

# Bisphenol AF Is a Full Agonist for the Estrogen Receptor ER $\alpha$ but a Highly Specific Antagonist for ER $\beta$

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**BACKGROUND:** Bisphenol AF has been acknowledged to be useful for the production of CF<sub>3</sub>-containing polymers with improved chemical, thermal, and mechanical properties. Because of the lack of adequate toxicity data, bisphenol AF has been nominated for comprehensive toxicological characterization.

**OBJECTIVES:** We aimed to determine the relative preference of bisphenol AF for the human nuclear estrogenic receptors ER $\alpha$  and ER $\beta$  and the bisphenol A-specific estrogen-related receptor ERR $\gamma$ , and to clarify structural characteristics of receptors that influence bisphenol AF binding.

**METHODS:** We examined receptor-binding activities of bisphenol AF relative to [<sup>3</sup>H]17 $\beta$ -estradiol (for ER $\alpha$  and ER $\beta$ ) and [<sup>3</sup>H]bisphenol A (for ERR $\gamma$ ). Functional luciferase reporter gene assays were performed to assess receptor activation in HeLa cells.

**RESULTS:** We found that bisphenol AF strongly and selectively binds to ERs over ERR $\gamma$ . Furthermore, bisphenol AF receptor-binding activity was three times stronger for ER $\beta$  [IC<sub>50</sub> (median inhibitory concentration) = 18.9 nM] than for ER $\alpha$ . When examined using a reporter gene assay, bisphenol AF was a full agonist for ER $\alpha$ . In contrast, it was almost completely inactive in stimulating the basal constitutive activity of ER $\beta$ . Surprisingly, bisphenol AF acted as a distinct and strong antagonist against the activity of the endogenous ER $\beta$  agonist 17 $\beta$ -estradiol.

**CONCLUSION:** Our results suggest that bisphenol AF could function as an endocrine-disrupting chemical by acting as an agonist or antagonist to perturb physiological processes mediated through ER $\alpha$  and/or ER $\beta$ .

**KEY WORDS:** bisphenol A, bisphenol AF, endocrine disruptor, estrogen receptors, receptor antagonist, receptor binding. *Environ Health Perspect* 118:1267–1272 (2010). doi:10.1289/ehp.0901819 [Online 28 April 2010]

Bisphenol AF (also referred to as hexafluoro-bisphenol A) is a homolog of bisphenol A (BPA) (Figure 1). Bisphenol AF has a symmetrical chemical structure of HO–C<sub>6</sub>H<sub>4</sub>–C(CF<sub>3</sub>)<sub>2</sub>–C<sub>6</sub>H<sub>4</sub>–OH and is designated as 1,1,1,3,3,3-hexafluoro-2,2-bis(4-hydroxyphenyl)propane by IUPAC (International Union of Pure and Applied Chemistry) nomenclature. Bisphenol AF-containing polymers such as polycarbonate copolymers, polyimides, polyamides, and polyesters are used in high-temperature composites, electronic materials, and gas-permeable membranes. Bisphenol AF is also used in many other specialty polymer applications, including plastic optical fibers and waveguides. Although industrial production of bisphenol AF seems to be increasing considerably, no data are available on annual production or concentrations of bisphenol AF in environmental substrates.

In 2008, the U.S. National Institute of Environmental Health Sciences nominated bisphenol AF for comprehensive toxicological characterization based on the lack of adequate toxicity data [National Toxicology Program (NTP) 2008a]. In this nomination report, the NTP noted concern regarding potential exposure of the general population to bisphenol AF. Structural dissimilarities between bisphenol AF and BPA are determined by the presence of a trifluoromethyl (CF<sub>3</sub>) or methyl

(CH<sub>3</sub>) group, respectively. The potential toxicity of bisphenol AF is of concern in part because its CF<sub>3</sub> group is much more electronegative (and potentially reactive) than is the CH<sub>3</sub> group of BPA.

Various “low-dose effects” of BPA have recently been reported *in vivo* for reproductive organ tissues in mice and rats. For example, *in utero* exposures to very low levels of BPA have been shown to increase the size and weight of the fetal mouse prostate (Gupta 2000; Nagel et al. 1997), and low-dose exposures have also been reported to decrease daily sperm production and fertility in male mice (Gupta 2000; vom Saal et al. 1998). Many lines of evidence have recently indicated that low doses of BPA affect the central nervous system as well (vom Saal and Welshons 2005; Welshons et al. 2003, 2006). All of these low-dose effects of BPA have been attributed to effects on steroid hormone receptors such as estrogen receptor (ER) and androgen receptor (AR) (Welshons et al. 2003; Xu et al. 2005). In the report by the NTP (2008b) on the potential for BPA exposure to affect human reproduction or development, “some concern” was indicated as the level of concern for potential effects on the brain, behavior, and the prostate gland.

BPA exhibits extremely weak binding activity for ER and AR. Based on the idea that

BPA may interact with nuclear receptors (NRs) other than ER and AR, we screened a series of NRs and eventually discovered estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) as the BPA target receptor (Takayanagi et al. 2006). BPA binds to ERR $\gamma$  very strongly [dissociation constant ( $K_d$ ) = 5.5 nM] with high constitutive basal activity (Liu et al. 2007; Okada et al. 2008; Takayanagi et al. 2006). Strong binding of BPA to ERR $\gamma$  was further demonstrated by direct X-ray crystallographic analysis of this complex (Matsushima et al. 2007, 2008). Moreover, using real-time PCR (polymerase chain reaction), we recently demonstrated that human ERR $\gamma$  mRNA is expressed abundantly in the placenta, prostate, and fetal brain (Takeda et al. 2009).

Our efforts to explore the target receptor of BPA suggested that it is essential to examine endocrine chemicals for interactions with all 48 human NRs. We previously reported that bisphenol AF binds to ER $\alpha$  more strongly than does BPA, and that the receptor selectivity of bisphenol AF is seven times higher for ER $\alpha$  than for ERR $\gamma$  (Okada et al. 2008). There are two subtypes of estrogen receptors, ER $\alpha$  and ER $\beta$ , with distinctly different physiological distributions and functions. Because effects of a number of chemicals have been reported to differ between ER $\alpha$  and ER $\beta$  (Harris et al. 2003; Manas et al. 2004), it is important to examine the effects of bisphenol AF on both ERs. In the present study, we evaluated the binding activity and functional biological activity of bisphenol AF for ER $\beta$  and found that bisphenol AF is a potent ligand that functions as an antagonist on ER $\beta$ .

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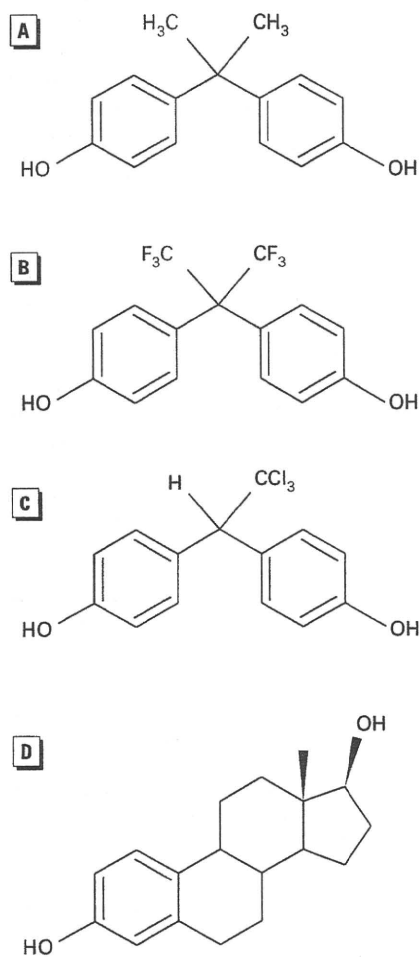
The authors declare they have no actual or potential competing financial interests.

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## Materials and Methods

**Test compounds.** We obtained 17 $\beta$ -estradiol (CAS no. 50-28-2; 98.9%) from Research Biochemicals International (Natick, MA, USA), and BPA (CAS no. 80-05-7; purity 99%) and bisphenol AF (CAS no. 1478-61-1; purity 99%) from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 4-Hydroxytamoxifen (4-OHT; CAS no. 68047-06-3; purity 98%) and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

**Preparation of glutathione S-transferase-(GST)-fused NR ligand-binding domain (LBD) protein.** cDNA clones of ER $\alpha$  and ER $\beta$  were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). GST-fused receptor LBDs expressed in *Escherichia coli* BL21 $\alpha$  (GST-ER $\alpha$ -LBD, GST-ER $\beta$ -LBD, and GST-ERR $\gamma$ -LBD) were purified on an affinity column of glutathione-Sepharose 4B (GE Healthcare BioSciences Co., Piscataway, NJ, USA) followed by gel filtration on a Sephadex G-10 column (15  $\times$  10 mm; GE Healthcare BioSciences).



**Figure 1.** Chemical structures of (A) BPA, (B) bisphenol AF, (C) 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), and (D) 17 $\beta$ -estradiol.

