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Estrogen receptor-based fluorescence polarization assay for bisphenol analogues and molecular modeling study of their complexation mechanism

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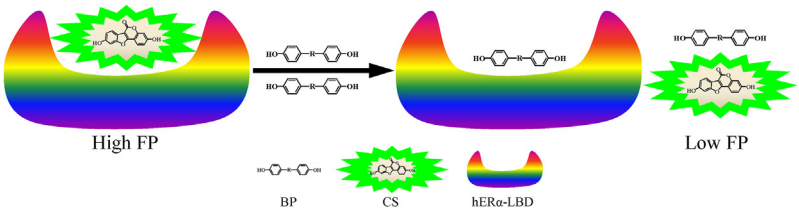
HIGHLIGHTS

- A fluorescence polarization assay for bisphenol compounds (BPs) was developed.
- BPs exhibited dose-dependent binding to human estrogen receptor α.
- BPs adopted distinct binding modes owing to their structural characteristics.
- Molecular modeling showed potential for predicting affinities of putative ligands.
- The proposed method can be applied for preliminary screening of BPs.

GRAPHICAL ABSTRACT



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ABSTRACT

A fluorescence polarization (FP) assay based on estrogen receptor was developed for the determination of bisphenol compounds (BPs). The human estrogen receptor α ligand binding domain (hERα-LBD) and coumestrol were employed as recognition element and fluorescent probe, respectively. Competitive displacement of tracer from receptor suggested that BPs exhibited dose-dependent binding to hERα-LBD. In order to elucidate the structural basis for the interaction between BPs and hERα-LBD, molecular dynamics simulations were performed to explore their complexation mechanism. The docked bisphenol compounds adopted agonist/antagonist conformations with varying positions and orientations in the hydrophobic binding pocket, depending on their structural characteristics of bridging moieties. Interestingly, the calculated binding energies were generally correlated with the experimentally measured affinities, indicating a potential advantage of the molecular modeling approach in predicting the binding potencies of putative ligands. Considering that the real samples may contain more than one BP, the established FP assay can potentially be used as a pre-screening method to determine the total amounts of bisphenol compounds.

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1. Introduction

Exposure to endocrine disrupting chemicals (EDCs) has been confirmed to induce adverse health effects [1,2]. As one of the most abundant EDCs, bisphenol A (BPA) is commonly used in the production of polycarbonate plastics and epoxy resins [3,4]. Plenty of researches have revealed the potential impact of BPA on human health due to its endocrine disrupting property [5–7]. Therefore, several countries and regions have restricted the use of BPA in food packaging materials [8,9]. Meanwhile, there is still a significant controversy regarding BPA toxicity, which has become a major public policy issue. Recent studies of the National Toxicology Program (NTP) pointed out the potential BPA effects on the hormone-mediated neurologic and behavioral development [10]. In the past decades, a series of bisphenol analogues were synthesized and applied as substitutes for BPA [11–13]. Unfortunately, some of them have been reported to exhibit endocrine-mediated cytotoxicity, genotoxicity and reproductive toxicity [14–16]. Base on structural similarity to BPA, these analogues may act via alteration of the endocrine system, thereby resulting in adverse effects close to or even greater than that of their parent compound [17,18].

As environmental xenoestrogens, bisphenol compounds (BPs) share some structural and physicochemical features with natural ligands, allowing them to target a variety of nuclear receptors (NRs) including androgen receptor (AR), estrogen receptors (ERs), glucocorticoid receptor (GR), peroxisome proliferator-activated receptor γ (PPARγ), etc [19–23]. It can be speculated that the total effect of bisphenol compounds may be mediated by the synergistic actions through various metabolic pathways [24,25]. Among the NRs mentioned above, estrogen receptor α and β are the main targets of EDCs and play a crucial role in the regulation of complex physiological functions [26,27]. Both subtypes have been characterized by their ability to bind endogenous estrogens (e.g., 17β-estradiol), phytoestrogens (e.g., genistein) and xenoestrogens (e.g., BPA) [28,29]. Reporter gene assays also indicate that several bisphenol compounds can induce estrogen receptor signal transduction pathways *in vitro* [30].

