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## Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of BPA substitutes

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**Keywords:** bisphenol A, endocrine disruptor, estrogen receptor, nuclear receptor, crystal structure.

## Abstract

Bisphenol A (BPA) is an industrial compound and a well-known endocrine-disrupting chemical (EDC) with estrogenic activity. The widespread exposure of individuals to BPA is suspected to affect a variety of physiological functions including reproduction, development and metabolism. Here we report that the mechanisms by which BPA and two congeners, BPAF and BPC, bind to and activate estrogen receptors ( $ER\alpha$  and  $ER\beta$ ) differ from that used by estradiol ( $E_2$ ). We show that bisphenols act as partial agonists of ERs by activating the N-terminal activation function 1 (AF-1) whatever their effect on the C-terminal activation function 2 (AF-2) that ranges from weak agonism (BPA) to antagonism (BPC). Crystallographic analysis of the interaction between bisphenols and ERs reveals two discrete binding modes reflecting the different activities of compounds on ERs. BPA and  $E_2$  bind to ERs in a similar fashion, whereas, with a phenol ring pointing towards the activation helix H12, the orientation of BPC accounts for the marked antagonist character of this compound. Based on structural data, we developed a protocol for *in silico* evaluation of the interaction between bisphenols and ERs or other members of the nuclear hormone receptor (NR) family such as ERR $\gamma$  and AR which are two known main targets of bisphenols. Overall, this study provides a wealth of tools and information which could be used for the development of BPA substitutes devoid of NR-mediated activity and more generally for environmental risk assessment.

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## **Introduction**

Bisphenols form a large family of chemicals which are commonly used in the manufacture of numerous consumer products. By far, the most widely used bisphenol (> 3 million tons/year) is bisphenol A (BPA; 4-[2-(4-hydroxyphenyl)propan-2-yl]phenol) which is utilized in the manufacture of items such as plastics, food can linings, dentistry sealants or thermal paper. Many other bisphenols are used in a variety of industrial applications as for example the BPAF (4-[1,1,1,3,3,3-hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol) in the fabrication of electronic materials, gas-permeable membranes and plastic optical fibres or the BPC (4-[2,2-dichloro-1-(4-hydroxyphenyl)ethenyl]phenol) in the manufacture of fire-resistant polymers (Fig. 1A). Several studies have shown that BPA is released from consumer products leading to its detection in food, drinking water, wastewater, air and dust (1). Others studies have identified BPA in human serum, urine, adipose and placental tissues, and umbilical cord blood (2, 3). The major source of consumer exposure is likely to be through food and drinks in contact with BPA-containing materials (1) although a recent study has shown that BPA can be also absorbed by the skin (4). Finally, BPA is a significant contaminant of wastewater and biosolids from sewage treatment plants which may affect wildlife at environmentally relevant concentrations (5). BPA has been shown to produce a range of adverse effects in laboratory animals, with major concerns regarding reproductive targets and embryonic development (6-8). More recently it has been hypothesized that early exposure to BPA could also play a role in the onset of obesity and other metabolic syndromes (9). In this regard, a large body of data about endocrine disrupting chemicals (EDCs) underlines the importance of exposure during early stages of development, which could result in numerous biological defects in adult life (10).

The molecular basis behind the deleterious effects of BPA is poorly understood and a large controversy has been created within the field of endocrine disruption about the low doses effects and the possible consequences of such exposures (11, 12). Although the two estrogen receptors (ER $\alpha$

and ER $\beta$ ) are considered as the main targets of BPA (13, 14), several other cellular targets have been proposed for this compound. We and others have previously demonstrated that BPA or its halogenated derivatives also activate the pregnane X receptor (PXR) (15, 16), the estrogen related receptor  $\gamma$  (ERR $\gamma$ ) (17), or the peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) (18, 19) and inhibits the androgen receptor (AR) (20) or the thyroid hormone receptor (TR) (21, 22). BPA has also been reported to interact with the G protein-coupled estrogen receptor (GPR30) (23) so that the net effect of BPA could be due to synergistic actions through different pathways.

ER $\alpha$  and ER $\beta$  are members of the nuclear hormone receptor (NR) family acting as ligand-inducible transcription factors (24, 25). Their activity is regulated by 17 $\beta$ -estradiol (E<sub>2</sub>) which plays important roles in the growth and maintenance of a diverse range of tissues such as the mammary gland, uterus, bone, the cardiovascular and the central nervous systems. The interaction of E<sub>2</sub> with ERs initiates a series of molecular events including the recruitment of members of the steroid receptor coactivator (SRC) family that culminate in the transcription of target genes (26). Like other members of this family, ERs contain three major functional domains, including a N-terminal A/B domain that harbors a transcriptional activation function (AF-1), a DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) hosting a ligand-dependent transcriptional activation function (AF-2). The LBD is crucially involved in most of the receptor functions because of its capacity of hormone binding, dimerization and interaction with coregulatory complexes. The LBD also contributes to the modulation of the N-terminal AF-1 through interdomain crosstalk so that both AF-1 and AF-2 domains can recruit a range of coregulatory proteins and act individually or in a synergistic manner (27, 28). The precise structural basis of this interdomain communication is unknown as no three-dimensional structure of an entire NR has been obtained.

In contrast, many crystal structures of ER LBDs have been determined in complex with natural and synthetic ligands revealing a conserved core of 12  $\alpha$ -helices (H1 to H12) arranged into a three-layered sandwich fold (29-31). This arrangement generates a mostly hydrophobic cavity in the lower

half of the domain to which hydrophobic ligands bind. In all hormone-bound structures, the ligand-binding cavity is sealed by the C-terminal helix H12. This conformation is specifically induced by the binding of hormones or synthetic agonists and is referred to as the “active conformation” because it favors the recruitment of coactivators to the so-called “AF-2 surface”. This surface formed by helices H3, H4 and H12 defines a hydrophobic binding groove for short LxxLL helical motifs found in coactivators.

In the following, we report on a study in which we combine biochemical, biophysical, structural and cell-based assays to provide insights as to how BPA and two derivatives, BPAF and BPC, bind to and activate ERs. Based on these data, we have built a computational tool to predict the ER binding and activation properties of bisphenols and further extended this bioinformatic approach to ERR $\gamma$  and AR which are two known main targets of bisphenols.