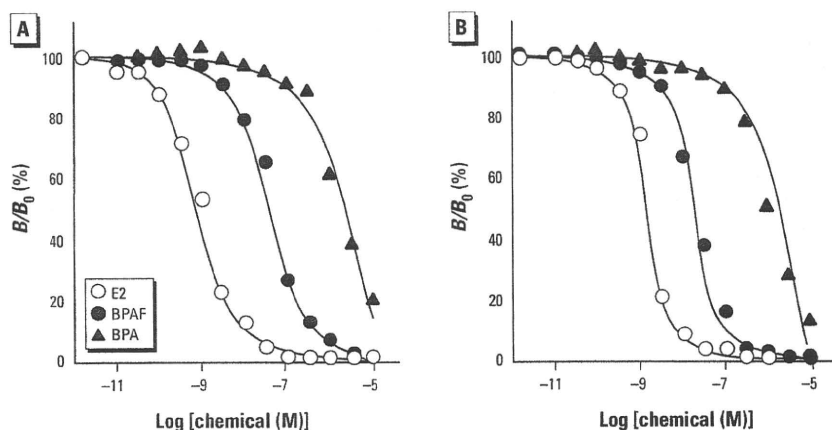
**Radioligand binding assays for saturation binding.** We conducted the saturation binding assays for ER $\alpha$  and ER $\beta$  essentially as reported by Nakai et al. (1999) using tritium-labeled ligand [<sup>3</sup>H]17 $\beta$ -estradiol (5.96 TBq/mmol; GE Healthcare UK Ltd., Buckinghamshire, UK). Receptor protein GST-ER $\alpha$ -LBD or GST-ER $\beta$ -LBD (0.3 nM) was incubated with increasing concentrations of [<sup>3</sup>H]17 $\beta$ -estradiol (0.1–30 nM) in a final volume of 100  $\mu$ L binding buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate(V), 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 10% glycerol; pH 7.4). Nonspecific binding was determined in a parallel set of incubations that included 10  $\mu$ M nonradiolabeled 17 $\beta$ -estradiol. After incubation for 2 hr at 20°C, free radioligand was removed by incubation with 0.4% dextran-coated charcoal (Sigma-Aldrich Inc.) in phosphate-buffered saline (PBS; pH 7.4) for 10 min on ice and then centrifuged for 10 min at 15,000 rpm.

We performed the saturation binding assay for ERR $\gamma$  as reported previously (Okada et al. (2008) using [<sup>3</sup>H]BPA (5.05 TBq/mmol; Moravek Biochemicals, Brea, CA, USA). Specific binding of tritium-labeled ligand was calculated by subtracting the nonspecific binding from the total binding. Receptor proteins that were expressed and purified were evaluated in a saturation binding assay to estimate  $K_d$  and

receptor density ( $B_{max}$ ), and only good-quality preparations with appropriate  $K_d$  and  $B_{max}$  were used for competitive receptor-binding assays.

**Radioligand binding assays for competitive binding.** Bisphenol AF, BPA, 17 $\beta$ -estradiol, and 4-OHT were dissolved in 0.3% DMSO in 1% bovine serum albumin (BSA; a blocker of nonspecific adsorption to the reaction vessels). HPTE was tested as a reference compound that acted as an ER $\alpha$  agonist and an ER $\beta$  antagonist. These chemicals were examined for their ability to inhibit the binding of [<sup>3</sup>H]17 $\beta$ -estradiol (5 nM in final) to GST-ER $\alpha$ -LBD (26 ng) and GST-ER $\beta$ -LBD (26 ng). The reaction mixtures were incubated overnight at 4°C, and free radioligand was removed with 1% dextran-coated charcoal by filtration. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences Japan, Tokyo, Japan). We calculated the half-maximal inhibitory concentrations ( $IC_{50}$ ) for 17 $\beta$ -estradiol from dose–response curves obtained using the nonlinear analysis program ALLFIT (DeLean et al. 1978). Each assay was performed in duplicate and repeated at least five times. For reconfirmation, we also performed the binding assay for ERR $\gamma$  using [<sup>3</sup>H]BPA (5 nM final concentration) and GST-ERR $\gamma$ -LBD (26 ng).

**Luciferase reporter gene assay.** HeLa cells were maintained in Eagle's minimum essential medium (MEM; Nissui, Tokyo, Japan)



**Figure 2.** Radioligand receptor-binding assays of bisphenol AF (BPAF), BPA, and 17 $\beta$ -estradiol (E2) to measure the ability of the compounds to displace [<sup>3</sup>H]17 $\beta$ -estradiol in recombinant human ER $\alpha$  (A) and ER $\beta$  (B).  $B/B_0$ , sample bound/maximum binding. The representative dose-dependent binding curves show the  $IC_{50}$  value closest to the mean  $IC_{50}$  from at least five independent assays. The  $IC_{50}$  values showed a between-experiment coefficient of variation of 5–12%.

**Table 1.** Receptor-binding characteristics of BPA and bisphenol AF for ER $\alpha$ , ER $\beta$ , and ERR $\gamma$ .

Compound	$IC_{50}$ (nM)		
	ER $\alpha$	ER $\beta$	ERR $\gamma$
17 $\beta$ -estradiol	0.88 $\pm$ 0.04	2.17 $\pm$ 0.12	NB
4-OHT	2.88 $\pm$ 0.15	3.17 $\pm$ 0.24	10.3 $\pm$ 0.8
BPA	1,030 $\pm$ 70	900 $\pm$ 70	9.70 $\pm$ 0.59
Bisphenol AF	53.4 $\pm$ 3.1	18.9 $\pm$ 0.84	358 $\pm$ 3.1
HPTE	59.1 $\pm$ 1.5	18.1 $\pm$ 1.9	36.4 $\pm$ 4.4

Abbreviations: HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; NB, not bound (no significant receptor binding at 10  $\mu$ M, the highest concentration tested).

in the presence of 10% (vol/vol) fetal bovine serum at 37°C. For luciferase assays, HeLa cells were seeded at  $5 \times 10^5$  cells per 6-cm dish for 24 hr and then transfected with 4  $\mu$ g reporter gene (pGL3/3xERE) and 3  $\mu$ g of ER $\alpha$  or ER $\beta$  expression plasmid (pcDNA3/ERs) by Lipofectamine Plus reagent (Invitrogen Japan, Tokyo, Japan) according to the manufacturer's protocol. Approximately 24 hr after transfection, cells were harvested and plated into 96-well plates at  $5 \times 10^4$  cells/well. The cells were then treated with varying doses of chemicals diluted with 1% BSA/PBS (vol/vol). After 24 hr, luciferase activity was measured with the appropriate reagent using a Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Light emissions were measured using a Wallace 1420 ARVOsx multilabel counter (PerkinElmer). Cells treated with 1% BSA/PBS were used as a vehicle control. Each assay was performed in triplicate and repeated at least three times. The assay for ERR $\gamma$  was carried out as previously reported (Okada et al. 2008).

To measure the antagonistic activity of bisphenol AF for ER $\beta$ , we examined four concentrations (0.01, 0.1, 1.0, and 10  $\mu$ M) of bisphenol AF for a serial concentration of 17 $\beta$ -estradiol ( $10^{-12}$  to  $10^{-5}$  M in the final solution). Also, a serial concentration of bisphenol AF ( $10^{-12}$  to  $10^{-5}$  M in the final

solution) was assayed in the presence of 10 or 100 nM concentrations of 17 $\beta$ -estradiol, which normally elicit full activation of ER $\beta$ .

## Results

**Strong binding activity of bisphenol AF to ER $\beta$  receptor.** We selected receptor protein preparations suitable for the competitive receptor-binding assay based on Scatchard plot analyses of saturation-binding assays. Receptor populations with the appropriate dissociation constant ( $K_d$ ) and receptor density ( $B_{max}$ ) were used for each radioligand receptor-binding assay. Because all of the NRs are secreted protein preparations, observed  $B_{max}$  values were comparable with those calculated from their molecular weight.

BPA was a very weak ligand for ER $\alpha$  ( $IC_{50} = 1,030$  nM) based on its ability to inhibit [ $^3H$ ]17 $\beta$ -estradiol binding (Figure 2A, Table 1), as we previously reported (Okada et al. 2008). In the present study, we confirmed that BPA is also a very weak ligand for ER $\beta$  ( $IC_{50} = 900$  nM; Figure 2B, Table 1), indicating comparable interactions of BPA with ER $\alpha$  and ER $\beta$  despite the subtle structural differences between these ERs. In contrast, bisphenol AF was 20 times more potent than BPA as a ligand for ER $\alpha$  ( $IC_{50} = 53.4$  nM; Figure 2A, Table 1) and was approximately 48 times more potent for ER $\beta$

( $IC_{50} = 18.9$  nM; Figure 2B, Table 1). This high binding activity for ER $\beta$  suggests that the binding pocket of ER $\beta$  possesses specific structural elements that interact much more favorably with the CF $_3$  groups of bisphenol AF than with the CH $_3$  groups of BPA. We also assayed HPTE, an analog of BPA and bisphenol AF with the CCl $_3$  group. HPTE was almost equipotent to bisphenol AF in the assays for both ER $\alpha$  and ER $\beta$  (Table 1), but approximately 10 times more potent than bisphenol AF for ERR $\gamma$ .

**Receptor-binding selectivity of bisphenol AF and BPA.** We used the  $IC_{50}$  values shown in Table 1 (from the competitive receptor-binding assay for nuclear ER $\alpha$ , ER $\beta$ , and ERR $\gamma$ ) to estimate receptor selectivity ratios for BPA and bisphenol AF (Table 2). The results indicate that BPA is exclusively selective for ERR $\gamma$ , being 90–100 times more active for ERR $\gamma$  than for ER $\alpha$  or ER $\beta$ . In contrast, bisphenol AF receptor binding is much more selective for ER $\alpha$  and ER $\beta$  than for ERR $\gamma$  (6.70 times more selective for ER $\alpha$  than for ERR $\gamma$  and 18.94 times more selective for ER $\beta$  than for ERR $\gamma$ ; Table 2). Bisphenol AF binding is also about three times more potent for ER $\beta$  than for ER $\alpha$ .

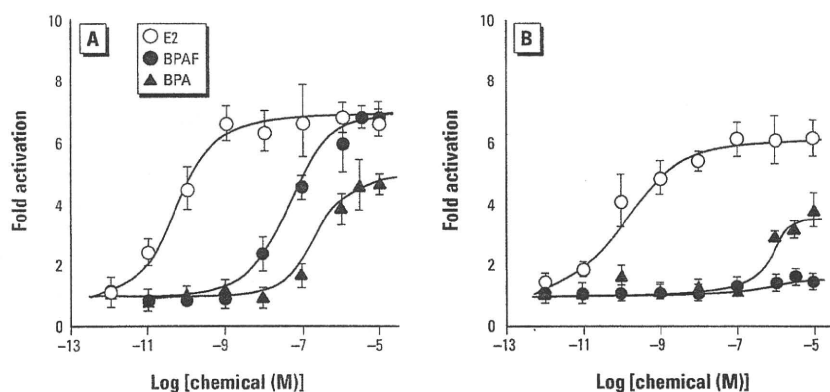
**Differential effects of bisphenol AF in the reporter gene assay.** We next examined reporter gene activity after bisphenol AF exposure in HeLa cells transiently cotransfected with an ER $\alpha$  or ER $\beta$  expression plasmid and an estrogen-response element (ERE)-luciferase reporter plasmid. Bisphenol AF fully activated ER $\alpha$  (increasing activity to ~ 7 times the baseline level) in a dose-dependent manner at concentrations of  $10^{-10}$  to  $10^{-5}$  M (Figure 3A). The half-maximal effective concentration ( $EC_{50}$ ) of bisphenol AF was 58.7 nM.