Estrogen receptors are allosterically regulated transcription factors that undergo ligand-dependent conformational selection [31]. As members of the nuclear hormone receptor superfamily, ERs contain three conserved functional domains, including an N-terminal A/B domain, a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD). The LBD folds into twelve helices, forming a ligand binding pocket (LBP) [32,33]. As a molecular switch, helix 12 (H12) locates at the C-terminal end of the LBD and plays a key role in regulating the link between coactivator and ligand binding sites [31,34]. Crystal structures of the human estrogen receptor α ligand binding domain (hERα-LBD) complexed with agonist diethylstilbestrol (DES) and selective antagonist 4-hydroxytamoxifen (OHT) reveal that these two ligands induce distinct conformations of H12 crucial for receptor activation [35]. Furthermore, the structure of the complex between hERα-LBD and BPA has also been analyzed crystallographically [36]. BPA induces an agonist conformation of hERα-LBD similar to that observed in the DES-bound structure, with H12 capping the LBP.

Although both *in vivo* and *in vitro* studies have confirmed the endocrine disrupting potencies of several bisphenol compounds [20,30,37–40], however, the underlying mechanism of their estrogenic activities is still largely unclear. In this work, bisphenol compounds were evaluate systematically for their ability to bind human estrogen receptor α. To establish a fluorescence polarization assay for BPs, the recombinant hERα-LBD protein was prepared in an *Escherichia coli* expression system firstly. The binding interactions between BPs and hERα-LBD were determined by using coumestrol (CS) as tracer. Subsequently, molecular dynamics simulations were performed to explore the binding modes of BPs with hERα-LBD in an attempt to unravel the molecular mechanism for their agonistic/antagonistic properties on estrogen receptor.

2. Materials and methods

2.1. Materials

Coumestrol (CS, ≥97.5%), 17β-estradiol (E_2, >97%), diethylstilbestrol (DES, >98%), 2,2-bis(4-hydroxyphenyl)propane (BPA, >99%), 2,2-bis(4-hydroxy-3-methylphenyl)propane (BPC, >98%), bis(4-hydroxyphenyl)sulfone (BPS, 99%), 2,2-bis(4-hydroxyphenyl) butane (BPB, ≥98%), 1,1-bis(4-hydroxyphenyl)ethane (BPE, ≥98%), 1,1-bis(4-hydroxyphenyl)-cyclohexane (BPZ, ≥98%), 1,1-bis(4-hydroxyphenyl)-1-phenylethane (BPAP, >98%), 1,3-bis(2-(4-hydroxyphenyl)-2-propyl)benzene (BPM, >98%), and 1,4-bis(2-(4-hydroxyphenyl)-2-propyl)benzene (BPP, >98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), TCI (Tokyo, Japan), and Aladdin (Shanghai, China). All other reagents used were of analytical grade.

2.2. Production of the soluble receptor protein

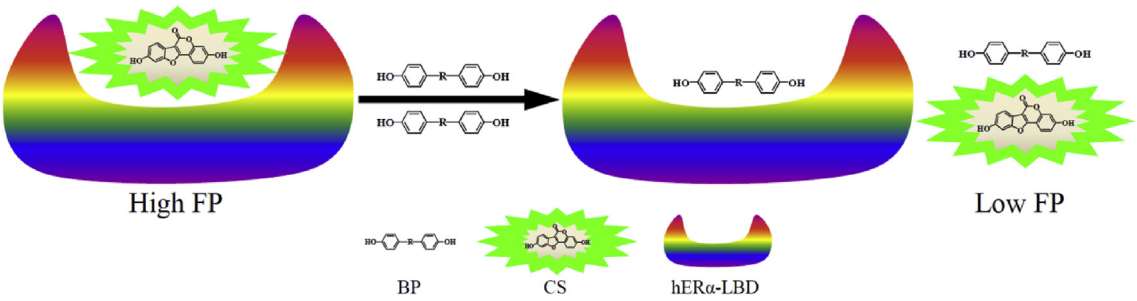
Human estrogen receptor α ligand binding domain (hERα-LBD, residues 282 to 595) was expressed as a glutathione S-transferase (GST) fusion protein. The coding region was synthesized *de novo* and inserted into *Bam*HI and *Xho*I restriction enzyme sites of the pGEX-4T-1 vector. Then the recombinant plasmid was transformed into *Escherichia coli* strain BL21 (DE3)pLysS. The cultures were induced overnight at 20 °C by adding IPTG (isopropyl-β-*d*-thiogalactoside) to 0.5 mM. Soluble GST-tagged protein in the supernatant was purified using a glutathione-Sepharose (GSH-Sepharose) affinity column. Homogeneity of the recombinant hERα-LBD was confirmed by SDS-PAGE analysis.