## Results and Discussion

**Bisphenols are Partial Activators of ERs and Potent Inducers of Cell Proliferation.** The agonistic potential of bisphenols (Fig. 1A) was monitored on ER $\alpha$  transcriptional activity and cell proliferation using breast cancer ER $\alpha$  positive MCF-7 reporter cells (MELN) (32). In these cells, bisphenols exert a partial potency on luciferase reporter activity (Fig. 1B) but act as full ER $\alpha$  agonists on cell growth (Fig. 1C). We then monitored the effect of bisphenols on ERs transcriptional activity using HeLa reporter cells stably expressing human ER $\alpha$  and ER $\beta$  (HELN ER $\alpha$  and ER $\beta$ ) (32) allowing for a direct comparison of the effect of compounds on the two ER subtypes in a similar cellular context. As shown in Fig. 1D, bisphenols exhibit almost similar activation capabilities of ER $\alpha$ , inducing 60-70% of the transactivation seen with E $_2$  (*SI Appendix*, Fig. S1). In contrast, the activation curves obtained with ER $\beta$  show different profiles with BPA being the most potent (80% activity) and BPC inducing only a 35% activity (Fig. 1E). Accordingly, bisphenols act as partial antagonists in the presence of E $_2$  (*SI Appendix*, Fig. S2). Transactivation assays (Fig. 1D and E) suggested that BPAF and BPC bind more avidly to both receptors than BPA. This observation was validated by competitive binding assays with

[<sup>3</sup>H]-E<sub>2</sub> (*SI Appendix*, Fig. S3). Together, these experiments show that bisphenols can be considered as Selective Estrogen Receptor Modulators (SERMs) (24) which activate partially luciferase reporter in MCF-7 and HeLa cells while being fully active on MCF-7 cell proliferation.

**The Activation Function 1 of ERs is Indispensable for Bisphenol Activity.** Having characterized the estrogenic potential of BPA, BPAF and BPC, we performed additional cell-based experiments aimed at assessing the relative contribution of ERs AF-1 and AF-2 to this activity. We first examined the agonistic properties of bisphenols using HELN cells stably transfected with ERs deleted of their N-terminal AB (AF-1) region ( $\Delta$ AB-ER $\alpha$  and  $\Delta$ AB-ER $\beta$ ) (32). Interestingly, deletion of the AB-domain strongly reduces the bisphenols-induced transcriptional activity of ER $\alpha$  and ER $\beta$  (Fig. 1D and E) so that in the presence of E<sub>2</sub>, BPC displays an almost full antagonistic activity (*SI Appendix*, Fig. S2). Next, we examined if the same phenomenon could be observed in a cellular response. For this purpose, we used the HELN ER cell lines whose proliferation is known to decrease upon E<sub>2</sub> treatment (32). In agreement with transcription data, bisphenols inhibit the proliferation of HELN ER cells as efficiently as E<sub>2</sub> (*SI Appendix*, Fig. S4A and B) but display a very weak efficacy in HELN  $\Delta$ AB-ERs cells (*SI Appendix*, Fig. S4C and D). This is in contrast with E<sub>2</sub> whose inhibition properties remain unaffected upon deletion of the ER AB-domains (*SI Appendix*, Fig. S4). The partial agonism of bisphenols is also observed in HeLa cells transiently transfected with another E<sub>2</sub>-regulated gene (pS2 promoter-luciferase) (*SI Appendix*, Fig. S5), as well as on the expression of the ER target gene GREB1 in HELN ERs cells (*SI Appendix*, Fig.S6). In HeLa cells, bisphenols clearly act as partial agonists compared to E<sub>2</sub>. In contrast in MCF-7 cells, expression of GREB1 and other endogenous E<sub>2</sub> regulated genes (pS2, RIP140 and progesterone receptor) is fully activated by bisphenols (*SI Appendix*, Fig. S7). These data reveal that bisphenols act as SERMs whose activity relies mostly on the AF-1 and depends on the cellular context. The ranking order of potency, with BPA>BPAF>BPC (Fig. 1D and E), likely originates from the differential synergy between AF-1 and AF-2 created by the various bisphenols.

**Bisphenols Render H12 Highly Dynamic and Disable the Activation Function 2.** To further characterize the capacity of bisphenols to induce the recruitment of coactivators to the AF-2 surface of ER $\alpha$ , we studied their effects on the interaction of the fluorescein-labeled NR box2 peptide of SRC-1 (SRC-1 NR2) with ER $\alpha$  LBD by fluorescence anisotropy (Fig. 2A). In keeping with their respective agonistic or antagonistic activities, E<sub>2</sub> and 4-hydroxytamoxifen (OHT) respectively strongly enhance and decrease the binding affinity of SRC-1 NR2 (apparent K<sub>d</sub> values are 0.07 ± 0.01 μM, 4.95 ± 1.12 μM and > 10 μM for the E<sub>2</sub>-, apo- and OHT-ER $\alpha$  LBDs, respectively). In contrast, we observed a weaker impact of bisphenols on the interaction with the coactivator-derived peptide and a progressive transition from weak agonist (BPA; K<sub>d</sub> = 1.61 ± 0.57 μM) to antagonist (BPC; K<sub>d</sub> > 8 μM).

As previously reported with RXR (33), we used fluorescence anisotropy measurements of a fluorescein moiety attached to the C-terminus of ER $\alpha$  LBD to monitor the effect of bisphenols on H12 dynamics. We showed that anisotropy is strongly enhanced upon addition of E<sub>2</sub>, reflecting the stabilization of ER $\alpha$  H12 in the active conformation (Fig. 2B). In contrast, binding of bisphenols or OHT slightly (BPA and BPAF) or markedly (BPC and OHT) decreases anisotropy, revealing a higher mobility of H12 in the presence of these compounds. These data fully support the above results and suggest that bisphenols fail to efficiently stabilize the active receptor conformation implying that they may act as weak AF-2 agonists or antagonists. To unambiguously characterize the functional profile of bisphenols, we monitored H12 dynamics in the various bisphenol-bound ER $\alpha$  complexes and in the presence of increasing concentrations of unlabeled SRC-1 NR2. Interestingly, addition of SRC-1 NR2 caused a clear dose dependent anisotropy increase of the ER $\alpha$  LBD bound to BPA and BPAF, indicating that peptide binding helps reducing H12 mobility by shifting the equilibrium towards the active conformation (Fig. 2C). In contrast, even high doses of SRC-1 NR2 failed to stabilize H12 in the presence of BPC or OHT, supporting the notion that, like OHT, BPC acts as an AF-2 antagonist preventing coactivator binding to ER $\alpha$  LBD. Taken as a whole, these data support the above cell-based assays and reveal that bisphenols fail to efficiently stabilize the proper LBD interaction surface

with coactivators. However, the graded effect of these compounds on H12 dynamics accounts for their differential impact on coactivator recruitment. BPA and BPAF allow some interaction, provided that coactivators are present in sufficient amount in the cellular environment, whereas BPC permanently prevents any interaction of ER $\alpha$  LBD with coactivators.