When we compared potencies for ER $\alpha$  activation versus ER $\alpha$  binding to determine receptor activation potency [expressed as  $EC_{50}$  (nM)/ $IC_{50}$  (nM)], we found a clear discrepancy between 17 $\beta$ -estradiol and bisphenol AF. As shown in Table 3, we estimated the receptor activation potency for 17 $\beta$ -estradiol to be 0.085 (0.075 nM/0.88 nM based on values from Figure 3A and Table 1, respectively). In contrast, the receptor activation potency of bisphenol AF [1.099 (58.7 nM/ 53.4 nM)] was approximately 13 times greater than that

**Table 2.** Receptor-binding selectivity of BPA and AF for ER $\alpha$ , ER $\beta$ , and ERR $\gamma$ .

Compound	Receptor-binding selectivity			Preferred receptor(s)
	ER $\alpha$ vs. ER $\beta$	ER $\alpha$ vs. ERR $\gamma$	ER $\beta$ vs. ERR $\gamma$	
17 $\beta$ -estradiol	2.47 ER $\alpha$	(ER $\alpha$ ) <sup>a</sup>	(ER $\beta$ ) <sup>a</sup>	ER $\alpha$
4-OHT	1.10 ER $\alpha$	3.58 ER $\alpha$	3.25 ER $\beta$	ER $\alpha$ ~ ER $\beta$
BPA	1.14 ER $\beta$	106.18 ERR $\gamma$	92.78 ERR $\gamma$	ERR $\gamma$
Bisphenol AF	2.83 ER $\beta$	6.70 ER $\alpha$	18.94 ER $\beta$	ER $\beta$
HPTE	3.27 ER $\beta$	1.63 ERR $\gamma$	2.01 ER $\beta$	ER $\beta$

HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1-trichloroethane. Data are *n*-fold strength of the preferred receptor compared with the nonpreferred receptor; for example, "2.47 ER $\alpha$ " means that 17 $\beta$ -estradiol binds to ER $\alpha$  2.47 times more strongly than to ER $\beta$ .  
<sup>a</sup>Because of inactivity of 17 $\beta$ -estradiol in ERR $\gamma$ , 17 $\beta$ -estradiol is active exclusively in ER $\alpha$  and ER $\beta$ .



**Figure 3.** Luciferase-reporter gene assays of bisphenol AF (BPAF), BPA, and 17 $\beta$ -estradiol (E2) for ER $\alpha$  and ER $\beta$  using reporter gene (pGL3/3xERE) and either ER $\alpha$  or ER $\beta$  expression plasmid (pcDNA3/ER $\alpha$  or pcDNA3/ER $\beta$ ) in HeLa cells. Concentration-dependent responses of 17 $\beta$ -estradiol, bisphenol AF, and BPA in the luciferase-reporter gene assay for ER $\alpha$  (A) and ER $\beta$  (B). For ER $\alpha$ , bisphenol AF displays full activation in a concentration-dependent manner, whereas for ER $\beta$  it displays extremely weak activity. 17 $\beta$ -Estradiol exhibits very strong activity, with approximately 4.5 times more activity induced at  $10^{-14}$  to  $10^{-5}$  M than at baseline.

**Table 3.** Binding affinities of 17 $\beta$ -estradiol, BPA, and bisphenol AF relative to their potencies for stimulating reporter gene activity by ER $\alpha$  and ER $\beta$  in HeLa cells.

Compound	$EC_{50}$ (nM)/ $IC_{50}$ (nM)	
	ER $\alpha$	ER $\beta$
17 $\beta$ -estradiol	0.085 (1.0)	0.041 (1.0)
BPA	0.308 (3.6)	0.770 (18.8)
Bisphenol AF	1.099 (12.9)	—

Values in the parentheses show the relative value of the  $EC_{50}/IC_{50}$  ratio (17 $\beta$ -estradiol = 1.0).

of 17 $\beta$ -estradiol (Table 3). This means that the concentration of 17 $\beta$ -estradiol required to stimulate a 50% response is about 13 times lower than the concentration required to occupy 50% of receptors, whereas the concentration of bisphenol AF required to stimulate a 50% response is about the same as that required to occupy 50% of receptors. This suggests that the receptor conformation induced by bisphenol AF is not as conducive to receptor activation as that induced by 17 $\beta$ -estradiol when measured in HeLa cells.

BPA was an extremely weak activator of both ER $\alpha$  (EC<sub>50</sub> = 317 nM) and ER $\beta$  (EC<sub>50</sub> = 693 nM) based on the luciferase reporter gene assay. The receptor activation potencies of BPA for ER $\alpha$  (0.308) and ER $\beta$  (0.770) were 3.6 and 18.8 times greater than the receptor activation potencies of 17 $\beta$ -estradiol for ER $\alpha$  and ER $\beta$ , respectively (Table 3). These suggest that, compared with 17 $\beta$ -estradiol, the concentration of BPA required to stimulate a 50% response is much higher than the concentration required to occupy 50% of receptors. In addition, as shown in Figure 3B, BPA exhibited a reduced ability to bring about full activation of ER $\beta$  (3.5 times greater activity relative to baseline in response to BPA vs. an increase to 6 times the baseline level in response to 17 $\beta$ -estradiol). This difference in efficacy indicates that BPA does not have the same ability as 17 $\beta$ -estradiol to induce activation conformation when measured in HeLa cells on this promoter.

**Antagonist activity of bisphenol AF on ER $\beta$ .** For ER $\beta$ , bisphenol AF was almost completely inactive, with very little increase in activity even at 10  $\mu$ M, the highest concentration tested (Figure 3B). Based on the strong receptor-binding activity of bisphenol AF for ER $\beta$  (IC<sub>50</sub> = 18.9 nM; Table 1), we expected

that bisphenol AF would also have a high receptor activation potency for ER $\beta$ . This unexpected inactivity in the reporter gene assay suggests that bisphenol AF binding disrupts the ER $\beta$ -LBD activation conformation, in which the  $\alpha$ -helix 12 (H12) of the receptor is normally positioned to recruit the coactivator protein conformation (Brzozowski et al. 1997; Ruff et al. 2000).

We therefore evaluated the antagonist activity of bisphenol AF against 17 $\beta$ -estradiol. When we examined 17 $\beta$ -estradiol, an endogenous agonist ligand of ER $\beta$ , in the presence of 0.01, 0.1, 1.0, and 10  $\mu$ M bisphenol AF, its activity (EC<sub>50</sub> = 0.075 nM) was gradually weakened. As shown in Figure 4A, the dose-dependent curves of 17 $\beta$ -estradiol shifted to the right with increasing concentrations of bisphenol AF, indicating that bisphenol AF effectively inhibits the interaction between 17 $\beta$ -estradiol and ER $\beta$ . When the results of Figure 4A were analyzed using a Schild plot, pA<sub>2</sub>, a measure of affinity of the antagonist for receptor, was calculated to be 7.87 from the dissociation equilibrium constant ( $K_B$  = 1.35  $\times$  10<sup>-8</sup> M).

The antagonist activity of bisphenol AF for 17 $\beta$ -estradiol/ER $\beta$  was further evidenced by assays in which we added serial concentrations of bisphenol AF (10<sup>-12</sup> to 10<sup>-5</sup> M) to a solution of 17 $\beta$ -estradiol maintained at a constant concentration. When 1  $\times$  10<sup>-8</sup> M 17 $\beta$ -estradiol was treated with bisphenol AF, the activity of 17 $\beta$ -estradiol was reduced in a dose-dependent manner in response to bisphenol AF concentrations ranging from 10<sup>-10</sup> to 10<sup>-5</sup> M (Figure 4B). We obtained a similar result for 1  $\times$  10<sup>-7</sup> M 17 $\beta$ -estradiol. These results demonstrate that bisphenol AF can antagonize the activity of 17 $\beta$ -estradiol on the ER $\beta$  receptor.

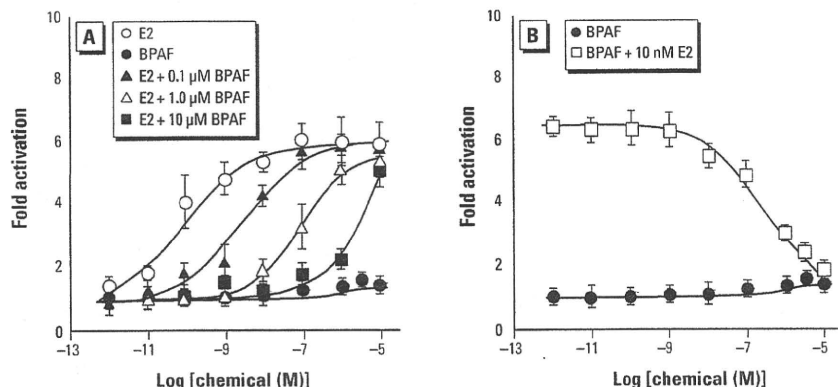
## Discussion

**Structural characteristics of bisphenols and ERs/ERR $\gamma$  receptors.** The differences in receptor selectivity between bisphenol AF and BPA are due to the CH<sub>3</sub>  $\leftrightarrow$  CF<sub>3</sub> substitution on the bisphenol backbone structure. Bisphenol AF is a hexafluoro derivative of BPA with the CH<sub>3</sub>  $\rightarrow$  CF<sub>3</sub> substitution on the backbone structure of 2,2-disubstituted propane CH<sub>3</sub>-C-CH<sub>3</sub>. BPA binds strongly to ERR $\gamma$ , but bisphenol AF binds to ERR $\gamma$  only weakly; we therefore judged that the binding pocket of ERR $\gamma$ -LBD possesses structural elements unfavorable for interaction with the trifluoro groups. The molecular size of CF<sub>3</sub> is almost the same as that of CH<sub>3</sub>, and thus there would be no structural repulsion or steric hindrance between these groups. However, because the CF<sub>3</sub> group is very electron rich, the structural elements standing face to face with CF<sub>3</sub> must also be electron rich, resulting in their electrostatic repulsion.

