

# In vitro and in silico assessment of the structure-dependent binding of bisphenol analogues to glucocorticoid receptor

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**Abstract** Widespread use of bisphenol A (BPA) and other bisphenol analogues has attracted increasing attention for their potential adverse effects. As environmental endocrine-disrupting compounds (EDCs), bisphenols (BPs) may activate a variety of nuclear receptors, including glucocorticoid receptor (GR). In this work, the binding of 11 BPs to GR was investigated by fluorescence polarization (FP) assay in combination with molecular dynamics simulations. The human glucocorticoid receptor was prepared as a soluble recombinant protein. A fluorescein-labeled dexamethasone derivative (Dex-fl) was employed as tracer. Competitive displacement of Dex-fl from GR by BPs showed that the binding affinities of bisphenol analogues were largely dependent on their characteristic functional groups. In order to further understand the relationship between BPs structures and their GR-mediated activities, molecular docking was utilized to explore the binding modes at the atomic level. The results confirmed that structural variations of bisphenol analogues contributed to different interactions of BPs with GR, potentially causing distinct toxic effects. Comparison of the calculated binding energies vs. experimental binding affinities yielded a good correlation

( $R^2 = 0.8266$ ), which might be helpful for the design of environmentally benign materials with reduced toxicities. In addition, the established FP assay based on GR exhibited the potential to offer an alternative to traditional methods for the detection of bisphenols.

**Keywords** Glucocorticoid receptor · Bisphenol analogues · Fluorescence polarization · Molecular docking

## Introduction

Bisphenol A is one of the highest volume chemicals produced around the world. During the past few decades, bisphenol A (BPA) has been widely used in the manufacturing of polycarbonate plastics and epoxy resins [1]. Owing to the migration of BPA from consumer products, it has been detected in various environmental matrices and human tissues [2, 3]. Numerous studies suggested that BPA might be harmful to health due to its endocrine disrupting properties [4, 5]. Hence, several countries have banned the production and usage of BPA on food packaging and contact materials [6, 7]. In recent years, many BPA analogues have been synthesized and used as substitutes for the parent compounds. Unfortunately, because of the structural similarity of these chemicals, they have also been found to act via endocrine disruption just like BPA. Some of the bisphenol analogues reveal adverse effects close to or even greater than that of BPA [8–10].

A number of bioassays based on reporter gene expression and cell proliferation assessment have demonstrated that BPA and its analogues can bind to and activate the nuclear receptors, including estrogen receptors (ERs) [11], estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) [12], peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) [13], pregnane X receptor (PXR) [14], and glucocorticoid receptor (GR) [15]. Bisphenols have been also

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shown to inhibit androgen receptor (AR) [16] and thyroid hormone receptor (TR) [17]. In conclusion, the total effect of bisphenols may be caused by synergistic actions through various metabolism pathways.

Given the universal distribution of multiple bisphenols [18–20], it is urgent to set up a credible method for the simultaneous monitoring of these contaminants. Conventional analyses of bisphenols have mainly been carried out by instrumental determination, such as gas chromatography tandem mass spectrometry (GC-MS) [21], high-performance liquid chromatography (HPLC) [22], and liquid chromatography coupled with tandem mass spectrometry (LC-MS) [23], fluorimetry (LC-FL) [24], or electrochemical detection (LC-ED) [25]. Although chromatographic techniques are accurate and quantitative, they need to be equipped with precision instruments and may be costly and time-consuming. Recently, immunoassays have attracted considerable attention for the determination of bisphenols [26–28] and some ELISA kits have been commercialized [29]. Compared with the methods mentioned above, immunoassays require neither sophisticated equipment nor qualified personnel. Based on the antigen-antibody reactions, immunoassays are highly specific and sensitive. However, an antibody focuses on recognizing only one compound, making it impossible to detect the whole group of bisphenol analogues.