**Bisphenols Interact with ER *via* Two Binding Modes.** In an attempt to gain structural insight into the binding mode of BPA to ERs, we subjected the wild-type ER $\alpha$  LBD complexed with BPA to crystallization assays. After several rounds of unsuccessful trials, we used the recently reported ER $\alpha$  LBD mutant (Y537S) which has been shown to stabilize the agonist-bound conformation of ER $\alpha$  and in turn facilitate crystallization of weak agonists (34). To ensure that this mutation at the surface of the protein will not compromise the accuracy of our structural analysis, we cocrystallized ER $\alpha$ -Y537S LBD with E<sub>2</sub> and SRC-1 NR2. Comparison of the obtained structure with that of the corresponding wild-type receptor (PDB code 1GWR) indicated a very high degree of similarity both in the overall structure (r.m.s. deviation of 0.48 Å for 230 backbone atoms) and in the details of the protein – ligand interactions (*SI Appendix*, Fig. S8A and B). These data complement other comparisons that were made earlier with a non-steroidal ligand (34, 35). Additional characterization of the Y537S mutant *via* transient transfection of HeLa cells, Thermofluor<sup>®</sup> and fluorescence anisotropy indicated that the mutation stabilizes the active conformation of the receptor without modifying the relative potencies of compounds (*SI Appendix*, Fig. S9 and S10). Prior comparative studies on the ER $\alpha$ -Y537S mutant established that it had somewhat elevated affinity for E<sub>2</sub> (36).

Subsequently, we crystallized ER $\alpha$ -Y537S LBD in complex with BPA or BPAF. Owing most likely to the stronger antagonistic character of BPC, crystals with this compound could be obtained using the wild-type construct but not with the H12-stabilized ER $\alpha$  mutant. Details of structure determination and refinement are summarized in *SI Appendix* Table S1. The structures with BPA and BPAF display the canonical active conformation with H12 capping the ligand binding pocket (LBP) and the SRC-1 peptide bound to the AF-2 surface (Fig. 3A). In agreement with the above functional data,

the structure with BPC displays an antagonist conformation similar to that observed in the OHT-bound structure (PDB code 3ERT) with H12 occupying the coactivator binding groove (Fig. 3A). All compounds could be precisely placed in their respective electron density (Fig. 3B to F), revealing two discrete orientations of the bisphenols in the LBP. As shown in Fig. 3C, BPA adopts a binding mode reminiscent of that used by E<sub>2</sub> (Fig. 3B) with the two phenol groups hydrogen bonded to three polar residues located at the two ends of the pocket, namely H524 (H11) on one side and E353 (H3) and R394 (H5) on the other side. The remaining contacts involve 51 van der Waals interactions (4.2 Å cut-off) in the E<sub>2</sub> complex but only 42 in the complex with BPA, this difference accounting, at least in part, for the weaker affinity of the bisphenol for ERs. In the complex with BPC, the ligand is positioned in the pocket so as to draw the phenol ring B into an alternate position as compared with that of the corresponding ring in BPA. A rotation by 180° around the main axis of phenol ring A, which remains anchored to E353 and R394, orients ring B towards H12 (Fig. 3C and F). A molecular modeling approach reveals that the “BPA-like” mode of binding would position one of the two chlorine atoms of BPC in very close proximity of A350 in helix H3 (*SI Appendix*, Fig. S11), thus explaining the “antagonist orientation” adopted by BPC. Finally, BPAF displays an intermediate situation with each subunit of the ER $\alpha$  homodimer containing one BPAF molecule with the “agonist, BPA-like” or “antagonist, BPC-like” positioning (Fig. 3D and E). The observation that two distinct orientations of BPAF are found in each monomer rather than occurring randomly is intriguing and suggests the existence of a regulatory crosstalk between the two subunits where, as recently reported, ligand and/or coregulator binding to one monomer can affect ligand and coregulator binding to the second monomer of a dimer (37). However, such a situation is not observed in the E<sub>2</sub> or BPA complexes which yet crystallize in the same crystal form as that with BPAF and are therefore engaged in similar packing contacts. This apparent discrepancy could indicate that there are some chemical requirements for a ligand to promote such allosteric regulation, thereby providing new perspectives for drug design.

**Key Contacts are Missing in the ER – Bisphenol Complexes.** We next considered how the two binding modes of bisphenols may contribute to the destabilization of the AF-2 surface. By comparing our four structures, we observed that previously recognized ligand – H11 stabilizing interactions observed in the E<sub>2</sub>-bound structure are altered in the bisphenol-containing complexes (34, 35). For example, E<sub>2</sub> makes an important stabilizing interaction with G521 (3.95 Å) which is severely weakened in the structures with bisphenols in the “BPA-like” conformation (4.65 Å) or completely abolished in the structures with bisphenols in the “BPC-like” conformation (> 5.20 Å) (Fig. 4A). We also noticed significant differences in the geometry of the interaction between H524 and the hydroxyl moieties of E<sub>2</sub> or bisphenols in the “BPA-like” orientation (Fig. 4A) and an absence of interaction of this residue with bisphenols in the “BPC-like” orientation (Fig. 4A). These suboptimal or complete lack of interactions induce a substantial reorientation of the H524 imidazole ring which, as previously reported by Nettles et al. 2008, perturbs a key hydrogen bond network involving residues from loop L6-7, H3 and H11. Last but not least, we observed that in the E<sub>2</sub>-bound structure, the 18-methyl group of E<sub>2</sub> is in contact with L525 and imposes a conformation that strengthens a network of van der Waals interactions involving T347, L525 and L536 (Fig. 4B). This cluster of interactions is of utmost importance because it holds together helices H3, H11 and the loop preceding H12, thereby stabilizing the AF-2 surface. In the structure with BPA, the stabilizing interaction between the bisphenol and L525 is absent. This renders L525 more dynamic so that its side chain adopts different conformations in the two subunits of the homodimer (Fig. 4C). The situation is even worse in the structure with BPC where the side chain of T347 rotates by 180° to form a hydrogen bond with the hydroxyl moiety from the phenol ring B of BPC (Fig. 4D). The complete disruption of the hydrophobic cluster by BPC probably accounts for the marked antagonistic character of this bisphenol. This massive loss of stabilizing contacts provoked by bisphenols renders the lower part of the ERα LBP more dynamic (Fig. 4E). Indeed, this portion of LBDs has been previously shown to have some of the characteristics of a molten globule and a large part of the agonistic properties of a ligand relies in its

capacity to stabilize this region encompassing the H3/H11 docking surface for H12 in the active conformation (38, 39). These findings reveal the mechanisms by which bisphenols interact with ER $\alpha$  and highlight how key secondary structural elements sense and allosterically convey ligand activities to the AF-2 surface through modifications of H12 positioning and/or dynamics. In this respect, it is noteworthy that the structural effects observed in the background of a H12-stabilized ER $\alpha$  mutant and in a crystalline context would be more pronounced with the wild-type receptor in solution as indicated by fluorescence anisotropy data (Fig. 2).