In our previous study (Matsushima et al. 2007, 2008), we found that the ERR $\gamma$  binding sites for BPA CH<sub>3</sub> groups were Phe435 and Met306. Because the aromatic phenyl and S-CH<sub>3</sub> groups of Phe435 and Met306 are electron rich, conditions would be unfavorable for binding of bisphenol AF's electron-rich CF<sub>3</sub> groups. Corresponding receptor residues in ER $\alpha$  are Leu525 and Leu384, respectively. Apparently, there would be no electrostatic repulsion between the bisphenol AF's CF<sub>3</sub> groups and the Leu residues. Such a release in structural stress must be very favorable for receptor activity and the selectivity of bisphenol AF for ER $\alpha$ .

In the present study, we found bisphenol AF to be a strong ligand for both ER $\alpha$  and ER $\beta$  receptors, although it shows a 3 times greater preference for ER $\beta$  over ER $\alpha$ . A much more important finding is that bisphenol AF functions in a different way for ER $\alpha$  and ER $\beta$ . Bisphenol AF is a full agonist for ER $\alpha$  but an antagonist for ER $\beta$ . The LBDs of ER $\alpha$  and ER $\beta$  share a high sequence identity (59%) and similar three-dimensional structures. We observed no obvious differences between ER $\alpha$  and ER $\beta$  in the ERE transcriptional assays in the presence of 17 $\beta$ -estradiol.

Among the amino acid residues lining the binding pockets of ER $\alpha$  and ER $\beta$ , two residues differ significantly: Leu384 in  $\alpha$ -helix 5 (H5) of ER $\alpha$  is replaced by Met336 in ER $\beta$ , and Met421 in loop 6-7 of ER $\alpha$  is replaced by Ile373 in ER $\beta$ . These two residues are most probably responsible for the discriminative affinity and reverse functional activity of bisphenol AF for ER $\alpha$  and ER $\beta$ . Furthermore, because bisphenol AF is an ER $\beta$  antagonist, the binding of bisphenol AF to the ER $\beta$  ligand-binding pocket must damage the ER $\beta$ -LBD activation conformation, in which the  $\alpha$ -helix 12 (H12) in LBD is



**Figure 4.** Effects of bisphenol AF (BPAF) on the agonist activity of 17 $\beta$ -estradiol (E2) in the luciferase-reporter gene assays for ER $\beta$ . (A) Concentration-dependent luciferase-reporter activities of 17 $\beta$ -estradiol by fold activation in the presence and absence of bisphenol AF (0.1, 1, or 10  $\mu$ M); these concentrations of bisphenol AF clearly weaken the agonist activity of 17 $\beta$ -estradiol for ER $\beta$ . (B) Concentration-dependent effects of bisphenol AF on the agonist activity of 10 nM 17 $\beta$ -estradiol; the agonist activity of 10 nM 17 $\beta$ -estradiol was clearly inhibited by bisphenol AF in a dose-dependent manner. Bisphenol AF itself sustained extremely weak activity for ER $\beta$ . In these assays, the reporter gene (pGL3/3 $\times$ ERE) and ER $\beta$  expression plasmid (pcDNA3/ER $\beta$ ) were measured in HeLa cells.

positioned to recruit the coactivator proteins conformation (Brzozowski et al. 1997; Ruff et al. 2000). Bisphenol AF binding to LBDs of ER $\alpha$  and ER $\beta$  are being analyzed in light of the crystal structures in studies in progress in our laboratory.

**Bisphenol AF as a candidate of potential endocrine disruptor.** Bisphenol AF is a potent estrogen agonist for ER $\alpha$  and a potent estrogen antagonist for ER $\beta$ . ER $\alpha$  and ER $\beta$  are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues. ER $\alpha$  is expressed primarily in the uterus, liver, kidneys, and heart (Couse and Korach 1999), whereas ER $\beta$  is expressed primarily in the ovaries (Couse and Korach 1999), prostate (Couse and Korach 1999), lungs (Kuiper et al. 1997), and gastrointestinal tract and bladder (Nilsson et al. 2001). Coexpression of both receptors occurs in the mammary glands (Pettersson and Gustafsson 2001), epididymis (Pau et al. 1998), thyroid (Pau et al. 1998), adrenals (Pau et al. 1998), bone (Arts et al. 1997; Brandenberger et al. 1997), and certain regions of the brain (Couse and Korach 1999). [For additional information, see Nuclear Receptor Signaling Atlas (2010).] 17 $\beta$ -Estradiol plays a critical role in many physiological processes in both females and males. These include normal growth, development, and cell-type-specific gene regulation in tissues of the reproductive tract, central nervous system, and skeleton (Couse and Korach 1999; Nilsson et al. 2001; Pettersson and Gustafsson 2001). Bisphenol AF is a potent binder of ER $\alpha$  and ER $\beta$  and thus would perturb these physiological processes, perhaps providing significant adverse influences for the central and peripheral systems.

**Effects of the bisphenol trihalogenated methyl group on receptor actions.** Bisphenol AF is an agonist for ER $\alpha$  and an antagonist for ER $\beta$ . Similar results have been reported for HPTE, a bisphenolic metabolite of methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane]. HPTE behaved as an ER $\alpha$  agonist and an ER $\beta$  antagonist with estrogen-responsive promoters in HeLa cells (Gaido et al. 1999). We confirmed these results in our assay systems as well. HPTE was a strong binder of ER $\alpha$  with IC<sub>50</sub> = 59.1 nM and of ER $\beta$  with IC<sub>50</sub> = 18.1 nM (Table 1). As reported previously by Gaido et al. (1999) and Nettles et al. (2004), HPTE acts as a full agonist for ER $\alpha$  but a strong antagonist for ER $\beta$ . However, bisphenol AF and HPTE differ in their receptor preference for ERR $\gamma$ . HPTE was approximately 10 times more potent than bisphenol AF for ERR $\gamma$  binding, although both chemicals were most strongly bound to ER $\beta$  (Tables 1, 2). As an antagonist for ER $\beta$ , bisphenol AF (pA<sub>2</sub> = 7.87) was somewhat stronger than HPTE, the pA<sub>2</sub> of which

was reported to be 7.52 (Gaido et al. 1999). However, both bisphenol AF and HPTE are significantly potent as ER $\beta$  antagonists.

Chemical structures of bisphenol AF and HPTE differ, with one of two CF<sub>3</sub> groups of bisphenol AF replaced by CCl<sub>3</sub> in HPTE, and the other by H (Figure 1). However, these compounds are similar in that both have trihalogenated methyl groups that may produce different activities for ER $\alpha$  and ER $\beta$  via their interactions with the ligand-binding pockets of each ER, namely, Leu384 in H5 of ER $\alpha$   $\leftrightarrow$  Met336 in ER $\beta$ , and Met421 in loop 6–7 of ER $\alpha$   $\leftrightarrow$  Ile373 in ER $\beta$ .

Methoxychlor is a chlorinated hydrocarbon pesticide structurally similar to DDT (dichlorodiphenyltrichloroethane) and thus is sometimes referred to as dimethoxy or methoxy DDT. It had been used to some degree as a replacement for DDT to protect crops, ornamentals, livestock, and pets against various insects, because it was believed to be metabolized more quickly than DDT, thus reducing or preventing bioaccumulation (Kapoor et al. 1970). Methoxychlor is uterotrophic in the ovariectomized rat and can cause adverse developmental and reproductive effects in mice and rats (Alm et al. 1996; Cummings 1997; Hall et al. 1997). However, HPTE is approximately 100 times more active at ERs than is methoxychlor. To date, the use of methoxychlor has been banned in many countries, including the United States, Japan, and the European Union. All these issues clearly raise concerns that not only HPTE but also bisphenol AF may be a potential endocrine disruptor affecting either ER $\alpha$  or ER $\beta$ , or both.

## Conclusions

BPA binds strongly to ERR $\gamma$  but very weakly to ER $\alpha$  and ER $\beta$ . In contrast, bisphenol AF binds very weakly to ERR $\gamma$  but strongly to ER $\alpha$  and ER $\beta$ . These differences in receptor selectivity reflect subtle but distinct structural differences resulting from the CH<sub>3</sub>  $\leftrightarrow$  CF<sub>3</sub> substitution on the bisphenol backbone structure. The trifluoromethyl group is much more electronegative than the methyl group. These results suggest that apparently minor structural differences among chemicals and NRs may have pronounced effects on binding affinity and selectivity. Thus, the present study emphasizes the crucial importance of accurate evaluation of receptor responses to understanding interactions between endocrine-disrupting compounds and diverse human NRs. Taken together, these results clearly indicate the importance of examining the degree and ways in which bisphenol AF may influence the physiological roles of ER $\alpha$  and ER $\beta$ . Given that bisphenol AF and BPA function as endocrine disruptors, these chemicals would work differently via different NRs.

