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Highly potent binding and inverse agonist activity of bisphenol A derivatives for retinoid-related orphan nuclear receptor RORγ

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HIGHLIGHTS

- We identified two potent RORγ binders, di-isopropyl-BPA and di-*sec*-butyl-BPA.
- Di-*sec*-butyl-BPA has emerged as a considerably potent binder (IC_{50} = 146 nM).
- They suppressed the basal constitutive transcriptional activity induced by RORγ.

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ABSTRACT

The plastic chemical bisphenol A (BPA) has recently been suspected to be a base structure of endocrine disrupting chemicals, which achieve their adverse effects by interfering with human nuclear receptors. For instance, BPA, bisphenol AF, and tetrabromo- or tetrachloro-BPA (X_4-BPA) have been characterized as binders for ERRγ, ER, and PPARγ, respectively. This ongoing string of findings has led to apprehension that some other BPA derivatives might also perturb important human nuclear receptors. The retinoid-related orphan receptor RORγ has been strongly suspected to be a target of highly hydrophobic chemical substances because of its extreme affinity for lipophilic sterols. In the present study, we tested a series of BPA derivatives for their ability to bind to RORγ, and identified two distinctly potent derivatives having isopropyl or *sec*-butyl groups at positions adjacent to the BPA-4-hydroxyl group. In particular, di-*sec*-butyl-BPA has emerged as a considerably potent ligand (IC_{50} = 146 nM). In the reporter gene assay, these compounds suppressed the basal constitutive transcriptional activity originally induced by wild-type RORγ. The present results strongly suggested that RORγ, and perhaps also RORα and RORβ, binds highly hydrophobic and sterically hindered chemical substances, inducing some unspecified physiological and biochemical disruptions.

1. Introduction

Although the estrogenic properties of bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, were first reported in 1936 (Dodds and Lawson, 1936), BPA has enjoyed long and widespread use as one of the key building blocks in consumer products that incorporate polycarbonate and epoxy resins. We recently found that BPA has a strong ability to disrupt normal endocrine functions via nuclear receptors (NRs). BPA binds very strongly to one of the human NRs, estrogen-related receptor γ (ERRγ) with K_d = 5.5 nM (Takayanagi et al., 2006; Matsushima et al., 2007; Okada et al., 2008). Since this constitutively active ERRγ is strongly expressed in the placenta and also in the fetal brain (Takeda et al., 2009), the adverse effects of BPA are a particularly great concern with respect to fetuses, infants, and children, who are especially vulnerable to chemicals. Also, we found that bisphenol AF (BPAF) functions as a full agonist for the estrogen receptor α (ERα), but as a highly specific antagonist for ERβ (Matsushima et al., 2010).

Riu et al. (2011) recently reported that halogenated BPA derivatives, such as tetrabromobisphenol A (tetrabromo-BPA) and tetrachlorobisphenol A (tetrachloro-BPA), bind to the peroxisome proliferator-activated receptor γ (PPARγ) and function as partial agonists. All these discoveries strongly suggest that BPA and its derivatives might function as latent endocrine disruptors via diverse NRs. It should be noted that so-called new-generation bisphenols have been enthusiastically designed and synthesized

Abbreviations: BP, bisphenol; ER, estrogen receptor; ERR, estrogen-related receptor; 25-HC, 25-hydroxycholesterol; LBD, ligand-binding domain; LBP, ligand-binding pocket; PPAR, peroxisome proliferator-activated receptor; RORγ, retinoid-related orphan receptors γ.

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in an effort to develop more sophisticated plasticwares and resins. Most of these bisphenols have not been examined for their environmental and human health and safety, and there is essentially no toxicity data on the exposure of, for example, pregnant women, fetuses, and newborns to these compounds.

Under these circumstances, there is much concern in regard to the retinoid-related orphan receptor RORγ as a potential target of highly hydrophobic chemical compounds because of its extreme affinity for sterols. Based on X-ray crystallographic studies, several selected chemicals, including cholesterols such as 25-hydroxycholesterol (25-HC), 20-hydroxycholesterol, and (3α,8α,22R)-cholest-5-ene-3,22-diol, have been shown to act as RORγ binders (Jin et al., 2010). Members of the ROR family, including RORα, RORβ, and RORγ belong to NR1, one of the groups of human NRs (Nuclear Receptor Nomenclature Committee, 1999), and their molecular mechanisms and physiological roles have been partly explored (Sun et al., 2000; Jetten and Joo, 2006; Dong, 2008; Jetten, 2009). If some BPA derivatives could bind to RORγ specifically, the physiological functions of RORγ might be decisively perturbed.