**Focused Virtual Screening of Bisphenols on NRs.** Having characterized the interaction of three bisphenols with ERs both at the functional and structural levels, we reasoned that this information could aid in the development of a computational tool to predict binding of any bisphenol to this receptor and the induced functional outcome. We took advantage of our server @TOME-2 (40) to select optimal ER $\alpha$  conformations for virtual screening. First, the various ER $\alpha$  crystal structures available in the Protein Data Bank (PDB) were partitioned into two groups according to their agonist or antagonist conformation. Within each group, we performed so-called “comparative docking” by which each ligand contained in a particular structure is transferred into the other structures of this group through protein-protein superimposition. This cross-docking allows exploring both a wide range of binding site conformations and ligand orientations, and builds up an array of optimal shape restraints to focus virtual screening. Implementation of an interface between the server @TOME-2 and the docking program PLANTS (41) allowed virtual screening of bisphenols using the complexes described above as anchoring models. Binding affinities were evaluated using several scoring functions including the recently developed DSX (42).

In our test case, we observed that BPAF adopts the two alternative BPA-like and BPC-like orientations in both the agonist and antagonist groups of ER $\alpha$  conformations, whereas BPC docks mostly in the BPC-like orientation whatever the conformation screened (results can be found at <http://atome.cbs.cnrs.fr/EDCNR.html>). These results are in full agreement with crystallographic data

(*SI Appendix*, Fig. S12A). In the case of BPA, the server predicted that it could adopt the two orientations (with however a majority of the poses in the BPA-like conformation), whereas the corresponding crystal structure shows only one orientation of the ligand (Fig. 3C). Note that these results were obtained before deposition of the various bisphenol-bound ER $\alpha$  structures to the PDB. The rough affinity predictions of BPA, BPAF and BPC for ER $\alpha$  nicely matched the experimental ones (<http://atome.cbs.cnrs.fr/EDCNR.html> and Fig. 3). Subsequently, we applied this screening approach to other bisphenols and found that the ranking order of affinity with BPAF, BPC, BPB>BPA, BPE, BPF>BPS agrees well with that obtained experimentally (*SI Appendix*, Fig. S13).

Then, we extended further this *in silico* approach to ER $\beta$ , AR and ERR $\gamma$  which are also known targets of bisphenols. Predictions indicated binding modes similar to those found in ER $\alpha$  (*SI Appendix*, Fig. S12B to D and <http://atome.cbs.cnrs.fr/EDCNR.html>). Interestingly, all studied bisphenols appeared to bind to AR exclusively in the antagonist BPC-like orientation, in agreement with the observation that these compounds act as AR antagonists (20) (*SI Appendix*, Fig. S14). This orientation appears to be stabilized by formation of a hydrogen bond between one hydroxyl group of the ligand and N705, a polar residue specific of AR (*SI Appendix*, Fig. S12C). This situation is mirrored with ERR $\gamma$  in which all bisphenols studied adopt the agonist BPA-like position due to a hydrogen bond with N346 from helix H7 (*SI Appendix*, Fig. S12D). This *in silico* result correlates both with functional data showing that bisphenols are ERR $\gamma$  activators (*SI Appendix*, Fig. S15) and the crystal structure of ERR $\gamma$  in complex with BPA (*SI Appendix*, Fig. S8C) (43, 44). Therefore, it appears that most bisphenols are rather weak binders ( $\mu$ molar range) of several NRs and that their binding mode varies according to their chemical structure as well as the receptor under scrutiny.

### **Concluding Remarks**

Deregulation of NR-mediated transcription accounts for the deleterious effects of many EDCs. Thus, characterization of the harmful interaction between receptors and environmental compounds both at the structural and functional levels, as well as the development of robust *in vivo*, *in vitro* and *in*

*silico* screening methods are important for assessment of the toxic potential of large numbers of chemicals. In addition, because of mounting restrictions on the use of many synthetic chemicals used in consumer products (e.g. BPA), especially in the European Union, Canada or the United States, there is a huge demand for alternative safer substitutes for industrial applications.

In this context, using complementary approaches we have dissected the mechanisms by which an important class of environmental endocrine disruptors interferes with ER signaling. We have found that bisphenols are SERMs that function in a cell- and tissue-selective manner (24). As a consequence, bisphenols might exert E<sub>2</sub>-like activities in some tissues but not in others, implying that cell, tissue or animal models used for assessing the risk to human health should be cautiously designed and the results carefully interpreted. Most of the methods employed in this study, including fluorescence anisotropy, thermal denaturation shift and cell-based assays have been implemented in a medium-throughput setting allowing for rapid assessment of the endocrine-disruptive potential of large numbers of EDCs. On the other hand, utilization of the previously described H12-stabilized ER $\alpha$  mutant (34) facilitating EDC-bound ER LBD crystallization will permit a rapid increase of our knowledge of the structural mechanisms and molecular interactions used by ERs and a wide range of structurally and chemically diverse compounds. To add to the tool box, we have developed a 3D structure-based computational method whose aim is to help evaluation of the interference of EDCs with hormonal signaling. Using this tool we have been able to discern with a high level of accuracy the docking modes of bisphenols in four different NRs, thereby allowing for the prediction of their activity profiles, and the ranges of binding affinities of these compounds. While currently restricted to virtual screening of bisphenols on four human NRs, future developments of the server will allow dealing with (i) other EDC families, (ii) an extended set of NRs, and (iii) other species including mouse, zebrafish and xenope. We believe that the structural insights gained at a near atomic resolution, together with the experimental and computational tools developed in this study, could facilitate

evaluation of the EDC activity of chemicals and aid in the design of novel compounds with the promise to separate their industrial characteristics from their unwanted toxic effects.

## **Materials and Methods**

**Reporter cell lines and culture conditions.** Luciferase, cell proliferation and whole-cell ER competitive binding assays have been performed using the stably transfected luciferase reporter MELN, HELN-ER $\alpha$ , -ER $\beta$ , - $\Delta$ AB-ER $\alpha$  and - $\Delta$ AB-ER $\beta$  cell lines as described in (32).

