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)
9	H	H	H	910	(35%)[a]	1900	(24%)[a]
1 (BPA)	Me	Me	H	(33%)[b]	(38%)[a]	1100	(25%)[a]
10	Et	Et	H	N.A.	86	(28%)[b]	93
11	*n*-Pr	*n*-Pr	H	N.A.	99	(43%)[b]	2200
12	*n*-Bu	*n*-Bu	H	N.A.	79	N.A.	180
13	$R^1,R^2 = -(CH_2)_4-$		H	N.A.	170	N.A.	270
14	$R^1,R^2 = -(CH_2)_5-$		H	N.A.	520	N.A.	560
15	H	H	Me	(23%)[b]	(26%)[a]	5500	N.A.
16	Me	Me	Me	(31%)[b]	1200	(19%)[b]	(25%)[a]
17	Et	Et	Me	N.A.	25	(15%)[b]	260
18	*n*-Pr	*n*-Pr	Me	N.A.	4.9	N.A.	140
19	*n*-Bu	*n*-Bu	Me	N.A.	14	N.A.	150
20	$R^1,R^2 = -(CH_2)_4-$		Me	N.A.	84	N.A.	770
21	$R^1,R^2 = -(CH_2)_5-$		Me	N.A.	230	N.A.	790
22	Et	Et	Et	N.A.	230	N.A.	1300
23	*n*-Pr	*n*-Pr	Et	N.A.	490	N.A.	2600
24	*n*-Bu	*n*-Bu	Et	N.A.	2900	N.A.	4500
25	Et	Et	*n*-Pr	N.A.	(17%)[a]	N.A.	(35%)[a]
26	*n*-Pr	*n*-Pr	*n*-Pr	N.A.	(38%)[a]	N.A.	(23%)[a]
27	*n*-Bu	*n*-Bu	*n*-Pr	N.A.	(15%)[a]	N.A.	(14%)[a]

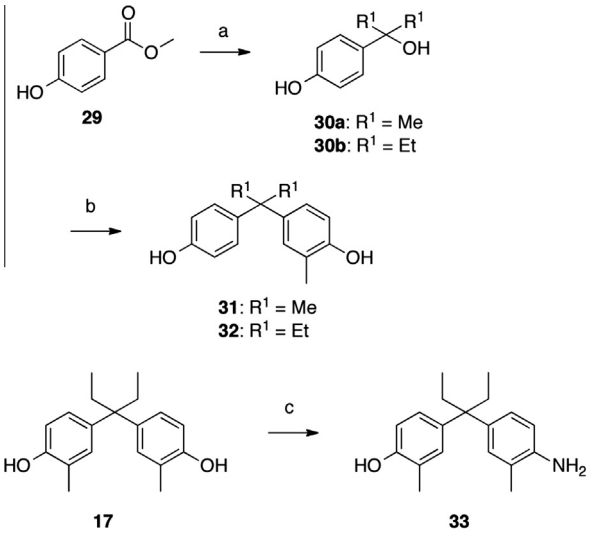
[a] % Inhibition at 3 μM.
[b] % To maximal activation of 17β-estradiol at 10 μM.

Table 2
Structure–activity relationships of bisphenol A analogs

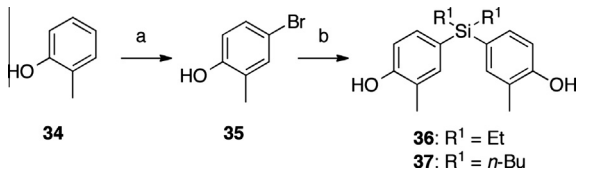


Compound	X	R^1	R^2	R^3	R^4	R^5	ERα		ERβ	
							EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)
1 (BPA)	C	Me	H	H	H	OH	(33%)[a]	(38%)[b]	1100	(25%)[b]
31	C	Me	Me	H	H	OH	(26%)[a]	(48%)[b]	(28%)[a]	(26%)[b]
16	C	Me	Me	Me	H	OH	(31%)[a]	1200	(19%)[a]	(25%)[b]
10	C	Et	H	H	H	OH	N.A.	86	(28%)[a]	93
32	C	Et	Me	H	H	OH	N.A.	110	(19%)[a]	380
17	C	Et	Me	Me	H	OH	N.A.	25	(15%)[a]	260
28	C	Et	Me	Me	Me	OH	N.A.	74	N.A.	300
33	C	Et	Me	Me	H	NH_2	N.A.	98	N.A.	43
36	Si	Et	Me	Me	H	OH	N.A.	72	N.A.	800
19	C	*n*-Bu	Me	Me	H	OH	N.A.	14	N.A.	150
37	Si	*n*-Bu	Me	Me	H	OH	N.A.	(30%)[b]	N.A.	N.A.