2.3. Fluorescence polarization assay

The fluorescence polarization assay was carried out by FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The excitation and emission wavelengths were 355 and 405 nm, respectively. In the direct binding assay, the recombinant protein was examined for its ability to bind coumestrol. The probe was titrated with various concentrations of protein and then the dissociation constant ($K_{d,CS}$) of coumestrol with hERα-LBD was obtained. In the competitive binding assay, hERα-LBD (250 nM) and coumestrol (10 nM) were mixed in a total volume of 290 μL. Then, 10 μL of the tested compound with a range of concentrations was added to bring the total volume to 300 μL. Each sample was subjected to the fluorescence polarization experiments after being incubated for 2 h at room temperature. The IC_{50} value (the concentration of the tested compound that inhibited the binding of probe with hERα-LBD by 50%) was calculated from the competition curves fitted using a four parameter logistic equation $Y = (A - D)/[1 + (X/IC_{50})^{B}] + D$, where Y and X correspond to the polarization value and the tested compound concentration, A and D are the polarization values at zero and an infinite concentration respectively, and B is the slope parameter [41]. The dissociation constant (K_d) of the tested compound with hERα-LBD was calculated according to the following equation: $IC_{50}/[\text{coumestrol}] = K_d/K_{d,CS}$. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA).

2.4. Molecular docking and binding energies calculation

The crystal structure of hERα-LBD complexed with 4-



Scheme 1. Schematic illustration of ER-based fluorescence polarization (FP) assay for bisphenol compounds. BP, bisphenol compound; CS, coumestrol; hERα-LBD, human estrogen receptor α ligand binding domain.

hydroxytamoxifen (OHT) was obtained from the Protein Data Bank (PDB ID: 3ERT) [35]. The initial structures of bisphenol compounds (BPs) were constructed by Gaussview and then optimized with Gaussian 09 using the B3LYP/6-31G(d) method. The molecular length and Connolly solvent-excluded volume (CSEV) of bisphenol compounds were calculated using AutoDockTools-1.5.6 and Chem3D Ultra 8.0, respectively. In the BP-hERα-LBD docking experiment, the grid box was generated at the center of the active region with adjusted box size according to the length of each BP. Automated docking was performed with optimized box parameters by AutoDockTools-1.5.6 and the predicted binding energies (kcal mol^{-1}) were calculated using scoring function. For each experiment, 10 independent docking runs were carried out and the one with the lowest binding energy was chosen for subsequent analysis. The intermolecular interactions of BPs with hERα-LBD were visualized by employing PyMol.

3. Results and discussion

3.1. Assessment of BPs binding potencies with hERα-LBD

In this work, the free and ER-bound tracer can be differentiated by monitoring the polarization signal (Scheme 1). In order to confirm whether the fluorescence polarization assay can be applied for the competitive binding experiment, two typical ligands, 17β-estradiol (E_2) and diethylstilbestrol (DES), were chosen to displace the tracer. As can be seen in Fig. 1, the addition of E_2 and DES resulted in decrease of polarization values, indicating that coumestrol (CS) was displaced from the CS-hERα-LBD complex. Base on the $K_{d,CS}$ of 255.50 nM previously measured in our lab, the IC_{50} values and dissociation constants (K_d) of hERα-LBD for E_2 and DES were determined and calculated (Table 1).

After the proposed method was established and validated, the binding affinities of bisphenol compounds (BPs) with hERα-LBD were determined. As shown in Fig. 1, the tested BPs exhibited dose-dependent binding to receptor protein. As illustrated in Table 1, the K_d values of these compounds are in the range of 2.81 μM–180.64 μM, reflecting their binding affinities to hERα-LBD. Therefore, it can be concluded that all the examined bisphenol compounds can bind to hERα-LBD as the functional ligands, in turn, might result in the activation of estrogen receptor. In recent years, several innovative techniques based on nanomaterials and electrochemical sensors have been developed for the sensitive detection of BPA [42–45]. Since these methods mainly focus on BPA rather than other analogues, they are not suitable for monitoring multiple BPs simultaneously. By employing receptor protein as a recognition element, the proposed fluorescence polarization assay can potentially be applied for preliminary screening of bisphenol