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## Distinction of the binding modes for human nuclear receptor ERR $\gamma$ between bisphenol A and 4-hydroxytamoxifen

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Bisphenol A (BPA) strongly binds to human estrogen-related receptor  $\gamma$  (ERR $\gamma$ ). BPA is an oestrogenic endocrine disruptor that influences various physiological functions at very low doses. BPA functions as an inverse-type antagonist of ERR $\gamma$  to retain its high basal constitutive activity by inhibiting the deactivating inverse agonist activity of 4-hydroxytamoxifen (4-OHT). We recently demonstrated that ERR $\gamma$  receptor residues Glu275 and Arg316 function as the intrinsic binding site of BPA's phenol-hydroxyl group. We also determined the chief importance of phenol-hydroxyl $\leftrightarrow$ Arg316 hydrogen bonding and the corroborative role of phenol-hydroxyl $\leftrightarrow$ Glu275 hydrogen bonding. However, there appeared to be a distinct difference between the receptor binding modes of BPA and 4-OHT. In the present study, using tritium-labelled or non-labelled BPA and 4-OHT, we evaluated in detail the receptor binding capabilities of wild-type ERR $\gamma$  and its mutants with amino acid alterations at positions 275 and 316. Both compounds exhibited a strong binding ability to wild-type ERR $\gamma$  due to the hydrogen bonding to Glu275 and Arg316. However, 4-OHT revealed significantly reduced occupancy for both wild-type and mutant receptors. The data obtained suggest that 4-OHT barely binds to ERR $\gamma$  due to the strong ability of Glu275 and Arg316 to recruit phenol compounds.

**Keywords:** bisphenol A/estrogen-related receptor  $\gamma$ /4-hydroxytamoxifen/receptor binding mode/receptor binding assay.

**Abbreviations:** BPA, bisphenol A; DCC, dextran-coated charcoal; DES, diethylstilbestrol; E2, 17 $\beta$ -estradiol; ER, estrogen receptor; ERE, estrogen response element; ERR, estrogen-related receptor; ERRE, ERR-response element; ERR $\gamma$ , estrogen-related receptor  $\gamma$ ; LBD, ligand-binding domain; LBP, ligand-binding pocket; NR, nuclear receptor; 4-OHT, 4-hydroxytamoxifen.

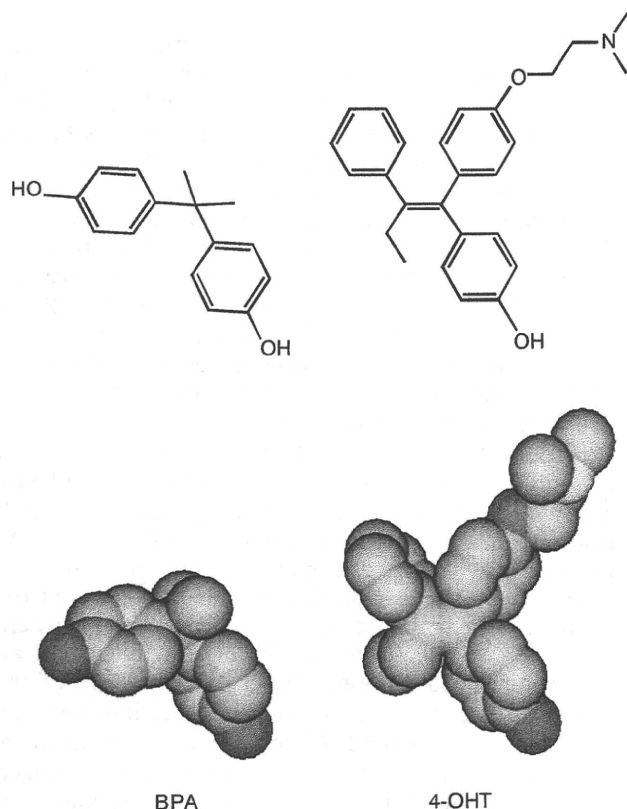
Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane], has recently been found to bind strongly to estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) with high constitutive basal activity (1). ERR $\gamma$  is one of the 48 human nuclear receptors (NRs) (2, 3), while BPA has long been recognized as an estrogenic chemical able to interact with human estrogen receptor (ER) (4–6). Various 'low-dose effects' of BPA have recently been reported *in vivo* for many organ tissues and systems in mice and rats (7–10). However, since the discovery of ERR $\gamma$ , it became an immediate and important requirement to evaluate whether the previously reported effects of BPA at low doses are mediated through ERR $\gamma$  and its specific target gene(s) (11).

BPA has the chemical structure of HO–C<sub>6</sub>H<sub>4</sub>–C(CH<sub>3</sub>)<sub>2</sub>–C<sub>6</sub>H<sub>4</sub>–OH with two phenol groups (Fig. 1). We have recently reported the crystallization and structural analysis of the BPA/ERR $\gamma$ -ligand binding domain (LBD) complex (12). In the complex, a single molecule of BPA stays at the ligand-binding pocket of each ERR $\gamma$ -LBD protein molecule, whose  $\alpha$ -helix 12 (H12) is stabilized in an activation conformation. The crystal structure of the complex suggests several essential interactions between the BPA and ERR $\gamma$ -LBD molecules. For instance, BPA's phenol-hydroxyl group is tethered by hydrogen bonds to the Glu275 and Arg316 residues in the ERR $\gamma$ -LBD (Fig. 2). These hydrogen bonds were also observed in the ERR $\gamma$ -LBD complex with 4- $\alpha$ -cumylphenol (13). Since 4- $\alpha$ -cumylphenol is a compound that lacks one of BPA's phenol-hydroxyl groups, the results clearly indicated that the hydrogen bonds of the phenol-hydroxyl group to the Glu275 and Arg316 residues are crucial for binding of the phenol compounds to ERR $\gamma$ . Indeed, by examining the ERR $\gamma$ -LBD analogues through site-directed mutagenesis, we demonstrated these residues as the intrinsic binding site of BPA's phenol-hydroxyl group (14).

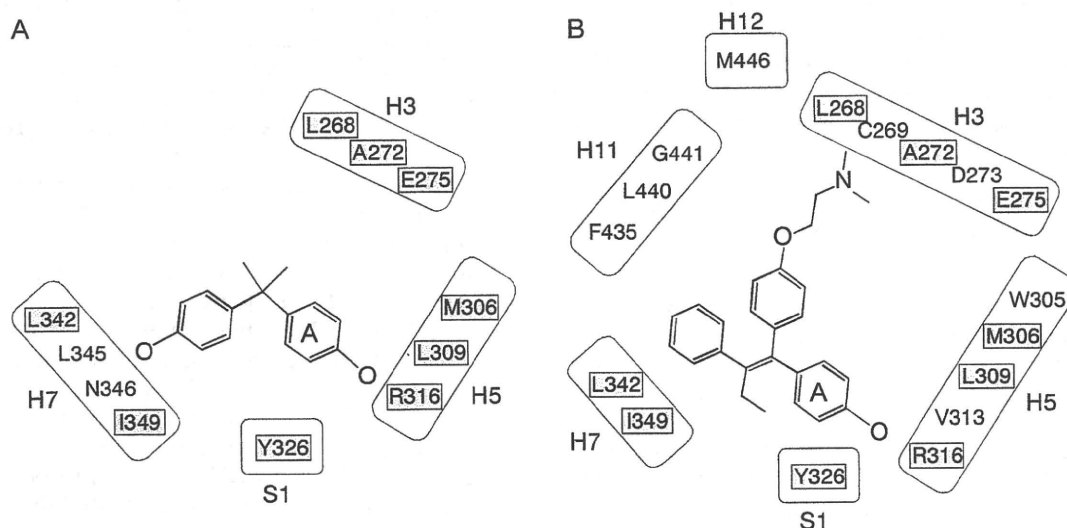
Similar hydrogen bonding was also found in the complex between 4-hydroxytamoxifen (4-OHT, Fig. 1) and ERR $\gamma$ -LBD (15). 4-OHT deactivates ERR $\gamma$  in, for example, the luciferase reporter gene assay (1, 16, 17). BPA inhibits such inverse agonist activity of 4-OHT. BPA reverses the deactivation to the originally high basal activation state in a dose-dependent manner, and thus acts as an inverse antagonist of ERR $\gamma$  (1). Although the phenol-hydroxyl group of 4-OHT shares the same site for its binding to ERR $\gamma$ , the difference in receptor binding modes between BPA and 4-OHT remains to be clarified.

In the previous study we elucidated the chief importance of phenol-hydroxyl $\leftrightarrow$ Arg316 hydrogen bonding

and the corroborative role of phenol-hydroxyl  $\leftrightarrow$ Glu275 hydrogen bonding (14). This result strongly suggested that the formation of these double hydrogen bonds is critical for phenol compounds to bind to ERR $\gamma$ . In the present study, to shed light on the structural significance of these double hydrogen bonds, we



**Fig. 1** Chemical structures of BPA and 4-OHT and their space-filling structures in the ligand-binding pocket of the ERR $\gamma$ . The space-filling structure of BPA originated from the X-ray crystal structure [Protein Data Bank with accession code 2E2R (12)], and that of 4-OHT was also from the deposited structure [2GPU, (30)].



**Fig. 2** Structural environments of BPA and 4-OHT in the ligand-binding pocket of the ERR $\gamma$ . The proximity of each amino acid residue (within the distance 4 Å) to BPA (A) and to 4-OHT (B) is shown in the boxes depicting the  $\alpha$ -helices. The amino acids in a grey backboard are the residues shared by both BPA and 4-OHT.

carried out a detailed comparison between BPA and 4-OHT for binding to ERR $\gamma$ -LBD in a site-directed point mutagenesis series. We here report that human NR ERR $\gamma$  binds highly specifically to BPA, but barely binds to the inverse agonist 4-OHT.

## Experimental procedures

### Chemicals

BPA was purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo). 4-Hydroxytamoxifen (4-OHT) was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). [ $^3$ H]BPA (5 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA) and [ $^3$ H]4-OHT (80 Ci/mmol) from American Radiolabelled Chemicals Inc. (St Louis, MO, USA).

### Plasmid construction and site-directed mutagenesis

A cDNA fragment encoding wild-type ERR $\gamma$ -LBD (residues 222–458) was generated by polymerase chain reaction (PCR) with specific primers using the human kidney cDNA library (Clontech Laboratories; Mountain View, CA, USA) and cloned into the vector pGEX-6p-1 (Amersham Biosciences, Piscataway, NJ, USA) at the *Eco*RI and *Xho*I sites. The resulting plasmids were designated as pGEX-ERR $\gamma$ -LBD.