[a] % To maximal activation of 17β-estradiol at 10 μM.
[b] % Inhibition at 3 μM.



Scheme 2. Reagents and conditions: (a) R^1MgBr, THF, 0 °C to rt, 66–84%; (b) *o*-cresol, H_2SO_4, 0 °C to rt, 16–18%; (c) *o*-toluidine–HCl, neat, 180 °C, 58%.



Scheme 3. Reagents and conditions: (a) NBS, CH_3CN, 0 °C to rt, 63%; (b) *n*-BuLi, $(R^1)_2SiCl_2$, THF, −78 °C to rt, 2–39%.

presence of 0.5 nM 17β-estradiol. Under these assay conditions, BPA (**1**) showed weak agonistic and antagonistic activities towards ERα at 3 μM, indicating that it is a weak ERα partial agonist under our assay conditions. With ERβ, BPA (**1**) showed agonistic activity with the EC_{50} value of 1100 nM. First, we investigated SARs regarding the central propyl group, as shown in Table 1. Removal of two methyl groups (**9**) increased the ERα-agonistic activity (EC_{50} 910 nM), with retention of the ERβ-agonistic activity, so **9** was an ERα and ERβ dual agonist. Introduction of longer linear alkyl chains at the central carbon (**10**–**12**) decreased the agonistic activities for both ERα and ERβ, but increased the antagonistic activities for both ERα and ERβ, compared with **1**. For example, compound **10** possessing two ethyl groups showed decreased agonistic activity for both ERs, but exhibited both ERα- and ERβ-antagonistic activities with the IC_{50} values of 86 and 93 nM, respectively. These results were consistent with expectation, that is, increase of bulkiness by introduction of longer alkyl chains at the central carbon led to a switch from agonist to antagonist. Cyclic alkyl analogs **13** and **14** also showed ERα/ERβ dual antagonistic activities.

Next, we investigated the effect of introduction of two methyl groups at the 3- and 3′-positions because all representative steroid surrogates with a diphenylmethane skeleton (Fig. 1) possess two methyl groups at the 3- and 3′-positions. The dimethyl analog of BPA **16** showed an increase of ERα-antagonistic activity and a decrease of ERβ-agonistic activity. In particular, dimethyl analogs with longer alkyl chains at R^1 and R^2 (**17**–**19**) showed increased ERα-antagonistic activity. Among them, *n*-Pr analog **18** showed potent ERα-antagonistic activity with an IC_{50} value of 4.9 nM, affording 28-fold selectivity over ERβ. Cyclic alkyl analogs **20** and **21** exhibited weaker antagonistic activity than the linear alkyl analogs **17**–**19**. These SARs regarding dimethyl analogs prompted us to synthesize alkyl analogs bearing Et or *n*-Pr groups at the 3- and 3′-positions. Indeed, the length of the alkyl chains at these positions was important for ERs-antagonistic activities, that is, the order of potency of antagonistic activities was: Me (**15**–**21**) > H (**9**–**14**) > Et (**22**–**24**) > *n*-Pr (**25**–**27**). Asymmetric mono-methyl analogs **31** and **32** showed lower activity than the corresponding dimethyl analogs **16** and **17**, respectively. Tetramethyl analog **28** also showed lower activities than the dimethyl analog **17**. These results indicated that dimethyl analogs are the most suitable to obtain potent antagonistic activity. Aniline **33** was distinctive among the compounds reported here, that is, it showed twofold ERβ-selective antagonistic activity.

As mentioned above, central alkyl chains were critical for potent ERs antagonistic activities, indicating that the hydrophobicity at the central moiety is important for strong antagonistic activity. On the other hand, sila-substitution (C/Si exchange) of existing drugs is an attractive approach to find new drug candidates with a clear intellectual property position: for example, silicon-containing analogues are more lipophilic and larger in molecular size than their carbon analogues.[34–37] Therefore, we designed sila-analogs **36** and **37** with the aim of increasing the hydrophobicity or molecular size. However, both sila-analogs **36** and **37** showed decreased activities compared with carbon derivatives **17** and **19**, respectively.