In comparison with antibody, receptor exhibits the advantage of broad-spectrum binding to a series of ligands. In this work, glucocorticoid receptor was chosen as a recognition element for multiple bisphenols. GR is a steroid hormone-activated transcription factor belonging to the nuclear receptor superfamily. Like other members of this family, GR contains three major functional domains, including an N-terminal activation function-1 domain (AF-1), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The latter is composed of about 250 amino acids arranged into 3 layers of 12 alpha helices [30]. Bisphenol analogues share similar physicochemical properties with natural ligands, allowing them to activate the glucocorticoid receptor [31]. Molecular docking studies revealed that the interaction mode and binding energy of BPA were similar to that of dexamethasone and cortisol, two known agonists of GR [32]. However, the molecular basis behind the deleterious effects of other BPA analogues is poorly understood.

In the present work, the binding between a series of bisphenol analogues and GR was investigated by using fluorescein-labeled dexamethasone as a probe. The recombinant glucocorticoid receptor was expressed in *Escherichia coli*. To develop a competitive binding assay, the dissociation constant ( $K_{d,probe}$ ) of Dex-fl with GR needs to be determined first. Then, the  $IC_{50}$  values (the concentrations of BPs that inhibited the binding of GR by 50%) and dissociation constants ( $K_{d,BP}$ ) of GR for 11 BPs were measured by the optimized fluorescence polarization (FP) assay. It is based on the coupling of receptor and tracer, capable of modulating the

fluorescence polarization emission on the basis of the binding of Dex-fl to GR. Molecular docking was performed to examine the binding modes between BPs and GR at the atomic level. Additionally, the binding energies were calculated to correlate with the experimental data.

## Materials and methods

### Materials

4,4'-Isopropylidenediphenol (BPA), 4,4'-(Hexafluoroisopropylidene)diphenol (BPAF), 4,4'-(1-Phenylethylidene)bisphenol (BPAP), 2,2-Bis(4-hydroxyphenyl)butane (BPB), 2,2-Bis(4-hydroxy-3-methylphenyl)propane (BPC), 1,1-Bis(4-hydroxyphenyl)ethane (BPE), 4,4'-Dihydroxydiphenylmethane (BPF), 4,4'-(1,3-Phenylenediisopropylidene)bisphenol (BPM), 4,4'-(1,4-Phenylenediisopropylidene)bisphenol (BPP), 4,4'-Sulfonyldiphenol (BPS), and 4,4'-Cyclohexylidenebisphenol (BPZ) were purchased from Aladdin (Shanghai, China) and Sigma-Aldrich (St. Louis, MO, USA). The structures of the BPs above are shown in Table 1. Dexamethasone fluorescein was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). PageRuler Prestained Protein Ladder was purchased from Thermo Fisher Scientific (San Jose, CA, USA). All other reagents used were of analytical grade.

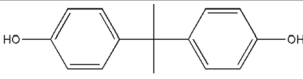
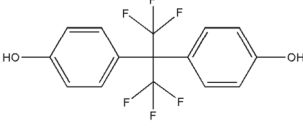
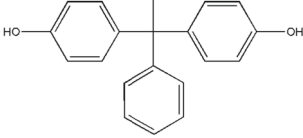
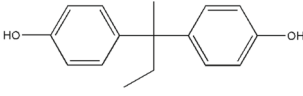
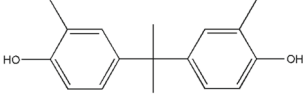
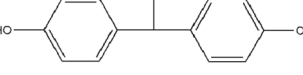
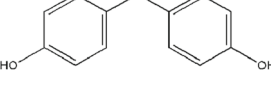
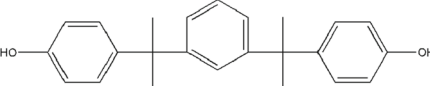
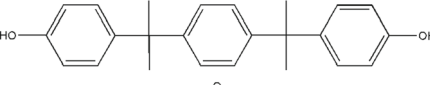
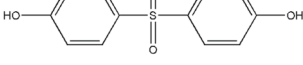
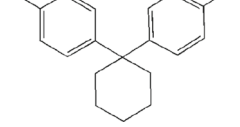
### Preparation of the receptor protein

The expression and purification of recombinant glucocorticoid receptor were carried out as described [33]. Briefly, the human GR (residues 1 to 777) was expressed as a fusion protein from the  $6 \times$  His-modified pET28a vector in *E. coli* strain Rosetta (DE3). Cells were grown in LB media and induced with IPTG and dexamethasone. The  $6 \times$  His tag was cleaved by thrombin to obtain the target protein with a molecular weight of 85.7 kDa.