**Structure determination.** The ER $\alpha$  LBD and ER $\alpha$ -Y537S LBD mutant were cloned into the pET-32a vector and expressed in BL21(DE3) cells. Protein domains were purified using nickel affinity column and size exclusion chromatography. The purified ER $\alpha$ -Y537S LBD was mixed with E<sub>2</sub>, BPA or BPAF and SRC-1 NR2, and ER $\alpha$  LBD was mixed with BPC. All complexes were crystallized using the vapor diffusion method. Data were collected on the ID14-1, ID23-2 or ID29 beamlines at the ESRF, Grenoble, France. Data were processed as described in the *SI Appendix* Materials and Methods.

**Fluorescence anisotropy measurements.** H12 dynamics was monitored using the fluorescein-labeled ER $\alpha$  LBD prepared following the previously described protocol (45). Assays were performed using a Safire<sup>2</sup> microplate reader (TECAN) at a protein concentration of 0.140  $\mu$ M. The excitation wavelength was set at 470 nm, with emission measured at 530 nm. SRC-1 NR2 was added to protein samples containing 5  $\mu$ M of ligand to a final concentration of 10  $\mu$ M. Then, samples were diluted successively with 20 mM Tris pH 8.0, 180 mM NaCl, 5 mM DTT, and 10% glycerol supplemented with 0.140  $\mu$ M of protein and 5  $\mu$ M of ligand. Details of the experimental procedures and associated references are given in the *SI Appendix* Materials and Methods.

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## Figure legends

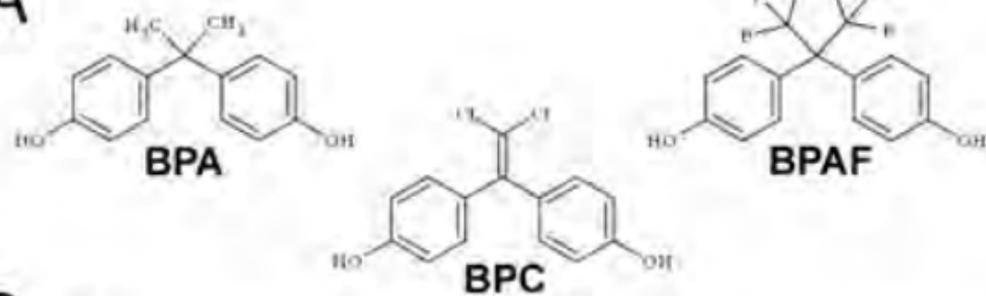
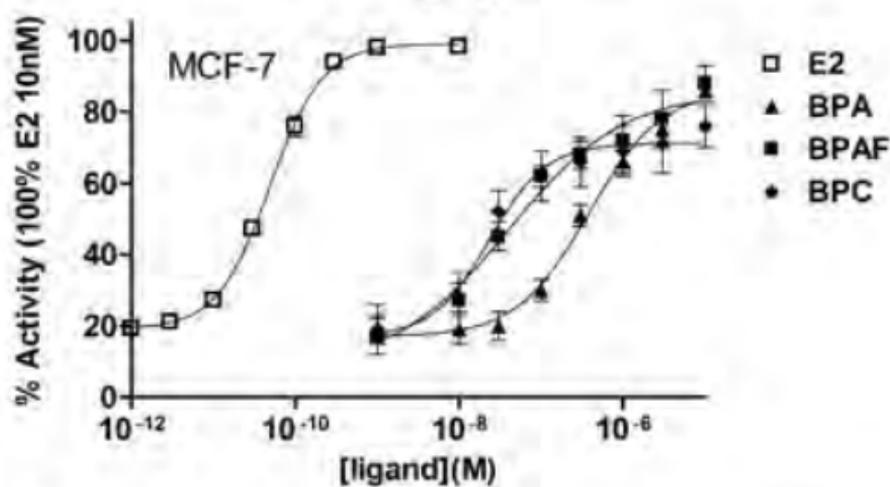
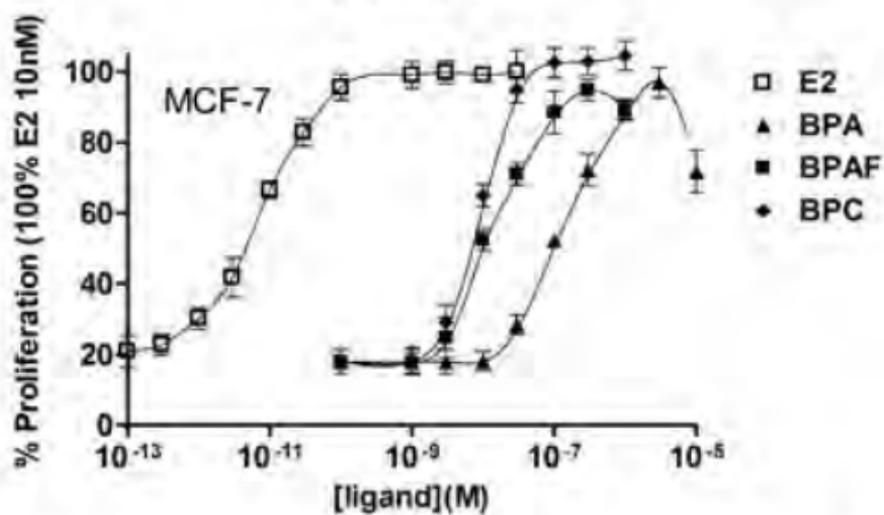
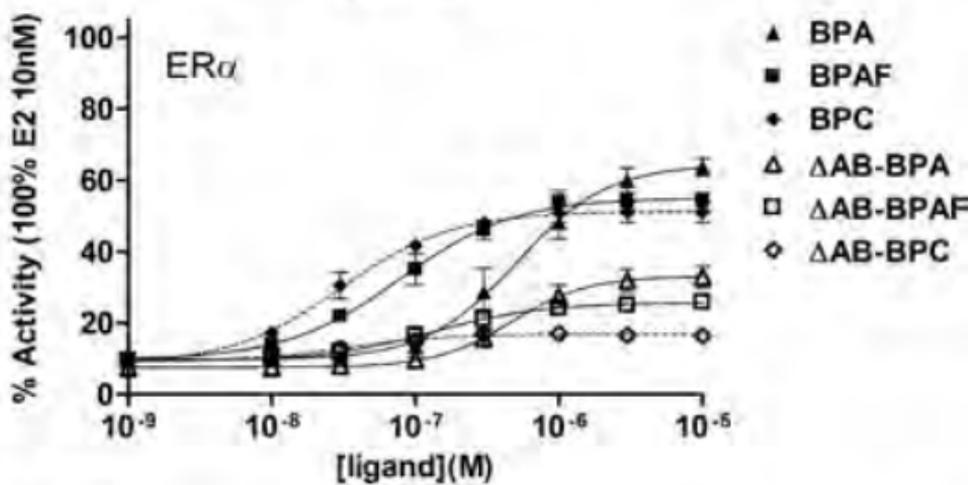
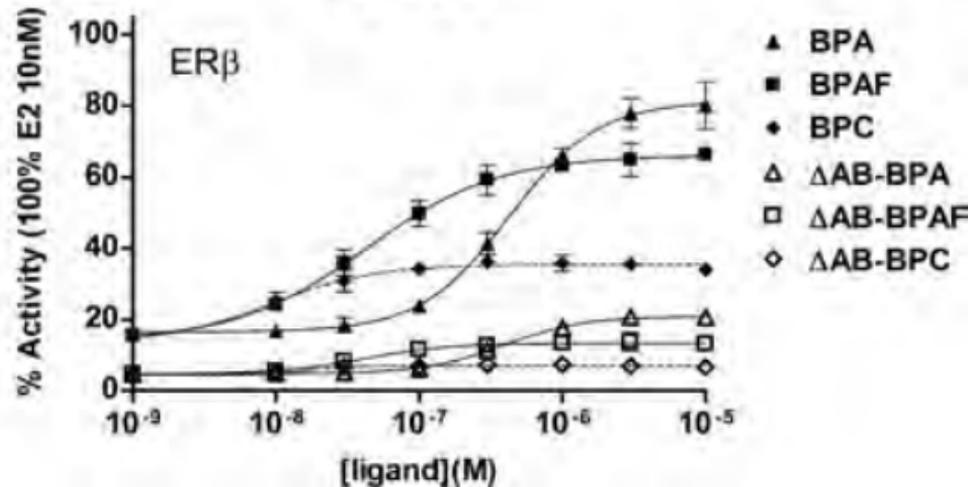
**Figure 1.** Dose response curve for bisphenols in reporter cell lines. (A) Chemical structures of some bisphenols used in this study. (B) MELN, (D) HELN-ER $\alpha$  and - $\Delta$ AB-ER $\alpha$  and (E) HELN-ER $\beta$  and - $\Delta$ AB-ER $\beta$  luciferase assays of BPA, BPAF and BPC. (C) Proliferative response of BPA, BPAF and BPC in MELN cells. The maximal luciferase and proliferation activity (100%) was obtained with 10 nM E<sub>2</sub>. Values were the mean  $\pm$  SD from three separate experiments.