Overall, we identified several important SARs, and discovered the *n*-Pr analog **18**, which possessed the most potent ERα-antagonistic activity (IC_{50} 4.9 nM) and the greatest selectivity for ERα over ERβ (28-fold) among the compounds examined.[38]

X-Ray crystal structures of two complexes, that is, a complex consisting of ERα LBD mutant (Y537S) and BPA, and a complex consisting of wild-type ERα LBD and bisphenol C (BPC), have recently been reported.[39] BPC is reported to act as an ER antagonist.[39] Both BPA and BPC bind to the LBD, as 17β-estradiol does (Fig. 4). A common feature of BPA and BPC is the hydrogen-bonding interaction between their phenol moieties and amino acid residues E353 and R394. However, there are differences in the binding modes of the two complexes, that is, (1) one of the phenol rings of BPC is positioned in an alternative pocket, different from that into which the corresponding ring of BPA binds, and (2) the chlorine atoms in BPC are directed toward M421, which forms a hydrogen bond with the phenol moiety in the case of BPA. As a result, the structures of ERα bound with BPA and 17β-estradiol display the canonical active conformation with helix 12 capping the ligand-binding pocket and the steroid receptor coactivator-1 (SRC-1) peptide bound to the transcriptional activation function (AF-1) surface, whereas the structure of ERα with BPC displays an antagonist conformation similar to that observed in the hydroxytamoxifen-bound structure, with helix 12 occupying the coactivator binding groove. To examine the binding mode of the most potent and selective antagonist **18**, this molecule was computationally docked into ERα (PDB ID: 3UUC) using AutoDock 4.2. The docking model of the complex of **18** with the active site of ERα shows the hydrogen-bonding interaction with the phenol and E353, as seen in the X-ray crystal structures of BPA and BPC. The most noteworthy result of the docking simulation was that the bulky *n*-Pr chains at the central carbon are directed toward M421, like the chlorine atoms of BPC. These X-ray crystal data and the docking simulation are consistent with our hypothesis that the diphenylmethane skeleton of BPA acts as a steroid surrogate, and introduction of bulky substituents causes a switch from agonist to antagonist. The precise molecular mechanism of the selectivity of **18** for ERα over ERβ is unclear. However, there is a a difference in amino acid sequences between ERα and ERβ in the region of the ligand-binding pocket, that is, M421 in ERα corresponds to I328 in ERβ. Because M421 is a key amino acid for the interaction with ERα ligands, this difference in amino acid sequence between ERα and ERβ might result in different interactions with **18**, possibly via changes in the size or electrostatic char-