In the present study, by using tritium-labeled 25-HC, we first attempted to establish a receptor binding assay system for RORγ. The regression analysis of the saturation binding assay revealed that [^{3}H]25-hydroxycholesterol ([^{3}H]25-HC) indeed binds to RORγ considerably strongly with the dissociation constant K_d = 8.35 nM. Then, we further performed a competitive receptor-binding assay using [^{3}H]-25-HC as a tracer. Eventually, we discovered 2,2-bis(3-*sec*-butyl-4-hydroxyphenyl)propane, namely, di-*sec*-butyl-bisphenol (di-*sec*-butyl-BPA), and 2,2-bis(4-hydroxy-3-isopropyl-phenyl)propane, namely, di-isopropyl-bisphenol (di-isopropyl-BPA), as potential endocrine disruptor candidates against RORγ. We here describe the discrimination of a series of BPA derivatives in the binding assay and also the reporter-gene assay to test the activity of selected binders.

2. Methods

2.1. Materials

25-HC, T0901317, and 1,1-bis(4-hydroxyphenyl)ethane (BPE) were purchased from Sigma–Aldrich Co. (St. Louis, MO). The following compounds were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan): bisphenol A (BPA), 2,2-bis-(4-hydroxyphenyl)hexafluoropropane or bisphenol AF (BPAF), 1,1-bis(4-hydroxyphenyl)-1-phenylethane or bisphenol AP (BPAP), bis-(4-hydroxyphenyl)-diphenylmethane or bisphenol BP (BPBP), bis(4-hydroxyphenyl)-2,2-dichlorethylene or bisphenol C (BPC), di-isopropyl-BPA or bisphenol G (BPG), 1,3-bis(2-(4-hydroxyphenyl)-2-propyl)benzene or bisphenol M (BPM), 1,4-bis(2-(4-hydroxy-phenyl)-2-propyl)benzene or bisphenol P (BPP), 2,2-bis(2-hydroxy-5-biphenylyl)propane or di-phenyl-bisphenol A (di-phenyl-BPA) (di-phenyl-BPA is occasionally termed bisphenol PH (BPPH)), bis(4-hydroxyphenyl)sulfone or bisphenol S (BPS), 1,1-bis(4-hydroxyphenyl)-cyclohexane or bisphenol Z (BPZ), 2,2-bis(3-methyl-4-hydroxyphenyl)propane or di-methyl-bisphenol A (di-methyl-BPA), 2,2-bis(3-*sec*-butyl-4-hydroxyphenyl)propane or di-*sec*-butyl-BPA, and 2,2-bis(3-cyclohexyl-4-hydroxyphenyl)propane or di-cyclohexyl-bisphenol A (di-cyclohexyl-BPA). 2,2-Bis(3-*tert*-butyl-4-hydroxyphenyl)propane or di-*tert*-butyl-bisphenol A (di-*tert*-butyl-BPA) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the best grade available.

2.2. Preparation of receptor protein GST-fused RORγ-LBD

RORγ-LBD cDNA was amplified from a human kidney cDNA library (Clontech Laboratories, Mountain View, CA) by PCR using gene-specific primers and cloned into pGEX6P-1 (Amersham Biosciences, Piscataway, NJ). The glutathione *S*-transferase (GST)-fused receptor protein expressed in *E. coli* BL21α was purified on an affinity column of glutathione-Sepharose 4B (GE Healthcare Bio-Sciences Co., Piscataway, NJ) to obtain GST-RORγ-LBD. The glutathione used for elution of GST-RORγ-LBD from the column was removed by gel filtration on a column of Sephadex G-10 (15 mm × 100 mm; GE Healthcare Bio-Sciences) equilibrated with phosphate buffered saline (PBS) (pH 7.4), and the protein content (955 μg/mL) was estimated by the Bradford method using a Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan).

2.3. Saturation receptor binding assay for RORγ

The saturation binding assay of [^{3}H]25-hydroxycholesterol ([^{3}H]25-HC; 80 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) for the human RORγ was performed by using the GST-fused RORγ-LBD (0.1 μg). [^{3}H]25-HC was tested under the presence (final concentration of 10 μM) or absence of non-radiolabeled 25-HC. Free [^{3}H]25-HC was removed with 1% dextran-coated charcoal (DCC) (Sigma) by sufficient incubation followed by centrifugation (10 min, 14,000 rpm) at 4 °C. The radioactivity of the receptor–ligand complex was determined by a liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA). The specific binding of [^{3}H]25-HC was calculated by subtracting the non-specific binding from the total binding.

2.4. Competitive receptor binding assay for RORγ

The RORγ receptor-binding assay using purified GST-RORγ-LBD was carried out by the previously reported method with some modifications (Nakai et al., 1999; Okada et al., 2008). Sample solutions of varied concentrations (1.0×10^{-11} to 1.0×10^{-5} M) were mixed with the receptor protein and [^{3}H]25-HC (5 nM, final), and the resulting mixture was incubated for 2 h at 4 °C. Free radio-ligand was removed by treatment with 100 μl of 1% DCC in PBS (pH 7.4) for 10 min at 4 °C. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences, Boston, MA). The IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves evaluated by the nonlinear analysis program ALLFIT (De Lean et al., 1978). Each assay was repeated at least three times.