ERR $\gamma$  mutants were generated using *PfuTurbo*<sup>®</sup> DNA Polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions using pGEX-ERR $\gamma$ -LBD as a template. The mutations were introduced by PCR mutagenesis in a two-step reaction essentially as reported (14, 18). Each mutant LBD was amplified and cloned into the vector pGEX-6p-1 at the *Eco*RI and *Xho*I sites. All PCR products were verified for their accuracy in the sequences.

### ERR $\gamma$ -LBD protein expression

Two glutathione *S*-transferase (GST)-fused receptor proteins—the wild-type and mutant GST-ERR $\gamma$ -LBD—were expressed in *Escherichia coli* BL21 as described previously (1). The receptor protein was purified using an affinity column of glutathione-sepharose 4B (GE Healthcare BioSciences Co., Piscataway, NJ, USA). After incubation for 1 h at 4°C, the column was washed three times with PBS containing 0.2% (v/v) Triton X-100 and once with sonication buffer. Fusion protein was eluted with 1 M Tris-HCl (pH 8.0) containing 20 mM reduced glutathione, which was removed by gel filtration on a column of Sephadex G-10 (15  $\times$  100 mm, GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 8.0). The purity was confirmed by SDS-PAGE using 12.5% polyacrylamide



gel. The protein concentrations were determined by the Bradford method (19).

#### Radio-ligand receptor binding assays

**Saturation binding.** A saturation binding assay was conducted (20) using [ $^3$ H]4-OHT or [ $^3$ H]BPA. The reaction mixture was incubated at 4°C for 2 h with the receptor proteins—GST-fused wild-type ERR $\gamma$ -LBD or its mutants—in 100  $\mu$ l of binding buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mM CHAPS and 2 mg/ml  $\gamma$ -globulin]. The assay was performed with or without the addition of unlabelled BPA or 4-OHT (final concentration of  $1 \times 10^{-5}$  M) to quantify the specific and nonspecific binding. After incubation with 100  $\mu$ l of 1% dextran-coated charcoal (DCC) (Sigma) (21) in PBS (pH 7.4) for 10 min at 4°C, free radioligand was removed by the direct vacuum filtration method using a 96-well filtration plate (Millipore, Bedford, MA, USA) for the B/F separation. The specific binding of [ $^3$ H]BPA or [ $^3$ H]4-OHT was calculated by subtracting the non-specific binding from the total binding, and the results were examined by Scatchard plot analysis (22). The assay was carried out at least in triplicate.

**Competitive binding.** Competitive binding assays were performed in the presence of GST-fused wild-type ERR $\gamma$ -LBD or its mutants at the most appropriate concentration of each. Reaction mixtures were incubated with either [ $^3$ H]BPA or [ $^3$ H]4-OHT (5 nM in final) at 4°C for 2 h, and free radioligand was removed by the method described above after incubation with 100  $\mu$ l of 1% DCC in PBS (pH 7.4) for 10 min at 4°C. To estimate the binding affinity, the IC<sub>50</sub> values (the concentrations for the half-maximal inhibition) were calculated from the dose-response curves evaluated by the nonlinear analysis program ALLFIT (23). Each assay was performed in duplicate and repeated at least three times.

## Results

#### Critical structural elements to detain 4-OHT and BPA to ERR $\gamma$

For the receptor binding assays, the ERR $\gamma$ -LBD (residues 222–458) was expressed in *E. coli* as a protein fused with glutathione *S*-transferase (GST). Mutations were introduced by the PCR mutagenesis method for the original nucleotide triplet codons GAG of Glu275 and CGG of Arg316 (24). To evaluate the ligand-binding ability of mutant receptors, a saturation binding assay was first performed using GST-ERR $\gamma$ -LBD and [ $^3$ H]4-OHT. In this assay for the mutant receptors, especially when no specific binding was measurable under the same experimental conditions for the wild-type ERR $\gamma$  receptor, the assay was carried out repeatedly for the specified times using varied concentrations of the receptor GST-ERR $\gamma$ -LBD or radio-ligand [ $^3$ H]4-OHT.

In the previous study using [ $^3$ H]BPA, it was found that Glu275 and Arg316 are necessary for holding BPA and 4-OHT in ERR $\gamma$ , but with different degrees of involvement in the hydrogen bonding (14). This result was further evidenced in the present study using [ $^3$ H]4-OHT and a series of mutant receptors, in which site-directed mutations in the LBD of ERR $\gamma$  were carried out for positions 275 and 316. When Glu275 and Arg316 were simultaneously mutated to Ala, the resulting (Ala, Ala)-ERR $\gamma$  mutant receptor exhibited no specific binding of [ $^3$ H]4-OHT, as reported for [ $^3$ H]BPA (14). As the X-ray crystal structure has suggested (12, 15), the results clearly indicated that Glu275 and Arg316 are crucial for the binding of 4-OHT and BPA, whose phenol-hydroxyl groups are

indeed engaged in hydrogen bonding with the side chain carboxyl (Glu) and guanidino groups (Arg).

#### Differential ability of hydrogen bonds evidenced by [ $^3$ H]4-OHT

When the Glu275→Ala substitution was achieved, the resulting mutant receptor (275Ala)-ERR $\gamma$  was found to exhibit considerably decreased binding potency for 4-OHT. [ $^3$ H]4-OHT showed significantly diminished binding ability, with dissociation constants of 28.8 nM (35% of the binding affinity for the wild-type ERR $\gamma$ ) (Table I). [ $^3$ H]BPA's dissociation constant was 17.8 nM (32% of the binding affinity for the wild-type ERR $\gamma$ ). The Arg316→Ala substitution resulted in a further reduction of activity. The dissociation constants were 210 nM (only 4.8% of the binding affinity for the wild-type ERR $\gamma$ ) for [ $^3$ H]4-OHT [171 nM (3.3%) for [ $^3$ H]BPA] (Table I).

The present results clearly indicate that the hydrogen bonds between the phenol-hydroxyl group of 4-OHT or BPA and the Glu275 and Arg316 residues are necessary for capturing these chemicals in the binding pocket of ERR $\gamma$ -LBD. However, it is also clear that the hydrogen bond between 4-OHT/BPA and the Arg316 is much more important than that between 4-OHT/BPA and the Glu275.

Receptor binding results were also obtained by a competitive binding assay using [ $^3$ H]4-OHT as a tracer (Table II). BPA and 4-OHT elicited almost the same results for the wild-type ERR $\gamma$ , with IC<sub>50</sub> values of 13.8 and 10.3 nM, respectively. For (275Ala)-ERR $\gamma$ , the IC<sub>50</sub> values were 365 nM (3.6% of the binding affinity for the wild-type ERR $\gamma$ ) and 356 nM (3.9%), respectively. However, for (316Ala)-ERR $\gamma$  with the Arg→Ala mutation, the competitive binding assay could not be carried out because of the extremely small specific binding in the saturation-binding assay. These results clearly indicate that the hydrogen bonding to the Arg316 residue is much more important for capturing BPA and 4-OHT than that to the Glu275 residue.

Table I. Receptor binding potency of ERR $\gamma$  and its mutants by tritium-labelled BPA and 4-OHT.

Amino acid residues of ERR $\gamma$ receptors <sup>a</sup>		Dissociation constant (K <sub>D</sub> , nM)	
Position 275	Position 316	[ $^3$ H]BPA	[ $^3$ H]4-OHT
Glu	Arg	5.70 ± 0.88	10.1 ± 0.29
(Wild-type)			
<i>Ala</i>	Arg	17.8 ± 2.74	28.8 ± 3.28
<i>Asp</i>	Arg	22.0 ± 2.86	42.0 ± 6.73
<i>Gln</i>	Arg	23.4 ± 3.34	29.4 ± 3.74
<i>Leu</i>	Arg	NSB <sup>b</sup>	NSB
Glu	<i>Ala</i>	171 ± 39.5	210 ± 38.3
Glu	<i>Lys</i>	22.5 ± 4.26	29.4 ± 3.79
Glu	<i>Leu</i>	NSB	NSB
<i>Ala</i>	<i>Ala</i>	NSB	NSB
<i>Arg</i>	<i>Glu</i>	59.7 ± 6.79	77.3 ± 9.38
<i>Ala</i>	<i>Glu</i>	NSB	NSB
<i>Arg</i>	<i>Ala</i>	54.3 ± 6.82	74.8 ± 6.15

<sup>a</sup>Specifically mutated residues are designated in italics.

<sup>b</sup>NSB means 'no specific binding' in the saturation binding assay.

Table II. Receptor binding potency of BPA and 4-OHT in the competitive binding assay for ERR $\gamma$  and its mutants by tritium-labelled 4-hydroxytamoxifen [ $^3\text{H}$ ] 4-OHT.

Amino acid residues of ERR $\gamma$ receptors <sup>a</sup>		Receptor binding potency IC <sub>50</sub> (nM)	
Position 275	Position 316	BPA	4-OHT
Glu (Wild-type)	Arg	13.8 $\pm$ 2.55	10.3 $\pm$ 0.07
<i>Ala</i>	Arg	365 $\pm$ 16.1	356 $\pm$ 58.9
<i>Asp</i>	Arg	179 $\pm$ 24.1	184 $\pm$ 27.1
<i>Gln</i>	Arg	256 $\pm$ 21.2	222 $\pm$ 26.7
<i>Leu</i>	Arg	Not carried out <sup>b</sup>	
Glu	<i>Ala</i>	Impossible to carry out <sup>c</sup>	
Glu	<i>Lys</i>	228 $\pm$ 24.4	293 $\pm$ 36.7
Glu	<i>Leu</i>	Not carried out <sup>b</sup>	
<i>Ala</i>	<i>Ala</i>	Not carried out <sup>b</sup>	
<i>Arg</i>	<i>Glu</i>	Impossible to carry out <sup>c</sup>	
<i>Ala</i>	<i>Glu</i>	Not carried out <sup>b</sup>	
<i>Arg</i>	<i>Ala</i>	Impossible to carry out <sup>c</sup>	

<sup>a</sup>Specifically mutated residues are designated in italics.

<sup>b</sup>Because of 'no specific binding' in the saturation binding assay, the competitive binding assay was not carried out.

<sup>c</sup>Because of the 'extremely small specific binding' in the saturation binding assay, the competitive binding assay could not be carried out.