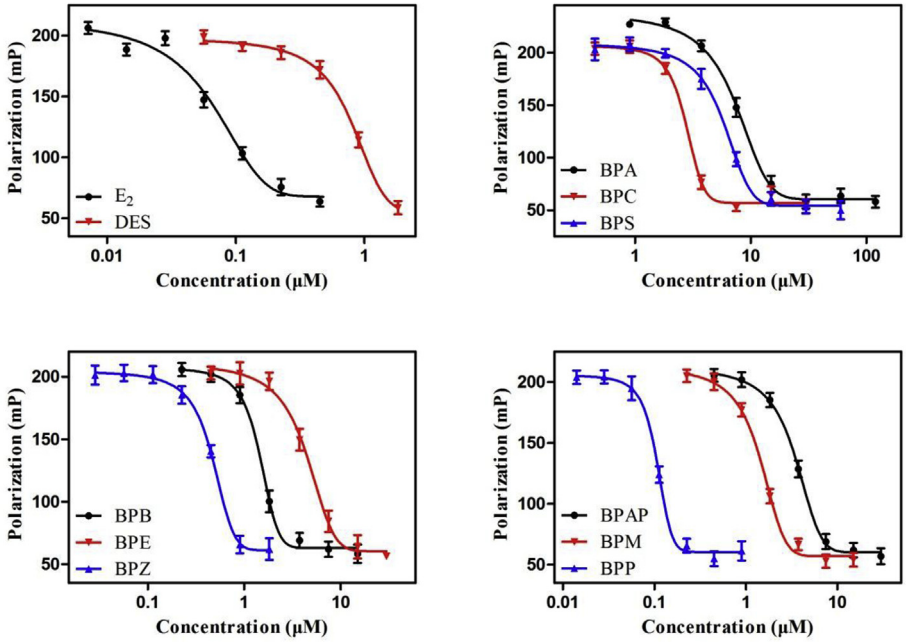


Fig. 1. Competitive binding of the tested compounds to hERα-LBD. Results are given as means ± SEM of three independent experiments.

Table 1
The molecular length, Connolly solvent-excluded volume (CSEV), IC_{50} values, dissociation constants (K_d), and binding energies for the tested compounds.

Compound	Length (Å)	CSEV (Å^3)	IC_{50} (μM)	K_d (μM)	Binding energy (kcal mol^{-1})
E_2	12.19	262.1	0.05	1.28	−9.47
DES	12.74	250.7	0.78	19.93	−8.65
BPA	9.99	205.5	7.07	180.64	−7.04
BPC	10.36	239.7	2.75	70.26	−7.56
BPS	9.87	181.3	5.78	147.68	−7.07
BPB	10.69	224.6	1.45	37.05	−7.61
BPE	9.57	186.5	4.23	108.08	−7.12
BPZ	9.54	245.9	0.47	12.01	−8.91
BPAP	9.85	256.6	3.35	85.59	−7.43
BPM	12.15	331.6	1.39	35.51	−7.96
BPP	10.84	329.6	0.11	2.81	−9.04

compounds in food and environmental samples.

3.2. Molecular basis for agonism and antagonism of BPs in hERα

To explore the binding modes of bisphenol compounds with hERα-LBD, they were docked into the active site pocket, as well as agonist (17β-estradiol, E_2) and antagonist (4-hydroxytamoxifen, OHT). Based on the crystal structure of hERα-LBD [32], the ligand binding pocket with a probe accessible volume of approximately 450 Å^3 can readily accommodate bisphenol compounds (~180–~330 Å^3). Additionally, the skeleton length of BPs (~10 Å) is well matched by the LBP, as summarized in Table 1. Molecular docking results showed that all the tested compounds completely fit into the cavity of hERα-LBD. As shown in Fig. 2, agonist and antagonist locate at the same site but demonstrate distinct binding modes. The structure of the hERα-LBD-E_2 complex reveals that the ligand binding domain adopts an active conformation, sealing agonist within the cavity and forming a hydrophobic groove to facilitate co-activator (CoA) recruitment. In contrast, the bulky side chain of OHT protrudes from the cavity between H3 and H11, preventing the alignment of H12 over the bound ligand and interrupting the signal transductions [32,35].