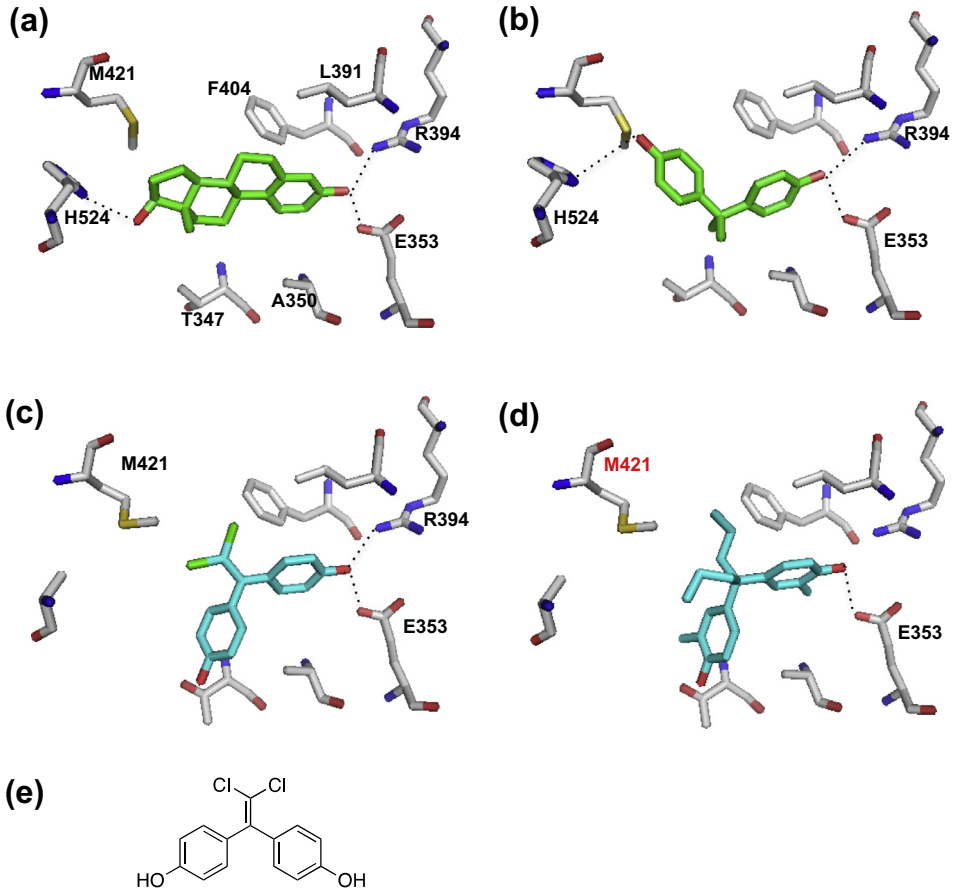


Figure 4. (a) X-ray crystal structure of ERα with 17β-estradiol (PDB ID: 3UUD); (b) X-ray crystal structure of ERα with BPA (PDB ID: 3UU7); (c) X-ray crystal structure of ERα with BPC (PDB ID: 3UUC); (d) docking simulation of ERα LBD and **18**; (e) chemical structure of BPC.

acter of the pocket occupied by the *n*-propyl groups, leading in turn to increased selectivity for ERβ.

Next, **16** and **17** were also docked into ERα (PDB ID: 3UUC) to explain the SARs of liner alkyl groups at the central carbon. A common feature of **16** and **17** was the direction of the liner alkyl groups, that is, the methyl groups or ethyl groups at the central carbon were directed toward M421, like the *n*-Pr groups of **18**. However, lowest binding energy derived from these docking studies was different, and it is noteworthy that the rank order of lowest binding energy (**18** < **17** < **16**) was corresponded to the order of ERα IC_{50} (**18** < **17** < **16**). Therefore, this docking study supported the SARs, that is, the length of the liner alkyl groups at the central carbon was very critical for ERα-antagonistic activity.

In 2003, BPA was reported to possess anti-androgen activity as well.[40] Therefore, we checked the AR-antagonistic activity of representative compounds by means of AR reporter gene assay using the pSG5-human AR reporter system.[37] Antagonistic activity was measured in the presence of 0.5 nM dihydrotestosterone, an AR agonist. BPA showed anti-androgen activity under our assay conditions with an IC_{50} value of 5900 nM (Table 3). Under this condition, **18** showed weaker AR-antagonistic activity (IC_{50} value of 25,000 nM) than BPA. Thus, **18** showed 28-fold and 5000-fold selectivity as an antagonist for ERα over ERβ and AR, respectively. These results indicated that lead compounds obtained via the multi-template approach can indeed be structurally modified to generate target-selective compounds. Other representative compounds **17**, **33**, **36** and **37** showed weak AR-antagonistic activity.

We believe that the multi-template approach for drug discovery, focusing on proteins with similar fold structures, is effective for generating lead compounds for multiple targets. Inspired by our previous studies on generation of steroid surrogates bearing a diphenylmethane skeleton, we speculated that the diphenylmethane derivative BPA would bind to the ligand binding domain of ERs like 17β-estradiol, acting as a steroid surrogate. Clear SARs for BPA was obtained, that is, introduction of alkyl chains at the central carbon atom led to a switch from agonist to antagonist, and introduction of two methyl groups at the 3- and 3′-positions increased the antagonistic activity and selectivity for ERα over ERβ. These structural developments of BPA resulted in the discovery of **18** as a potent and selective ERα-antagonist under our assay conditions. Although BPA is also reported to possess anti-androgen activity, **18** showed also showed high selectivity for ERα over AR, as compared with BPA. Docking studies suggested that **18** would bind to the antagonistic conformation of ERα. These results further confirm the versatility of the multi-template approach and the utility of the diphenylmethane skeleton. Based on its ERα-selective antagonistic activity, **18** is considered to be a candidate agent for treatment of breast cancer.

Table 3
AR-antagonistic activity of bisphenol A analogs

Compound	AR IC_{50} (nM)
1 (BPA)	5900
18	25,000
17	4500
33	5400
36	1900
37	8200

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