#### Glu275 and Arg316 as hydrogen-bonding anchors to tether the ligands

When Glu275 and Arg316 were each replaced by Leu instead of Ala, the resulting (275*Leu*)-ERR $\gamma$  and (316*Leu*)-ERR $\gamma$  mutant receptors were completely inactive, with no specific binding (Table I). Thus, it was impossible to carry out their competitive binding assays (Table II). When Glu275 was replaced by glutamine (Gln), the resulting (275*Gln*)-ERR $\gamma$  mutant receptor exhibited a sufficient specific binding (~70% of the total binding) for [ $^3\text{H}$ ]BPA, but just barely sufficient specific binding (~35% of the total binding) for [ $^3\text{H}$ ]4-OHT. The  $K_D$  values were 23.4 nM (~25% of the binding affinity for the wild-type ERR $\gamma$ ) for [ $^3\text{H}$ ]BPA and 29.4 nM (34%) for [ $^3\text{H}$ ]4-OHT (Table I). The IC<sub>50</sub> values of BPA and 4-OHT were 179 nM (5.1% of the binding affinity for the wild type) and 184 nM (6.2%), respectively (Table II). These results are rather worse than those obtained for (275*Ala*)-ERR $\gamma$ . It is therefore clear that Gln does not compensate for Glu, indicating that the carboxyl (COOH) group, but not the carboxyl amide (CONH<sub>2</sub>) group, is crucial to capturing BPA and 4-OHT.

For (275*Asp*)-ERR $\gamma$  with the Glu275 $\rightarrow$ Asp substitution, [ $^3\text{H}$ ]4-OHT exhibited just barely sufficient specific binding (~30% of the total binding) (data not shown). It was found that (275*Asp*)-ERR $\gamma$  affords almost the same results obtained for (275*Ala*)-ERR $\gamma$  and (275*Gln*)-ERR $\gamma$  (Tables I and II). In particular, BPA and 4-OHT are significantly weak for binding to this mutant receptor (4-8% of the binding affinity for the wild-type ERR $\gamma$ ) (Table II). All these results indicate that the  $\gamma$ -carboxyl group of Glu275 is crucially important to binding BPA and 4-OHT.

The importance of Arg316 was also demonstrated by another mutation, in which the basic Arg residue was replaced by lysine (Lys) with the amino group.

Prepared (316*Lys*)-ERR $\gamma$  was found to be considerably weak for binding [ $^3\text{H}$ ]4-OHT ( $K_D$  = 29.4 nM) and [ $^3\text{H}$ ]BPA (22.5 nM) (Table I), since these activities are only ~25% that of the parent wild-type receptor ERR $\gamma$ . In the competitive binding assay using (316*Lys*)-ERR $\gamma$  and [ $^3\text{H}$ ]4-OHT, BPA and 4-OHT were significantly weak for binding to this mutant receptor (~5% of the binding affinity for the wild-type ERR $\gamma$ ) (Table II). Furthermore, the inactivity of (316*Leu*)-ERR $\gamma$  and the extremely weak activity of (316*Ala*)-ERR $\gamma$  (Tables I and II) definitely reveal the importance of the basic Arg residue for receptor activation. All these results indicate that Arg316 is a very important structural element for the binding of BPA and 4-OHT to the binding pocket of ERR $\gamma$ -LBD by hydrogen bonding.

Collectively, it is now clear that Glu275 and Arg316 are necessary for holding BPA and 4-OHT in ERR $\gamma$ , but with different degrees of involvement in the hydrogen bonding. The results clearly indicate the major importance of phenol-hydroxyl $\leftrightarrow$ Arg316 hydrogen bonding and the supportive role of the phenol-hydroxyl $\leftrightarrow$ Glu275 hydrogen bonding. The difference in their significance might be attributable to the importance and/or necessity of receipt of the phenol-hydroxyl group, even by using an assisting group to facilitate the receptor function. These results are coincident with those evidenced by [ $^3\text{H}$ ]BPA, as reported previously (14).

#### (Glu275, Arg316)-binding site arranged ideally to arrest the phenol-hydroxyl groups

In our previous study (14), hypothesizing that no amino acids other than 316Arg and 275Glu would play such an intrinsic role in capturing the phenol-hydroxyl group, we prepared a (*Arg, Glu*)-ERR $\gamma$  double mutant receptor, in which Arg and Glu were placed simply in opposite order. Due to the significance of the receipt of the phenol-hydroxyl group, for example, of BPA and 4-OHT, we expected that the Arg and Glu residues would be transferable. In fact, [ $^3\text{H}$ ]BPA was found to bind to this (*Arg, Glu*)-ERR $\gamma$  double mutant receptor (14). However, its binding potency was almost 10 times weaker than that to the wild-type receptor (Table I). In the present study, almost the same result was obtained for [ $^3\text{H}$ ]4-OHT, as shown in Table I. [ $^3\text{H}$ ]4-OHT bound to (*Arg, Glu*)-ERR $\gamma$  with a dissociation constant of 77.3 nM, showing only one-seventh the strength of the bond to the wild-type receptor (Table I).

The specific binding of [ $^3\text{H}$ ]BPA was ~65%. In contrast, [ $^3\text{H}$ ]4-OHT exhibited only a very small specific binding (<20%) in relation to the total binding. Thus, since [ $^3\text{H}$ ]4-OHT could only barely bind to (*Arg, Glu*)-ERR $\gamma$ , it was impossible to carry out the competitive binding assay (Table II).

As to the (*Arg, Glu*)-ERR $\gamma$  receptor, we made further Ala substitutions for 275Arg and 316Glu, respectively. The resulting (*Ala, Glu*)-ERR $\gamma$  mutant receptor with the 275Arg $\rightarrow$ Ala substitution was found to completely lack the binding capability for [ $^3\text{H}$ ]BPA and [ $^3\text{H}$ ]4-OHT (Table I). In contrast, the Arg-containing (*Arg, Ala*)-ERR $\gamma$  mutant receptor was still active

for [ $^3$ H]BPA and [ $^3$ H]4-OHT (Table I). In the saturation binding assay for (*Arg, Ala*)-ERR $\gamma$ , these tritium-labelled compounds exhibited similar results to those obtained for (*Arg, Glu*)-ERR $\gamma$ —the dissociation constant  $K_D$  values of 54.3 nM for [ $^3$ H]BPA and 74.8 nM for [ $^3$ H]4-OHT. However, for this (*Arg, Ala*)-ERR $\gamma$  receptor, [ $^3$ H]4-OHT also exhibited only a very small specific binding (<20%) of the total binding. Due to the very small amount of specific binding, we could carry out only the Scatchard plot analysis. It was impossible to carry out the competitive binding assay for the (*Arg, Glu*)-ERR $\gamma$  receptor by using [ $^3$ H]4-OHT as a tracer (Table II).

The fact that the Arg-containing (*Arg, Glu*)-ERR $\gamma$  and (*Arg, Ala*)-ERR $\gamma$  mutant receptors were still active, but (*Ala, Glu*)-ERR $\gamma$  with no Arg was completely inactive, led us to conclude that phenol-hydroxyl $\leftrightarrow$ 275Arg hydrogen bonding plays a primary role, while the role of phenol-hydroxyl $\leftrightarrow$ 316Glu hydrogen bonding is only supportive. Furthermore, our finding that (*Arg, Glu*)-ERR $\gamma$  and (*Arg, Ala*)-ERR $\gamma$  are almost equipotent (Table I) indicates that the supportive role of the phenol-hydroxyl $\leftrightarrow$ 316Glu hydrogen bond is actually almost insignificant. This together with the result that these mutant receptors have considerably lower affinity to BPA and 4-OHT is apparently due to the mismatched proximity to the phenol-hydroxyl group of BPA and that of 4-OHT. This assumption was proved recently by the X-ray crystal structure analysis of the BPA/(*Arg, Glu*)-ERR $\gamma$  complex (under submission). Although Glu275 and Arg316 in ERR $\gamma$  are interchangeable for keeping up the interaction with BPA and 4-OHT, ERR $\gamma$  appears to afford simultaneously an ideal space and the capability of arresting the phenol-hydroxyl groups by arranging the Glu and Arg residues at positions 275 and 316, respectively.

#### Discrepancy in potency to replace [ $^3$ H]4-OHT and [ $^3$ H]BPA

When the competitive receptor binding assay was carried out for BPA using [ $^3$ H]4-OHT as a tracer, the wild-type ERR $\gamma$  afforded a slightly weakened result as compared with that from the assay using [ $^3$ H]BPA. In the competitive receptor binding assay using [ $^3$ H]BPA as a tracer, BPA showed an  $IC_{50}$  value of 9.70 nM (14). In the assay using [ $^3$ H]4-OHT, however, the  $IC_{50}$  value of BPA was 13.8 nM, ~40% larger than that when using [ $^3$ H]BPA (Table II). These results clearly indicate that BPA has certain difficulty in displacing [ $^3$ H]4-OHT, probably due to the increased binding attachment points with [ $^3$ H]4-OHT as compared with [ $^3$ H]BPA.

On the other hand, in the assays using [ $^3$ H]4-OHT as a tracer for the mutant receptors that afforded the specific binding, (*275Ala*)-, (*275Asp*)-, (*275Gln*)-, (*316Lys*)- and (*Arg, Glu*)-ERR $\gamma$ , the  $IC_{50}$  values of the compounds were one order of magnitude larger (3–10 times larger) than those obtained from the assays using [ $^3$ H]BPA as a tracer. For example, for the (*275Ala*)-ERR $\gamma$  mutant receptor, BPA exhibited an  $IC_{50}$  value of 35.7 nM with [ $^3$ H]BPA (14), while this value was 10 times larger, 365 nM, with

[ $^3$ H]4-OHT (Fig. 3, Table II). It should be noted that the competitive binding assay measures the ability of the compound to replace the radiolabelled tracer in the ligand-binding pocket of the receptor. Thus, the results simply imply that, for BPA, to replace [ $^3$ H]4-OHT is more difficult than to replace [ $^3$ H]BPA.