2.5. Cell culture and reporter gene assay

The reporter gene assay was performed basically as reported by Okada et al. (2008). COS-7 cells were utilized for this assay for RORγ according to the method reported by Jin et al. (2010). Cells were maintained in Dulbecco's modified Eagle's Medium (DMEM; Nissui, Tokyo, Japan) within the presence of 10% (v/v) fetal bovine serum at 37 °C, then seeded at 5×10^5 cells/6 cm dish for 24 h and transfected with 2 μg of reporter gene (pGL4.23/4×RORE) and 1 μg of RORγ expression plasmid (pcDNA3.1/RORγ) by using Lipofectamine Plus reagent (Invitrogen Japan, Tokyo) according to the manufacturer's protocol. Approximately 4 h after transfection, cells were harvested and plated into 96-well plates at 2×10^4 cells/well. The cells were then treated with varying doses of test chemicals diluted with 1% BSA/PBS. After 24 h, luciferase activity was measured using a Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instruction. Light emission was measured using a Multilabel Counter (Wallac 1420 ARVOsx; PerkinElmer). Each assay was performed in duplicate and repeated at least three times.

2.6. Computational structural analysis of RORγ and ligands

All molecular modeling studies were carried out using the Molecular Operating Environment (MOE; Chemical Computing Group, Montreal, Canada) package. The protein structure of the complex of RORγ-LBD and 25-HC was downloaded from the RCSB PDB (Protein Data Bank) (http://www.pdb.org/pdb/home/home.do) (PDB accession code: 3L0L) and hydrogen atoms were added correctly. An MMFF94x force field was employed to optimize the structure of the hydrogen atoms added. Test chemicals in 2D molecular files were changed to 3D PDB files on Chem3D (CambridgeSoft, Cambridge, MA), and then transferred to the database for MOE. MOE-ASEDock 2010 was applied for the docking calculation of the chemical/RORγ-LBD complex.

2.7. Statistical analysis

Data are presented as the mean ± SD for indicated number of separate experiments. Curve-fitting and statistical analyses were conducted by use of the nonlinear analysis program ALLFIT (De Lean et al., 1978) for the binding assay or GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA) for the reporter gene assay. For comparison of more than two groups, statistical analyses were performed by ANOVA with Dunnett's multiple comparison post hoc test. *P*-values less than 0.05 were considered to be significant.

3. Results and discussion

3.1. Binding activity of BPA and BPAF for RORγ

We first attempted to establish a competitive binding assay system by using [^{3}H]25-HC as a tracer. To this end, the saturation-binding assay was carried out using [^{3}H]25-HC for the GST-RORγ-LBD protein expressed (Fig. 1A). Fig. 1B shows the Scatchard plot analysis, which exhibited a distinct single binding mode. We analyzed the saturation binding data with nonlinear

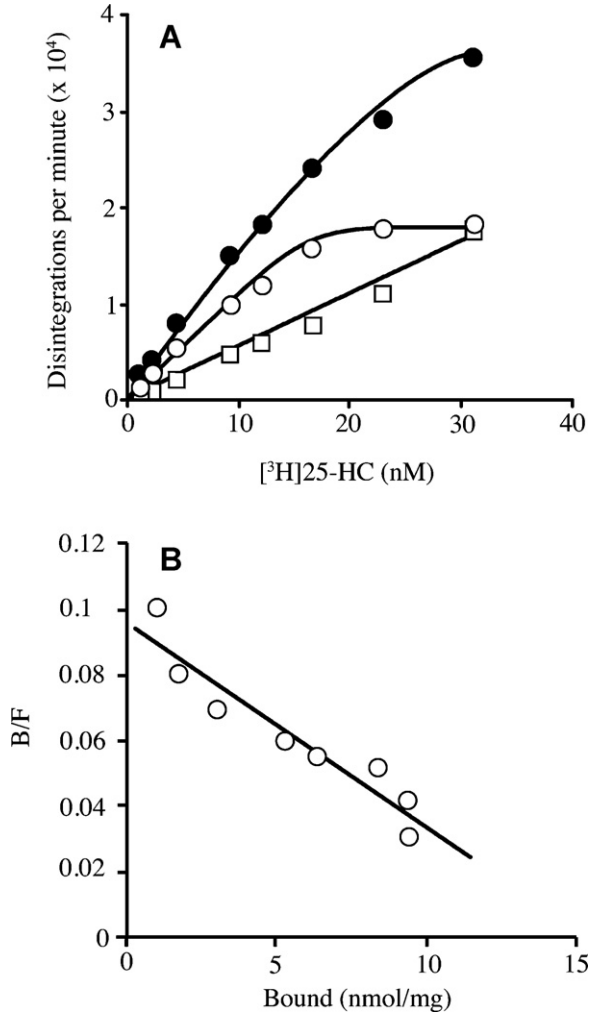

Fig. 1. Results of a saturation binding assay of 25-hydroxycholestreol (25-HC) for nuclear receptor RORγ. (A) Saturation binding curve of [^{3}H]25-hydroxycholesterol for the recombinant human GST-RORγ-LBD; total binding (closed circle), non-specific binding (open square), and specific binding (open circle) are shown. Estimation of the non-specific binding was carried out with excess unlabeled 25-HC (10 μM). (B) Binding data analyzed by a Scatchard plot analysis to estimate the dissociation constant (K_d) and the receptor density (B_{max}). Actual data were obtained by regression analysis of the specific binding curve, i.e., $K_d = 8.35 \pm 0.67$ nM and $B_{max} = 13.6 \pm 1.08$ nmol/mg protein. The saturation binding analysis was performed three times.