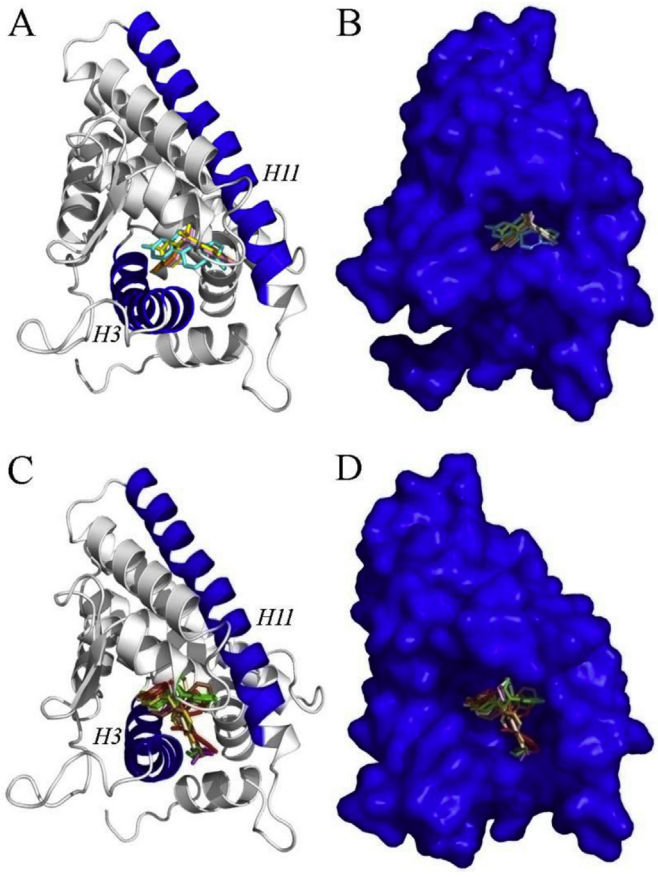


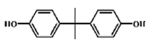
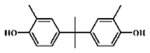
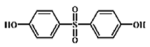
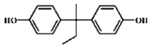
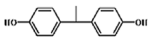
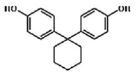
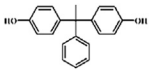
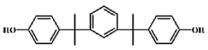
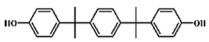
Fig. 2. Agonist (*top*) and antagonist (*bottom*) conformations of hERα-LBD displayed with cartoon (*left*) and surface (*right*), respectively. A, B: E_2, cyan; BPA, pink; BPC, yellow; BPS, orange. C, D: OHT, green; BPB, magenta; BPE, olive; BPZ, wheat; BPAP, brown; BPM, red; BPP, purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

With regard to the bisphenol compounds investigated in this work, they can be obviously classified into three categories according to their distinguishing structural characteristics and binding modes (Table 2). The compounds in class I adopt an agonist conformation that resembles E_2, whereas those in class II and III display an antagonist conformation similar to that of OHT, as can be seen in Fig. 2. The symmetrical structures of the bridging moieties in class I compounds may contribute to maintain the agonist conformation of the receptor. The two phenolic hydroxyl groups form hydrogen bonds with polar residues located at the two ends of the cavity, namely Glu353 in H3 and Gly521 and His524 in H11 (Fig. 3). By contrast, the dissymmetric bridging moieties in other classes of compounds (II and III) provoke a different conformation of hERα. One of their phenolic rings locates in the active site and form hydrogen bonds with several polar residues including Glu353, Leu387 and Arg394, as well as a water molecule. Meanwhile, the other phenolic ring protrudes from the pocket between H3 and H11, adopting an antagonist conformation that resembles OHT (Fig. 2). It is worth noting that the protruding phenolic hydroxyl group of class II compounds makes a hydrogen bond with Thr347, as can be observed in Fig. 3. However, the class III compounds with a benzene ring located at the bridging moieties may disturb the hydrophobic pocket, resulting in the loss of a hydrogen bond at this amino acid site. Therefore, the different binding modes for each class of compounds might be attributed to their distinguishing characteristics of bridging moieties.

3.3. Correlation analysis

As summarized in Table 1, the order of the calculated binding potencies for BPs with hERα-LBD is in general agreement with their binding affinities determined in FP assay. Comparison of the calculation values versus the aforementioned experimental data yields an R-squared value of 0.74 (Fig. 4), suggesting the potential application of the molecular modeling approach to predict the binding potencies between putative ligands and their respective target receptors. In conclusion, the present work provides valuable insights into the binding modes of bisphenol compounds to human estrogen receptor, and thus demonstrates a structural basis for the design of BPA substitutes devoid of endocrine toxicity.

Table 2
The docking results of bisphenol compounds to human estrogen receptor α.