### Direct binding assay

In the direct binding assay, the recombinant protein was tested for the ability to bind Dex-fl using fluorescence polarization. FP experiments were performed on a microplate reader (TECAN, infinite F500, Austria). The excitation and emission wavelengths were 484 and 520 nm, respectively [34]. Dex-fl (5 nM) was titrated with various concentrations of GR using buffer 10 mM Hepes pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.005% Tween-20. The increase of FP values upon the formation of GR-Dex-fl complexes was monitored. The dissociation constant ( $K_{d,probe}$ ) of Dex-fl with GR was obtained by nonlinear curve fitting using a one site binding

**Table 1** IC<sub>50</sub> values and dissociation constants ( $K_{d,BP}$ ) of 11 BPs

Chemical	Structure	IC <sub>50</sub> (μM)	K <sub>d,BP</sub> (μM)
BPA		18.82	31.66
BPAF		66.92	112.56
BPAP		7.12	11.98
BPB		14.82	24.93
BPC		6.92	11.64
BPE		31.65	53.24
BPF		61.79	103.93
BPM		2.81	4.73
BPP		1.81	3.04
BPS		25.19	42.37
BPZ		2.94	4.95

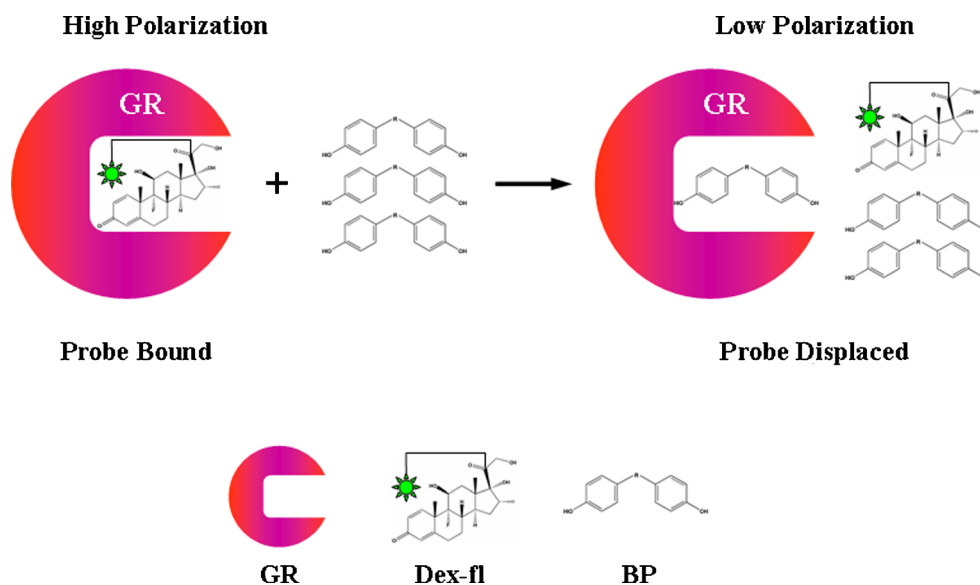
model  $Y = B_{\max} * X / (K_d + X)$ , where  $Y$  and  $X$  correspond to the polarization value and the probe concentration,  $B_{\max}$  is the maximal binding. Data analysis was performed using GraphPad Prism 5 (GraphPad Software, USA).