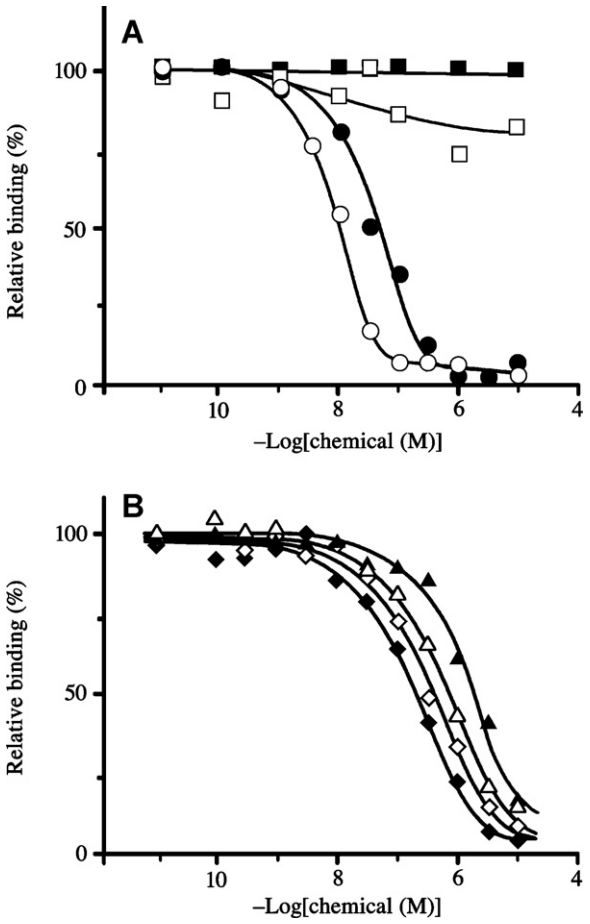

Fig. 2. Dose–response curves of the radioligand receptor binding assay for human RORγ. (A) RORγ receptor binding activities of 25-hydroxycholestreol (25-HC; open circle), T0901317 (closed circle), BPA (closed square), and BPAF (open square). The heterogeneous competitive binding assays were performed by using [^{3}H]25-HC and GST-RORγ-LBD, and at least five independent assays were carried out for each compound. (B) Dose–response curves of BPA derivatives possessing binding activity to human RORγ in the radioligand receptor-binding assay. RORγ receptor binding activities of di-phenyl-BPA (open triangle), di-*tert*-butyl-BPA (closed triangle), di-isopropyl-BPA (open rhombus), and di-*sec*-butyl-BPA (closed rhombus). Binding activities of the BPA derivatives examined by the competitive binding assay using [^{3}H]25-HC and GST-RORγ-LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

regression. The dissociation constant (K_d) was calculated to be 8.35 ± 0.67 nM, while the receptor density (B_{max}) was estimated to be 13.6 ± 1.08 nmol/mg protein with an almost 1:1 stoichiometry. The K_d value obtained for [^{3}H]25-HC in this study was almost equal to those reported by others (Kumar et al., 2010; Wang et al., 2010). These results clearly show that [^{3}H]25-HC can be utilized as a tracer in the competitive binding assay for RORγ receptor.

We first carried out a homologous competitive receptor-binding assay using [^{3}H]25-HC for GST-RORγ-LBD (Fig. 2A) (Table 1). The IC_{50} value of non-tritium labeled 25-HC was estimated to be 7.3 ± 1.0 nM in this competitive binding assay. This result indicates that used GST-RORγ-LBD is sound as a receptor protein and can be used for the heterogenous competitive receptor-binding assay.

We next tested the parent standard reference compounds in this study, namely, BPA and BPAF, in the competitive binding assay (Fig. 2A). BPA was highly specific for ERRγ, but almost completely inactive for RORγ. Also, BPAF, a strong binder for both ERα and ERβ, was also nearly inactive for RORγ. Since T0901317, a RORγ inverse agonist (Kumar et al., 2010), was evaluated adequately in this binding assay ($IC_{50} = 36.5 \pm 6.5$ nM), the present binding assay system was judged to be sound and reliable for evaluation of a full series of BPA derivatives, which we wanted to weigh for their binding potential to RORγ.

3.2. Binding activity of BPA derivatives for RORγ receptor

To evaluate a series of BPA derivatives for their ability to bind to RORγ receptor, we tested 15 representative compounds by the competitive binding assay established above. In this heterogeneous RORγ receptor-binding assay to use [^{3}H]25-HC, we set T0901317 as a reference compound in addition to non-tritium labeled 25-HC. Fifteen BPA derivatives were grouped into three different categories based on their structures (Fig. 3), i.e., (1) derivatives with substituents at the central sp^3-carbon moiety of BPA, (2) derivatives with substituents between two phenol structural cores, and (3) derivatives with substituents at the phenol-benzene rings.