Compound		Structure	Hydrogen bonds	Estrogenicity
Class I	BPA		Glu353, Gly521, His524	Agonist
	BPC		Glu353, Gly521, His524	Agonist
	BPS		Glu353, Gly521, His524	Agonist
Class II	BPB		Thr347, Glu353, Arg394, H_2O	Antagonist
	BPE		Thr347, Glu353, Leu387, Arg394, H_2O	Antagonist
	BPZ		Thr347, Glu353, Arg394, H_2O	Antagonist
Class III	BPAP		Glu353, Arg394, H_2O	Antagonist
	BPM		Glu353, Leu387, Arg394, H_2O	Antagonist
	BPP		Glu353, Leu387, Arg394, H_2O	Antagonist

4. Conclusion

In this work, the binding interactions between bisphenol compounds and hERα-LBD were investigated by fluorescence polarization coupled with molecular dynamics simulations. *In vitro* competitive displacement assay with coumestrol as tracer suggested that BPs exhibited dose-dependent binding to hERα-LBD. Additionally, the structural basis for the estrogenic activities of BPs was illustrated by molecular docking. Three classes of bisphenol compounds adopted distinct binding modes in the hydrophobic binding pocket owing to their structural characteristics of bridging moieties. Comparison between the calculated binding energies and the experimentally determined dissociation constants yields an R-squared value of 0.74, indicating a potential advantage of the

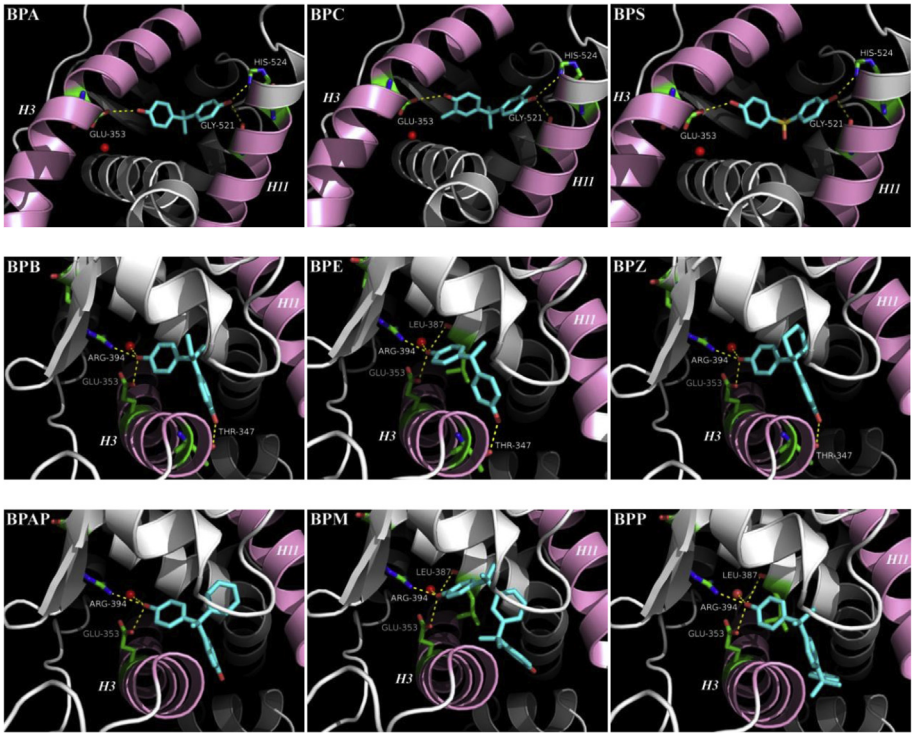


Fig. 3. Computational docking of bisphenol compounds to hERα-LBD. Red sphere, water molecule; yellow dashed lines, hydrogen bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

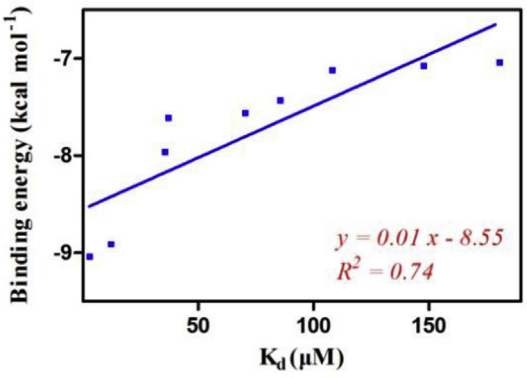


Fig. 4. Correlation between the calculated binding energies and the experimentally determined dissociation constants of bisphenol compounds.

molecular modeling approach in predicting the binding potencies of putative ligands. Considering that the real samples may contain more than one BP, the established FP assay can potentially be used as a pre-screening method to determine the total amounts of bisphenol compounds.

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