### Competitive binding assay

The affinities of BPs with GR were estimated by using Dex-fl as a probe. Then, 90 μL of Dex-fl (5 nM) and 20 μL of BPs

were mixed. Then, 90 μL of GR (10 nM) was added to bring the total volume to 200 μL and incubated for 30 min at room temperature. The displacement of bound Dex-fl was calculated from the decrease of FP values of GR-Dex-fl complexes with increasing BPs concentrations. The IC<sub>50</sub> values were obtained from the competition curves fitted using a four-parameter logistic equation (Sigmoidal model)  $Y = (A - D) / [1 + (X/IC_{50})^B] + D$ , where  $Y$  and  $X$  correspond to the polarization value and the BPs concentrations,  $A$  and  $D$  are the

**Fig. 1** Principle of GR-based fluorescence polarization competitive binding assay for BPs. GR glucocorticoid receptor, Dex-fl fluorescein-labeled dexamethasone, BPs bisphenols



polarization values at zero and an infinite concentrations respectively, and  $B$  is the slope parameter. The dissociation constants ( $K_{d,BP}$ ) of the tested compounds with GR were calculated according to the relationship  $IC_{50} / [Dex-fl] = K_{d,BP} / K_{d,probe}$ .

### Molecular dynamics simulations and binding energy calculation

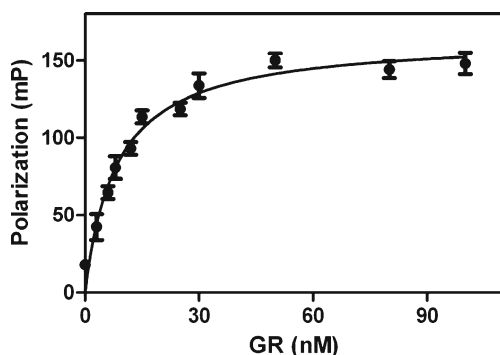
The crystal structure of human glucocorticoid receptor ligand-binding domain (residues 500 to 777) with the bound dexamethasone was available in Protein Data Bank (PDB ID: 4UDC) and served as a model to explore the binding modes between GR and BPs. The initial structures of Dex and 11 BPs were constructed using Gaussview and then optimized with Gaussian 09 by the B3LYP/6-31G(d) method. Automated ligand-receptor docking calculations were performed with AutoDock Vina to explore the interaction modes of GR and different ligands. Dex was first docked to GR. AutoDock

Tools were utilized to set the size and the center of the grid box, and to prepare the input.pdbqt file. By using the same docking parameters, the 11 BPs were also docked with GR. The predicted binding affinity ( $kcal\ mol^{-1}$ ) was calculated based on the scoring function used in AutoDock Vina. The Pymol program was used to analyze the molecular interaction between GR and ligands.

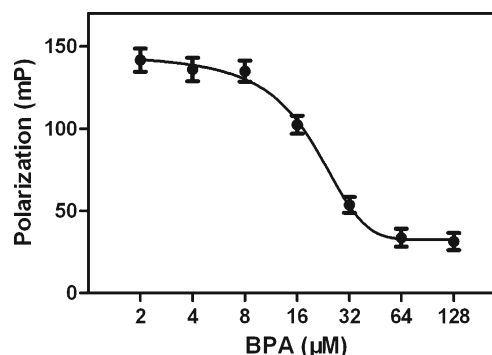
## Results and discussion

### Working principle

Fluorescence polarization assay, which is one of the most sensitive, robust, and widely used high-throughput screening (HTS) methods for the study of protein interactions, can detect changes in polarization caused by changes in the molecular mass of the labeled species [35]. In the present work, the free and GR-bound Dex-fl can be differentiated by the FP assay.