When category 1 compounds were assayed, it became evident that any substitutions at the central sp^3-carbon moiety of BPA failed to create a compound with the ability to bind RORγ. BPAP, BPBP, BPE, BPF, and BPZ were found to be almost completely inactive

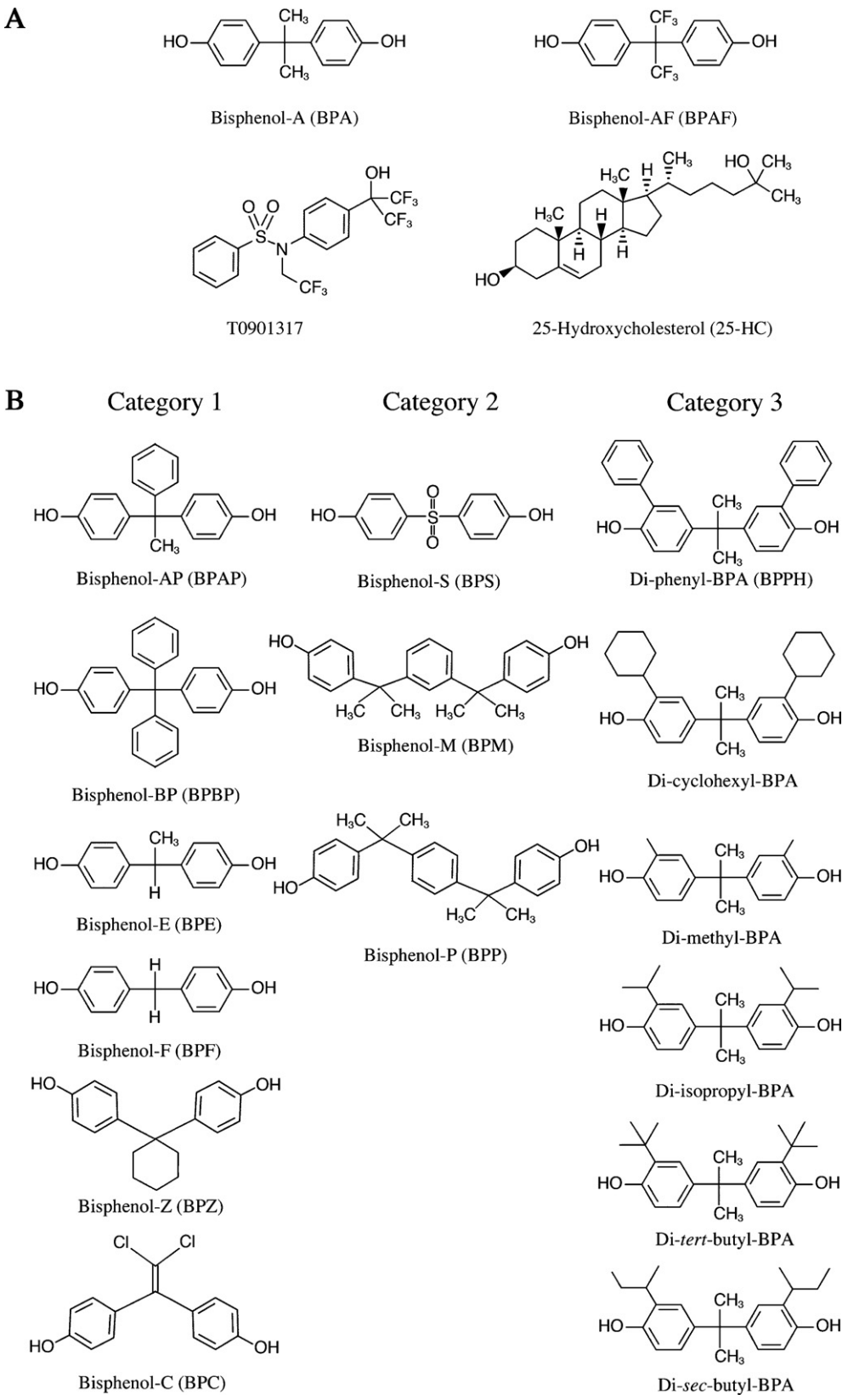

Fig. 3. Structural formulae of chemicals assayed in this study. (A) Chemical structure of BPA, BPAF, T0901317, and 25-hydroxycholestreol (25-HC). (B) Chemical structure and grouping of representative BPA derivatives and classification of 15 representative BPA analogs. Category 1: Derivatives with substituents at the central sp^3-carbon moiety of BPA. Category 2: Derivatives with substituents between two phenol structural cores. Category 3: Derivatives with substituents at the phenol-benzene rings. The chemicals are abbreviated as BPX, where X indicates the individual derivatives.

Table 1
Receptor binding affinity of bisphenol A (BPA) and its derivatives.