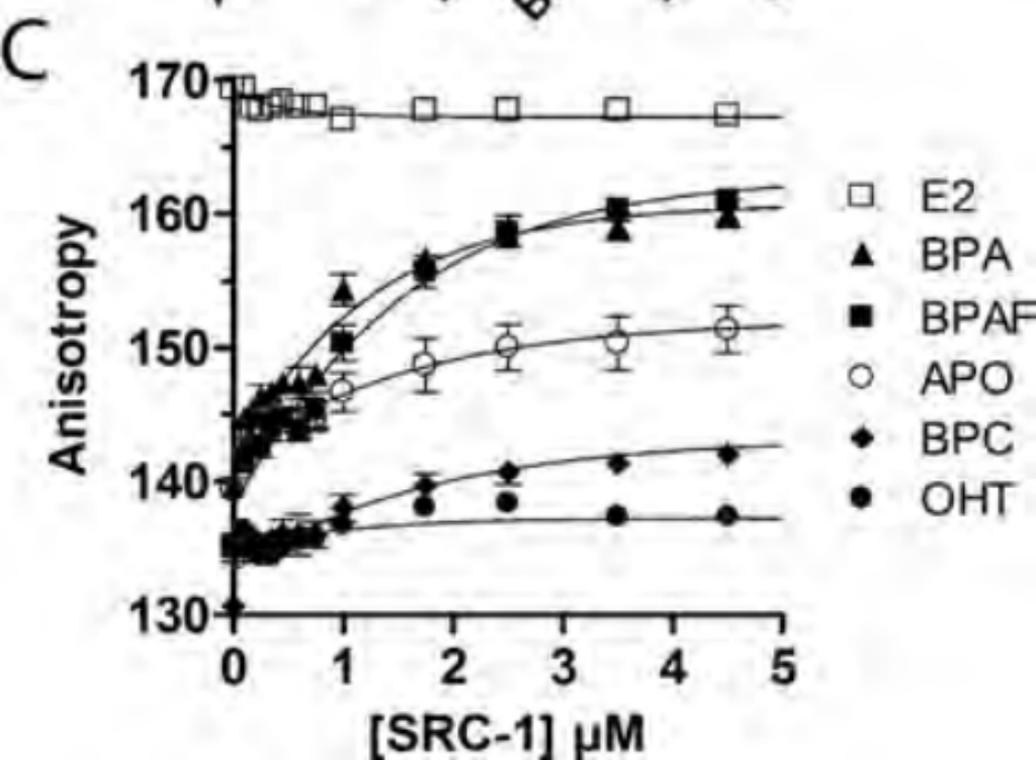
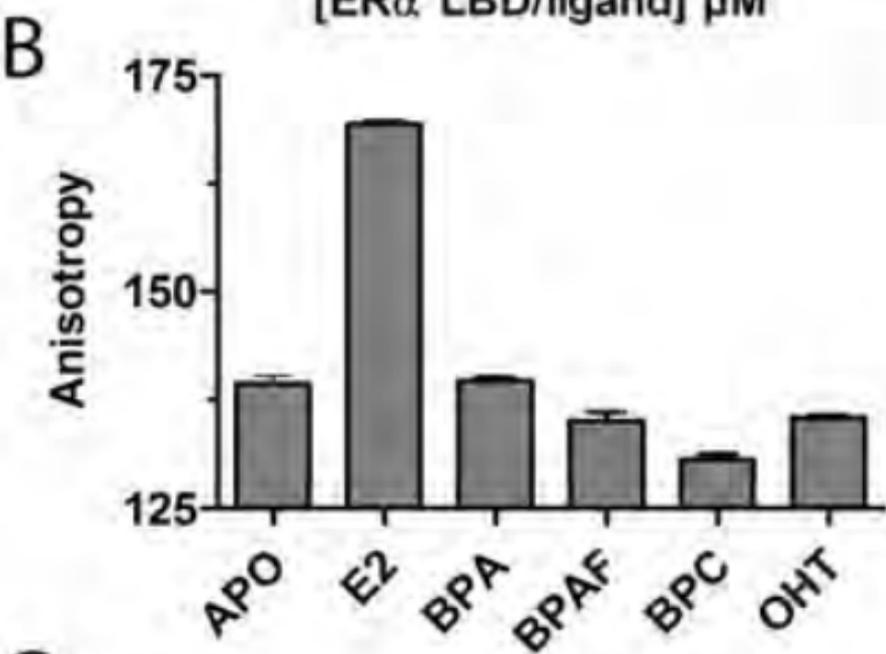
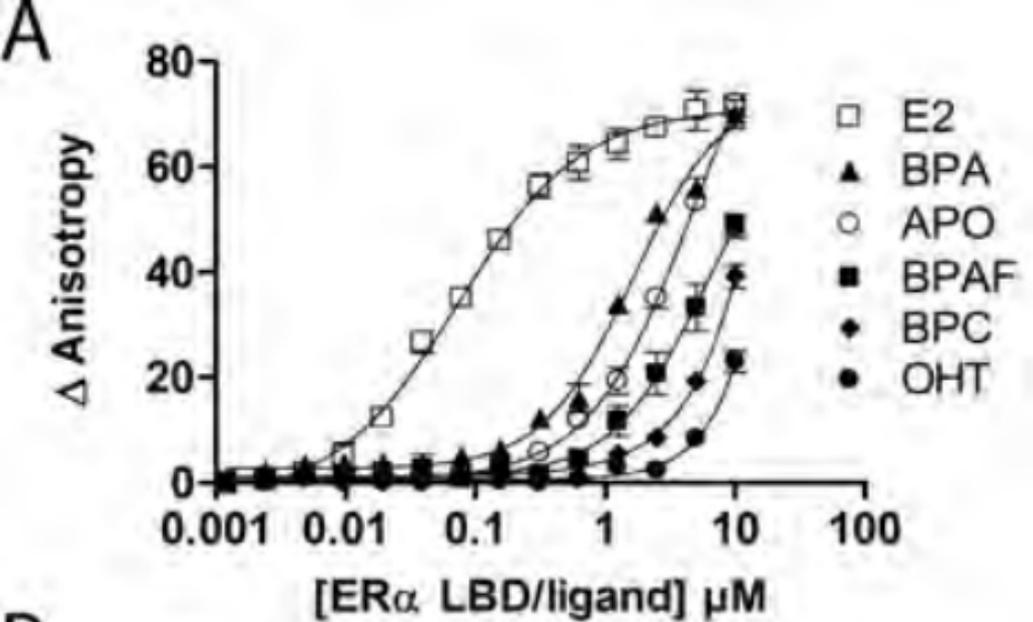
**Figure 2.** Bisphenol-induced coactivator recruitment and structural dynamics. (A) Titration of fluorescein-labeled SRC-1 NR2 peptide by ER $\alpha$  LBD in the absence of ligand or in the presence of E<sub>2</sub> (agonist), OHT (antagonist), BPA, BPAF or BPC. (B) Anisotropy measurements of fluorescein-labeled ER $\alpha$  LBD in the presence of saturating concentrations of bisphenols, E<sub>2</sub> or OHT. (C) Similar experiments performed in the presence of increasing concentrations of the coactivator-derived peptide SRC-1 NR2.