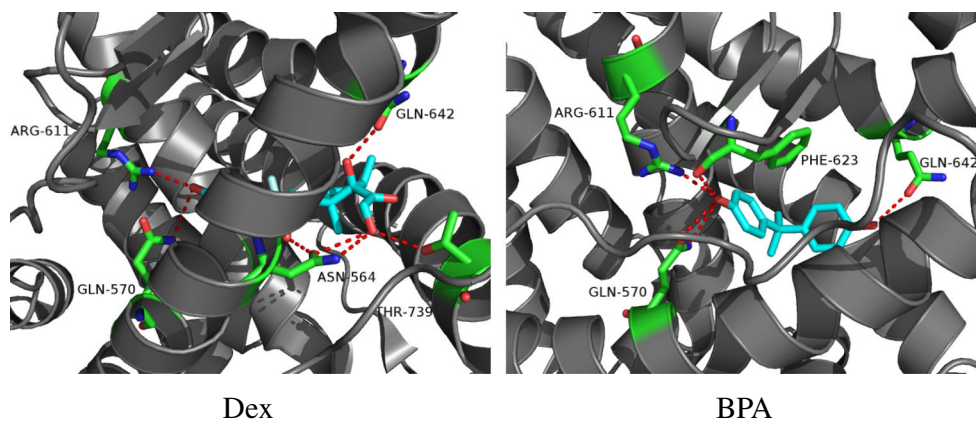


**Fig. 2** Direct binding of Dex-fl to GR. Results are given as means  $\pm$  SEM of three independent experiments



**Fig. 3** Competitive binding of BPA to GR. Results are given as means  $\pm$  SEM of three independent experiments

**Fig. 4** Binding modes of dexamethasone (*Dex*) and BPA to GR. Hydrogen bonds formed between ligands and GR are indicated in *dashed lines*



At the beginning of the reaction, the fluorescein-labeled dexamethasone and receptor protein form a GR-Dex-fl complex, which rotates slowly and produces a high polarization value. With the introduction of BPs to the solution, the added compounds and Dex-fl compete for the binding site of GR. The displaced Dex-fl, with decreasing molecular volume, rotates quickly and results in a low polarization value. Accordingly, the detection of BPs was performed by monitoring the FP signal, as illustrated in Fig. 1.

### Functional characterization of GR

In the present work, human glucocorticoid receptor was partially purified in order to maintain high BPs binding activity. Previous researches suggested that GR must be associated with a complex of chaperone proteins for ligand activation [36, 37]. Hence, further purification would result in decreased activity, primarily due to loss of accessory proteins and regulators. A fluorescein-labeled dexamethasone derivative, which can bind glucocorticoid receptor [38, 39], was employed as a tracer to evaluate the functionality of the recombinant protein. GR with a range of concentrations were titrated versus a fixed concentration of Dex-fl (5 nM) and kinetic measurements for receptor-ligand binding were carried out. Figure 2 illustrated the dependence of fluorescence polarization as a function of GR concentrations. It can be observed that the FP values increased from 18 to 148 mP once the protein was added, reflecting the binding of Dex-fl to GR. The FP values reached a plateau when the GR concentration was about 30 nM, indicating that Dex-fl bound to GR in a saturable manner. The dissociation constant of Dex-fl with GR obtained from the binding curve was  $8.41 \pm 0.97$  nM.

### Assessment of BPs binding affinities with GR

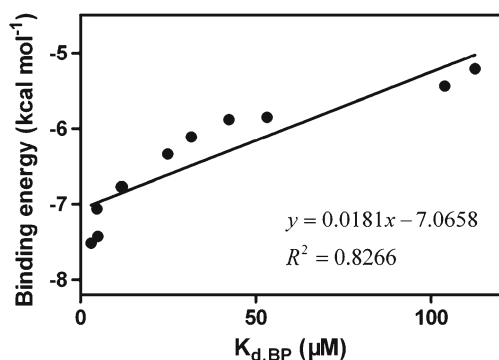
On the basis of the determination of  $K_{d,probe}$ , the binding potency of 11 BPs with GR was assessed quantitatively by competitive binding assay. A range of concentrations of BPs were

mixed with 5 nM Dex-fl and then added with 10 nM GR. Each sample was subjected to fluorescence polarization measurement after being incubated for 30 min at room temperature. If BPs could compete with probe for the protein binding site, they would displace Dex-fl from GR, leading to the decrease of FP values. As can be seen in Fig. 3 and in Fig. S1 in the Electronic Supplementary Material (ESM), all the bisphenol analogues, shared a common molecular structure of two hydroxyphenyl functionalities, exhibited dose-dependent binding to GR. The  $IC_{50}$  observed were in the order: BPP (1.81) < BPM (2.81) < BPZ (2.94) < BPC (6.92) < BPAP (7.12) < BPB (14.82) < BPA (18.82) < BPS (25.19) < BPE (31.65) < BPF (61.79) < BPAF (66.92). The dissociation constants ( $K_{d,BP}$ ) of the tested BPs with GR were calculated using the  $IC_{50}$  values obtained from the competition curves, as summarized in Table 1. In conclusion, BPs could bind to GR as the affinity ligands, resulting in the activation of receptor which would in turn adversely affect a series of physiological processes.