Chemicals	IC_{50} (nM)
25-HC	7.3 ± 1.0
T0901317	36.5 ± 6.5
BPA	N.B.
BPAF	N.B.
Category 1	
BPAP	N.B.
BPBP	N.B.
BPE	N.B.
BPF	N.B.
BPZ	N.B.
BPC	N.B.
Category 2	
BPS	N.B.
BPM	>3000
BPP	>3000
Category 3	
di-cyclohexyl-BPA	>3000
di-methyl-BPA	>3000
di-isopropyl-BPA	557 ± 58
di-phenyl-BPA	1100 ± 120
di-*sec*-butyl-BPA	146 ± 32
di-*tert*-butyl-BPA	2230 ± 250

The heterogeneous competitive binding assay was performed by using GST-fused RORγ-LBD and a tracer [^{3}H]25-HC. Assays were repeated at least three times and the data are shown with the mean S.D.
N.B. means that compounds did "not bound" even at their high concentration (1.0×10^{-5} M), where those compounds exhibited the displacement much less than 50%.

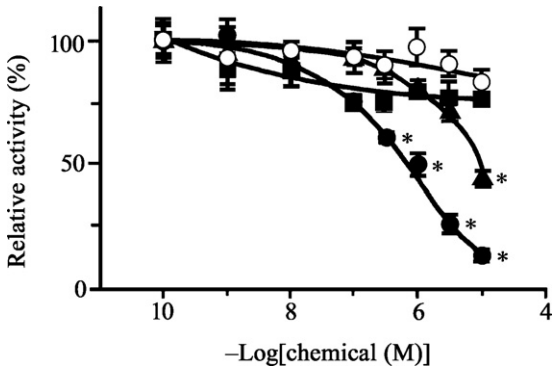

Fig. 4. Luciferase-reporter gene assay of the BPA derivatives for human RORγ. Deactivation of the fully activated human RORγ by 25-HC (open circle), T0901317 (closed circle), di-*sec*-butyl-BPA (closed triangle), and di-methyl-BPA (closed square). Data are from a single experiment performed in triplicate; two additional experiments gave similar results. Basal constitutive activity of RORγ was evaluated with the luciferase-reporter plasmid (pGL3/4×RORE), and the highest activity was estimated in a cell preparation of 1.0×10^5 COS-7 cells/well. Even high concentration of test chemicals (10 μM), significant cell toxicity did not observed in this assay. As a control, the level of transcription activity without treatment of any ligands was assigned as 100%. Relative transcription activity levels in treated samples are shown as the mean ± SD for nine independent experiments. Data points marked with asterisks are statistically significant as compared with the control transactivation (*$P < 0.05$).

(Table 1). Even at their high concentration (1.0×10^{-5} M), these compounds could not sufficiently displace [^{3}H]25-HC in RORγ: their displacement activity at this 10 μM concentration was far less than 50%. BPAP and BPBP have one and two phenyl groups as substituents, but these phenyl-benzene rings did not function as a structure that could reinforce the binding ability. Bisphenol C (BPC), which possesses an sp^2-dichloroethylene group at the central isopropyl moiety of BPA, also did not bind to RORγ. All these results indicate that, at the region corresponding to the sp^3-carbon moiety of BPA, the RORγ ligand binding pocket does not hold structural elements which bind BPA derivatives.

BPS, BPM, and BPP are compounds classified into the category 2 (Fig. 3). BPS, in which two phenol groups are cross-linked by a sulfone group (–SO_2–), was found not to be a binder of RORγ. BPS exhibited only about 10% activity at its 10 μM concentration. BPM and BPP are structural isomers, having a structure in which two phenol groups are cross-linked by 1,4-diisopropylbenzene (Fig. 3). The binding activities of BPM and BPP were also quite limited, showing only about 50% displacement of [^{3}H]25-HC at their 10 μM concentration. These results indicate that the introduction of an electron-rich substituent, such as a structural core made up of sulfone or benzene, cannot work to elicit the ability to bind to RORγ.

Category 3 compounds possessing substituents at the *ortho* position of the BPA's 4-hydroxyl group include di-isopropyl-BPA (Fig. 3). In contrast to the inactive compounds of category 1 and 2, di-isopropyl-BPA was found to exhibit specific and potent binding to RORγ. It showed an IC_{50} value of 557 ± 58 nM, exhibiting the full binding activity for this RORγ receptor (Fig. 2B). When di-*sec*-butyl-BPA was assayed, the activity was strengthened further. The IC_{50} value of di-*sec*-butyl-BPA was 146 ± 32 nM, approximately four times more potent than di-isopropyl-BPA (Table 1). This enhanced binding activity was probably due to its considerably high hydrophobicity. Thus, we next tested di-*tert*-butyl-BPA and di-phenyl-BPA.

It should be noted that di-phenyl-BPA was found to be almost equipotent to di-isopropyl-BPA (Fig. 2B). This means that the aryl group, or phenyl-benzene ring, does not interfere at all with the binding to RORγ-LBP. Although di-*tert*-butyl-BPA was slightly less potent than these compounds, it was judged to be almost equally potent for binding to the receptor. The *tert*-butyl group might work as an element to cause a steric hindrance against the adjacent phenol-hydroxyl group.