**Figure 3.** Two different binding modes of bisphenols. (A) The whole structure of the ER $\alpha$  Y537S LBD in complex with SRC-1 NR2 and BPA (cyan) superimposed on that of the wild-type ER $\alpha$  LBD bound to BPC (orange). The orange dashed line denotes residues not visible in the electron density map. (B-F) Interaction networks of E<sub>2</sub> (B), BPA (C), BPAF (D and E) and BPC (F) with ligand-binding pocket residues in ER $\alpha$ . Oxygen, nitrogen, sulfur, fluorine and chlorine atoms are colored in red, blue, yellow, cyan and green, respectively. Hydrogen bonds are indicated by black dashed lines. For clarity not all the protein – ligand interactions are depicted. The blue electron density represents a  $F_o - F_c$  simulated annealing omit map contoured at  $3\sigma$ .

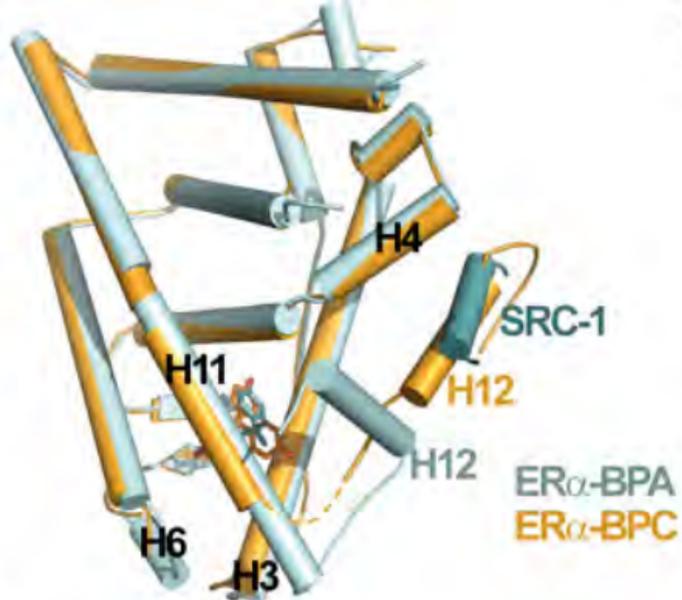
**Figure 4.** Bisphenol binding promotes ER $\alpha$  structural dynamics. (A) Differential interactions of bisphenols and E<sub>2</sub> with G521 and H524. (B) The interaction of E<sub>2</sub> with L525 strengthens a van der Waals interactions network involving T347 (H3), L525 (H11) and L536 (L11-12). (C) Due to a lack of contact with BPA, L525 is not stabilized and adopts two different conformations. (D) In the BPC-bound ER $\alpha$  structure, T347 rotates by 180° to form a hydrogen bond with the bisphenol, resulting in the disruption of the hydrophobic network. (E) Ribbon representation of ER $\alpha$  LBD in complex with E<sub>2</sub>

(red), BPA (green), BPAF (blue) and BPC (purple; dashed line denotes missing residues). Ligands are shown in yellow. The diameter of the ribbon is directly proportional to the temperature factor  $B$  and highlights the dynamics all along the polypeptide chain.