**Table 2** Molecular docking results of 11 BPs to GR

Chemical	Binding energy (kcal mol <sup>-1</sup> )	Hydrogen bonds
BPA	-6.11	Gln570, Arg611, Phe623, Gln642
BPAF	-5.21	Gln570, Arg611, Phe623, Gln642
BPAP	-6.77	Gln570, Arg611, Gln642
BPB	-6.34	Gln570, Met604, Arg611, Gln642
BPC	-6.77	Gln570, Met604, Arg611, Gln642
BPE	-5.85	Gln570, Met604, Arg611, Gln642
BPF	-5.44	Gln570, Met604, Arg611, Leu732
BPM	-7.06	Gln570, Arg611, Phe623, Gln642
BPP	-7.52	Asn564, Gln570, Arg611, Phe623
BPS	-5.88	Leu563, Asn564, Gln570, Arg611, Gln642
BPZ	-7.43	Leu563, Asn564, Thr739





**Fig. 5** Correlation between  $K_{d,BP}$  values and binding energies of BPs

### Molecular docking of Dex and BPs with GR

Molecular dynamics simulations are important tools for providing an atomic level insight to probe the recognition process of ligands toward receptors [40, 41]. In order to elucidate the binding modes between small-molecule ligands and glucocorticoid receptor, the interactions of Dex and 11 BPs with GR were modeled by molecular docking. Results show that the bottom half of the LBD completely encloses BPs in a hydrophobic ligand binding pocket. Along with hydrophobic interactions, hydrogen bonding and van der Waals forces appear to be key factors in ligand binding. As shown in Fig. 4 and Fig. S2 (see ESM), most of the BPs form a hydrogen bond with Gln642 to help stabilize their position in the binding pocket. The residue at this position has been reported to play a unique role in ligands recognition and binding [42, 43], which is further confirmed in this work. Hydrogen bonds that exist between BPs and other residues (such as Gln570, Met604, Arg611, and Phe623) can also help to enhance the binding force. Moreover, the helix 12 (Leu753) and the peptide loop (Phe749) preceding it interact directly with BPs through van der Waals forces. These interactions have a stabilizing effect on the orientation of the helix 12 in its active form, which is considered to modulate the functional response for ligand binding in GR [44–46]. Besides, several residues in the helix 11 (Leu732, Tyr735, Cys736, and Thr739) are also in direct contact with BPs. Despite the common molecular structure of two hydroxyphenyl, the characteristic functionalities, such as halogen, phenyl, sulfonyl, and cyclohexyl at the bridging alkyl moiety lead to the main structural differences of BPs. These chemical groups are positioned toward helix 12 and may be responsible for its conformational change. Previous research also indicates that various halogenation patterns result in different interactions of BPs with other nuclear receptors [47]. In summary, the structural variations of BPs distinctly affected their physicochemical properties and caused different binding modes and binding energies of BPs toward GR (Table 2). As shown in Fig. 5, the calculated binding energies were in good agreement with the measured  $K_{d,BP}$  values ( $R^2 =$

0.8266), demonstrating that the computational method could successfully predict the relative binding affinities of putative ligands with GR. Therefore, this work would provide valuable information for *in silico* pre-screening of BPA substitutes devoid of GR-mediated activities and for environmental risk assessment.

### Conclusion

In this work, the binding potency of BPA and its ten analogues with GR was evaluated by FP-based competitive binding assay. The tested compounds bound to GR with  $IC_{50}$  from 1.81 to 66.92  $\mu\text{M}$ , indicating that the binding affinities of bisphenols were largely dependent on their characteristic functional groups. Furthermore, the mechanism of toxicity action of BPs toward GR was illustrated by molecular dynamics simulations. Interestingly, the calculated binding energies correlated well with the experimentally determined dissociation constants of BPs, resulting in an  $R$ -squared value of 0.8266. These data may serve as a molecular basis for the BPs-dependent activation of GR. Considering that real samples may contain more than one BP, the proposed method can potentially be used as a high-throughput screening assay to determine the total concentrations of multiple bisphenols.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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