The chemical structures of the isopropyl and *sec*-butyl are –$CH(CH_3)_2$ and –$CH(CH_3)CH_2CH_3$, respectively. As a result, the binding assay clearly indicated that the branched ethyl group is much better than the branched methyl group to interact with the receptor residues in RORγ-LBP. This further suggested that non-branched single methyl group on the BPA backbone would result in poorer binding to the receptor. In fact, di-methyl-BPA was found to be essentially inactive. On the other hand, we highly expected that di-cyclohexyl-BPA would be superior to di-isopropyl-BPA and di-*sec*-butyl-BPA in binding to RORγ. This is because cyclohexyl has a chemical structure in which two of the branched ethyl groups are cross-linked by the methylene group, CH_2. However, di-cyclohexyl-BPA exhibited extremely limited activity, just as BPM and BPP did, in the competitive binding assay for RORγ. These results strongly suggested that the important structural feature for better interaction with the RORγ-LBP is not only the hydrophobicity, but also the size and flexibility of the groups attached to the phenol.

3.3. Inverse agonist activity of alkylated BPA derivatives for RORγ

To evaluate the biological activity of compounds identified as RORγ binders, we carried out a luciferase reporter gene assay. In our assay system, RORγ showed a full transcription activity with no ligand. Such a constitutive activity is characteristic for some NRs, and RORγ is one of the so-called self-activated NRs. When we administered 25-HC, which has been believed as a ligand of RORγ, it induced no effect at all within an ordinary range of concentration (10^{-10}–10^{-5} M) in this reporter gene assay. 25-HC did not cause any reduction and increase in the relative activity (Fig. 4).

Inverse agonists deactivate constitutive active NRs as seen, for instance, in the activity of 4-OHT against ERRγ (Greschik et al., 2004; Takayanagi et al., 2006). Since T0901317 has been reported to be an inverse agonist of RORγ, we tested this compound in our assay system. As shown in Fig. 4, T0901317 inhibited a RORγ's constitutive activity in a dose-dependent manner, indicating that T0901317 is a genuine inverse agonist of RORγ. T0901317 indeed deactivated

RORγ effectively as expected for inverse agonist and this is a reason for choosing only the compound T0901317 as a model for the reporter gene assay.

We next tested di-*sec*-butyl-BPA, which was identified as a potential RORγ binder in the present study. As the concentration of di-*sec*-butyl-BPA was increased, the transcription activity of RORγ clearly declined (Fig. 4), as observed for T0901317. This is a clear demonstration that di-*sec*-butyl-BPA is an inverse agonist of RORγ. Di-isopropyl-BPA, which is also identified as an RORγ binder, exhibited clear, but slightly weaker inverse agonist activity than di-*sec*-butyl-BPA. At the concentration of 1×10^{-5} M, di-isopropyl-BPA exhibited almost 60% diminished transcription activity. Although these two BPA derivatives were judged to be inverse agonists for RORγ, their inhibition abilities were somehow weaker than that of T0901317; both did not show complete suppression of the transcription activity (Fig. 4). However, the distinctiveness of their inverse agonist activity became clear when di-methyl-BPA was examined in the same reporter gene assay. Di-methyl-BPA was almost completely inactive even at the concentration of 1×10^{-5} M, although it was found to bind RORγ very weakly at that concentration (Fig. 4).

3.4. Structural characteristics of BPA derivatives in their binding to RORγ

In this study, we newly discovered that di-*sec*-butyl-BPA functions as an inverse agonist for RORγ. Since di-*sec*-butyl-BPA shows considerably strong binding to RORγ, we performed *in silico* docking modeling for this combination to better understand the structural characteristics of its binding to RORγ. To this end, we used the docking module ASE-dock on MOE. When the volume of RORγ's ligand binding pocket (LBP), 431.3 Å^3, was compared with that of BPA, 187.6 Å^3, RORγ-LBP was expected to retain or capture at least one molecule of BPA derivatives, but no more than one molecule of BPA derivatives because of the sizeable volume of substituents attached to the molecule. As a template of RORγ for docking modeling, 3L0L (PDB accession code) was utilized after removal of a docked ligand 25-HC.

When the docking modeling calculation was achieved for BPA derivatives and RORγ, it immediately became evident that RORγ can hold just one molecule of di-*sec*-butyl-BPA. In a calculated binding structure of the di-*sec*-butyl-BPA-RORγ complex, di-*sec*-butyl-BPA was found to be situated at almost the same position, where 25-HC sits. As shown in Fig. 5, di-*sec*-butyl-BPA covers the space originally occupied by 25-HC. One of the most impressive structural features of this complex is that both of the *sec*-butyl groups are surrounded by a variety of hydrophobic amino acid residues. These include, for instance, Gln286, Leu287, Ala327, Val361, Arg364, Arg367, and Ala368, for one of the *sec*-butyl groups, and Cys320, Leu324, Phe378, Phe388, Leu391, and Ile397 for the other one. Apparently, these hydrophobic groups function to retain the BPA derivatives in a binding pocket, and the *sec*-butyl groups in particular are a major force for the interaction with those hydrophobic groups.