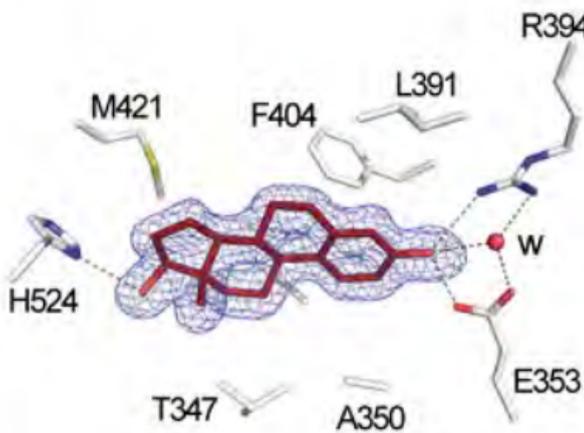
**A****B****C****D****E**



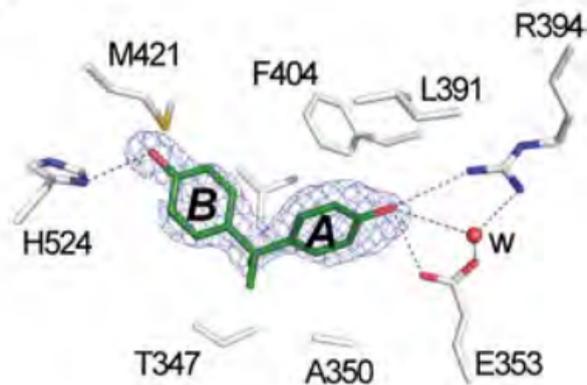
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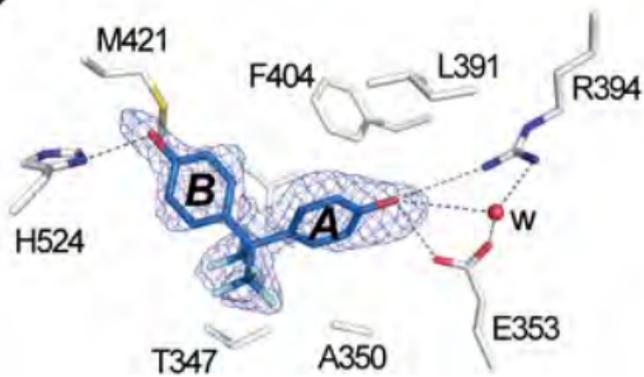
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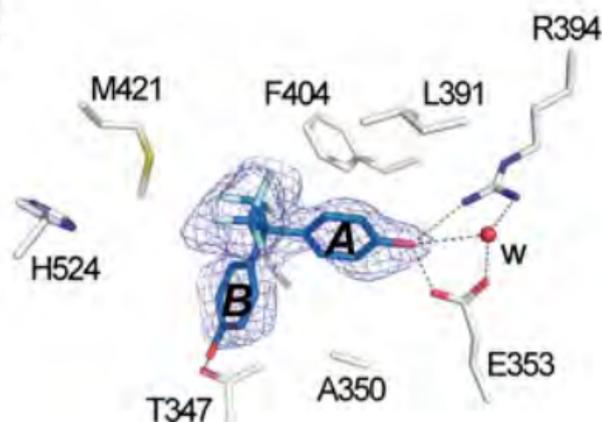
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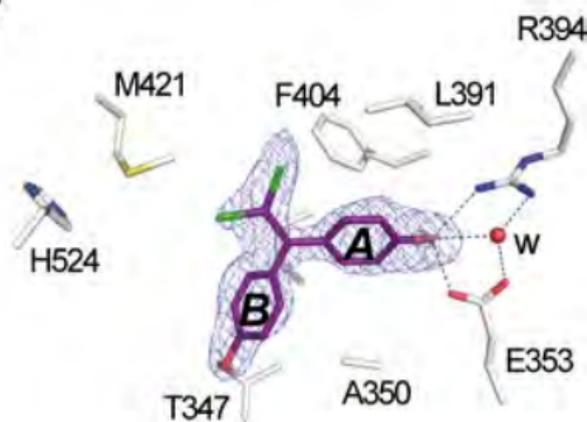
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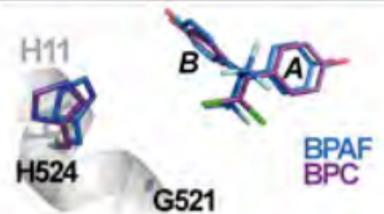
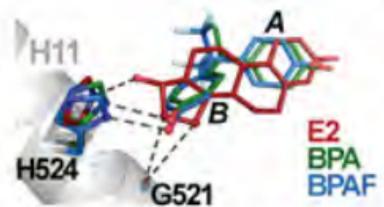
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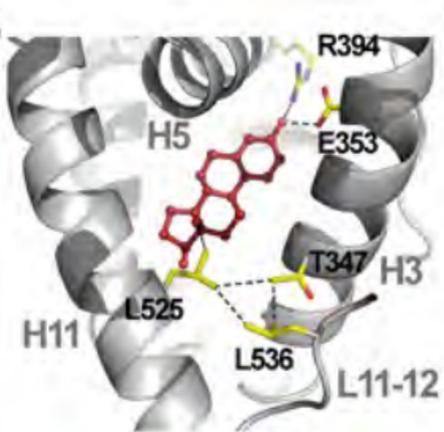
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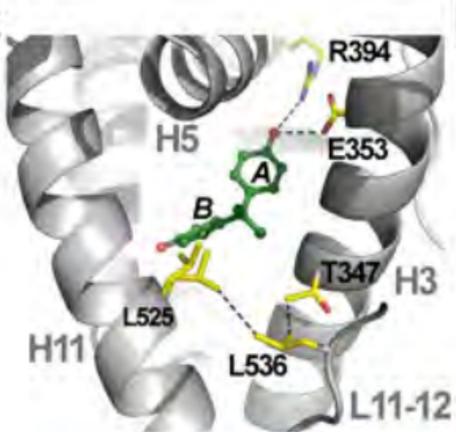
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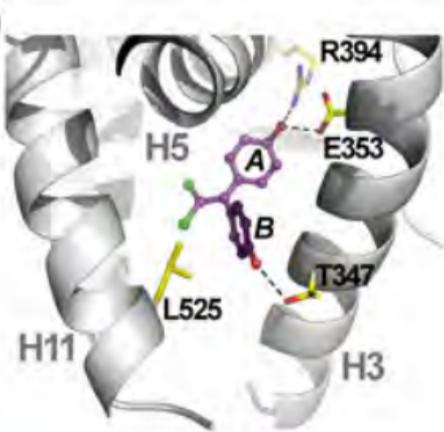
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