One of the characteristic structural features of 25-HC is the presence of two hydroxyl groups on the C3 carbon atom in the steroidal backbone and on the C25 carbon atom in the side-chain alkyl group. It should be noted that the hydroxyl group on C25 is in a hydrogen bond with the RORγ-Tyr502-phenol hydroxyl group via the water molecule (Fig. 5). BPA derivatives also have two hydroxyl groups at the symmetrical positions on their phenol groups. However, di-*sec*-butyl-BPA is not involved in hydrogen bonding, as seen for 25-HC-C25 hydroxyl in the docking modeling structure. As shown in Fig. 5, di-*sec*-butyl-BPA does not have any hydrogen bonds with Tyr502.

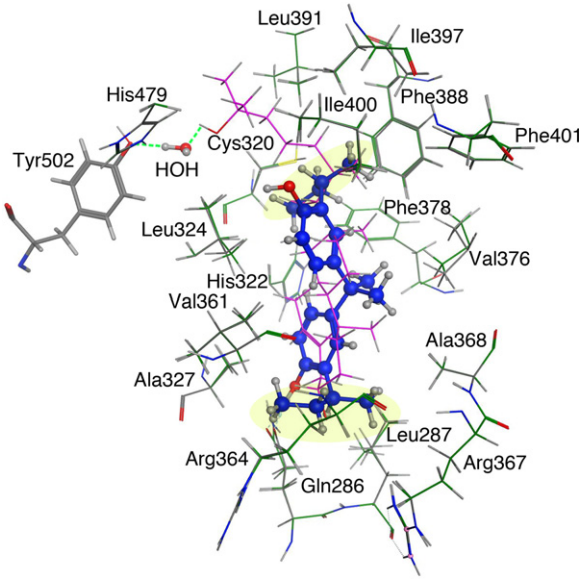

Fig. 5. Estimation of the binding structure of di-*sec*-butyl-BPA by docking modeling. The putative docking model of di-*sec*-butyl-BPA with RORγ-LBD was obtained by the docking calculation using ASE-Dock 2010 on the MOE package. Di-*sec*-butyl-BPA is indicated by a ball_and_stick model (blue) and 25-HC, the genuine ligand of the complex used in the docking calculation, is shown by a stick model (magenta). Two *sec*-butyl groups of di-*sec*-butyl-BPA are highlighted in yellow. The hydrogen bond between 25-HC and Tyr502 is displayed by a green dotted line.

Tyr502 is present in the helix 12 of RORγ-LBD, which is crucially important to form an activation conformation of RORγ. The H_2O-mediated hydrogen bond between the 25-HC-C25-hydroxyl and Tyr502-hydroxyl groups appears to be an anchor to retain this activation conformation of RORγ-LBD. Therefore, a lack of the hydrogen bonding in the di-*sec*-butyl-BPA binding model must be a cause of the inverse agonist activity of di-*sec*-butyl-BPA, eliciting a release of helix 12 from the receptor activation conformation.

We discovered several fully active bisphenol derivatives in this study. As mentioned above, in binding to RORγ, the most active derivative was di-*sec*-butyl-BPA, whereas non-substituted bare bisphenol A (BPA) was almost completely inactive. This very contrary result clearly indicates that the *sec*-butyl group at the 3-position of BPA-phenyl is a key structural element in the binding to RORγ. Among BPA derivatives assayed for RORγ in this study, the order of binding activity is di-*sec*-butyl > di-isopropyl ≥ di-phenyl > di-*tert*-butyl ≫ di-methyl, di-cyclohexyl. The striking activity difference between di-*sec*-butyl-BPA and di-cyclohexyl-BPA indicates that RORγ-LBP possesses a subtle structure that can distinguish the structural differences between the di-*sec*-butyl and di-cyclohexyl groups.

Recent advanced technologies for highly functional plastics are focusing especially on development of processing and manufacturing, such as raw materials, equipments, and technologies. It is marvelous to know that a number of BPA derivatives have been newly developed as raw materials for such highly-functional plastics, but most of those novel derivatives are free from any risk assessments. Our present findings that di-*sec*-butyl-BPA and di-isopropyl-BPA bind considerably strongly to RORγ and affect its receptor function clearly alert the riskiness of BPA derivatives in human. All the novel materials including BPA derivatives should be served for effective risk assessments.

4. Conclusion

In the present study, we discovered significantly potent RORγ-binding chemicals that act as inverse agonists. These RORγ-binders

were characterized by rather larger aliphatic groups of *sec*-butyl or isopropyl at the position adjacent to the BPA's hydroxyl group. Because of such structural features, careful attention should be focused on as-yet-to-be-identified chemicals for the possible disruption of the RORγ receptor functions. In particular, the repeated discoveries of BPA derivatives for various NRs as potential active ligands indicate the necessity for prompt inspection of BPA derivatives for all NRs.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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