#### Large difference in receptor occupancy between [ $^3$ H]4-OHT and [ $^3$ H]BPA

The results described above suggest that 4-OHT binds more solidly to ERR $\gamma$  than BPA. On the contrary, 4-OHT must have more difficulty fastening to the binding site because of the increased number of attachment points. This difference between [ $^3$ H]4-OHT and [ $^3$ H]BPA was clearly shown by the difference in their occupied receptor populations.

The maximal receptor density ( $B_{max}$ ) was estimated by Scatchard plot analysis for each mutant receptor in which specific binding was observed (Fig. 4). It is clear that the  $B_{max}$  values of [ $^3$ H]4-OHT are significantly

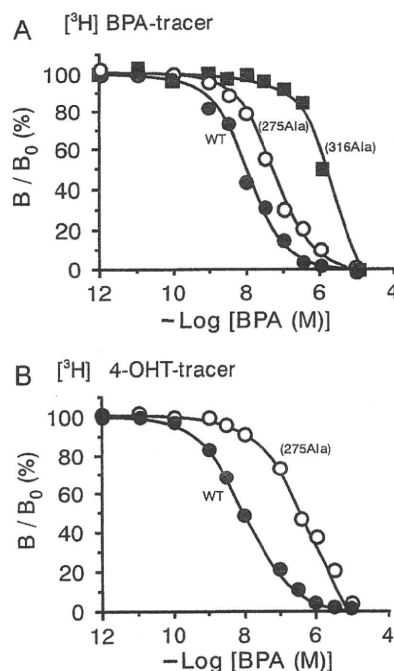
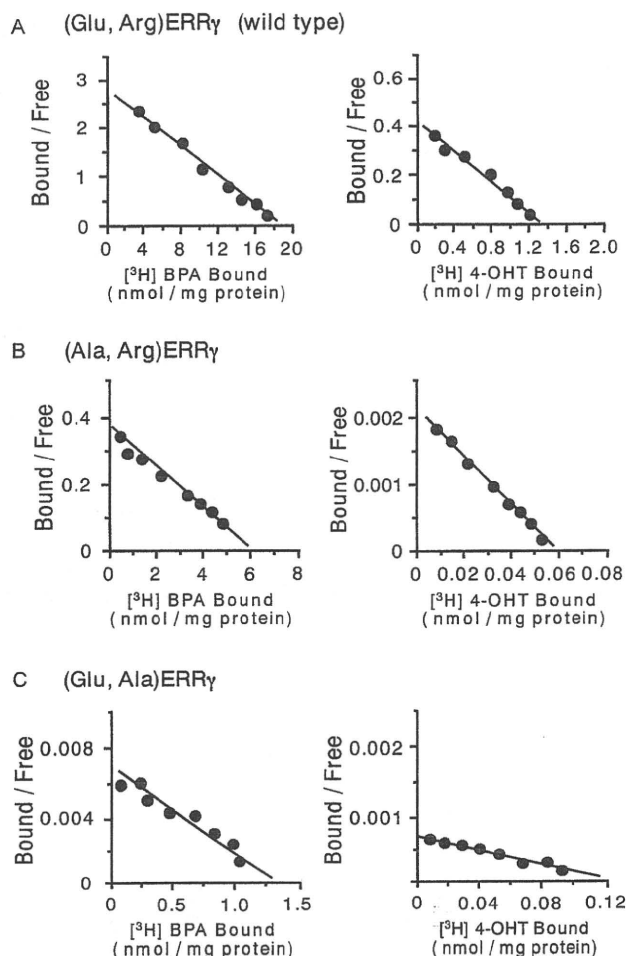


Fig. 3 Concentration-dependent curves of BPA for ERR $\gamma$ -LBD and its site-directed mutant derivatives by using tritium-labelled BPA (A) and 4-OHT (B). The receptor competitive binding assays were carried out to measure the ability to displace [ $^3$ H]BPA (A) and [ $^3$ H]4-OHT (B). Used receptors are wild-type ERR $\gamma$  (filled circle), (*275Ala*)-ERR $\gamma$  with the Glu275 $\rightarrow$ Ala substitution (open circle), and (*316Ala*)-ERR $\gamma$  with the Arg316 $\rightarrow$ Ala substitution (filled square). The graphs show representative dose-dependent binding curves, which give the  $IC_{50}$  value closest to the mean  $IC_{50}$  from at least five independent assays for BPA. The  $IC_{50}$  values showed a between-experiment coefficient of variation of 3–10%. All the receptors used are the ligand-binding domain (LBD) of the human ERR $\gamma$  and its mutant receptors. The  $IC_{50}$  values of BPA in the [ $^3$ H]BPA binding assay (A) were 9.70  $\pm$  0.59 nM for wild-type ERR $\gamma$ , 35.7  $\pm$  5.45 nM for (*275Ala*)-ERR $\gamma$  and 990  $\pm$  78.4 nM for (*316Ala*)-ERR $\gamma$  (14). Those in the [ $^3$ H]4-OHT binding assay (B) were 10.3  $\pm$  0.07 nM for wild-type ERR $\gamma$  and 365  $\pm$  16.1 nM for (*275Ala*)-ERR $\gamma$ , as shown in Table II. The assay for (*316Ala*)-ERR $\gamma$  was not feasible due to the extremely small amounts of specific binding observed for [ $^3$ H]4-OHT and the very weak binding potency of 4-OHT ( $IC_{50}$  = 818 nM) (14).



**Fig. 4** Scatchard plot analyses showing a single binding mode with a binding affinity constant ( $K_d$ ) and receptor density ( $B_{max}$ ). Analyses were carried out from the radioligand receptor saturation binding curves of [ $^3$ H]BPA (left-side figures) and [ $^3$ H]4-OHT (right-side figures) for the human ERR $\gamma$  ligand-binding domain (LBD) and its site-directed mutant derivatives. Those include the wild-type ERR $\gamma$  (A), (275Ala)-ERR $\gamma$  with the Glu275 $\rightarrow$ Ala substitution (B), and (316Ala)-ERR $\gamma$  with the Arg316 $\rightarrow$ Ala substitution (C). Note that the  $B_{max}$  value (nmol/mg protein on the abscissa) of [ $^3$ H]4-OHT is much smaller than that of [ $^3$ H]BPA in each receptor assay.

smaller than those of [ $^3$ H]BPA (Table III). As to the parent wild-type ERR $\gamma$  receptor, the [ $^3$ H]BPA's  $B_{max}$  value, 18.4 nmol/mg protein, is very compatible with the 18.8 nmol/mg protein that was estimated for the mature ERR $\gamma$  receptor protein based on its molecular weight (53 500). However, the [ $^3$ H]4-OHT's  $B_{max}$  value, 1.26 nmol/mg protein, was only 1/16th of the estimated value (Fig. 4, Table III). Obviously, these results imply that the feasibility of [ $^3$ H]4-OHT binding to ERR $\gamma$  is relatively small, as is also true for other mutant receptors.

## Discussion

### Structural requirements of ERR $\gamma$ to bind BPA and 4-OHT

Among the total 48 human NRs, 26 NRs have Arg at the position corresponding to ERR $\gamma$ 's 316 (14). In contrast to this Arg316, Glu275 is conserved among only five NRs: ER $\alpha$ , ER $\beta$ , ERR $\alpha$ , ERR $\beta$  and ERR $\gamma$ .

**Table III.** Receptor density of ERR $\gamma$  and its mutants by tritium-labelled BPA and 4-OHT.

Amino acid residues of ERR $\gamma$ receptors <sup>a</sup>		Receptor density ( $B_{max}$ , nmol/mg)	
Position 275	Position 316	[ $^3$ H]BPA	[ $^3$ H]4-OHT
Glu	Arg	18.4 $\pm$ 0.78	1.26 $\pm$ 0.055
(Wild-type)			
<i>Ala</i>	Arg	6.72 $\pm$ 0.62	0.059 $\pm$ 0.0030
<i>Asp</i>	Arg	12.4 $\pm$ 0.46	0.28 $\pm$ 0.024
<i>Gln</i>	Arg	7.81 $\pm$ 0.47	0.35 $\pm$ 0.041
<i>Leu</i>	Arg	NSB <sup>b</sup>	NSB
Glu	<i>Ala</i>	1.34 $\pm$ 0.16	0.12 $\pm$ 0.016
Glu	<i>Lys</i>	9.98 $\pm$ 0.76	0.18 $\pm$ 0.049
Glu	<i>Leu</i>	NSB	NSB
<i>Ala</i>	<i>Ala</i>	NSB	NSB
<i>Arg</i>	<i>Glu</i>	3.66 $\pm$ 0.29	0.095 $\pm$ 0.043
<i>Ala</i>	<i>Glu</i>	NSB	NSB
<i>Arg</i>	<i>Ala</i>	3.56 $\pm$ 0.38	0.30 $\pm$ 0.070

<sup>a</sup>Specifically mutated residues are designated in italics.

<sup>b</sup>NSB means 'no specific binding' in the saturation binding assay, and thus the  $B_{max}$  value was not obtained.

Since all these Glu275-containing NRs contain Arg316, it is very reasonable to assume that these (Glu275, Arg316)-containing NRs are able to bind to the phenol compounds. We have recently demonstrated that a number of 4-alkylphenols bind to ERR $\gamma$  considerably more strongly (11), and that their phenol-hydroxyl group is arrested by the Glu275 and Arg316 residues, as shown by the X-ray structural analysis of the phenol/ERR $\gamma$  complexes (unpublished data). Although the natural hormone of ER $\alpha$  and ER $\beta$  is 17 $\beta$ -estradiol, ERR $\alpha$ , ERR $\beta$  and ERR $\gamma$  are all orphan receptors whose endogenous ligand is not identified (25–28). It is highly likely that if the ERs have any endogenous ligands, they must be phenol compounds.

When the Glu275 $\rightarrow$ Ala substitution was accomplished, the resulting mutant receptor (275Ala)-ERR $\gamma$  was found to exhibit just about sufficient specific binding (~35% of the total binding) for [ $^3$ H]4-OHT, in contrast to the clearly sufficient specific binding (~65% of the total binding) for [ $^3$ H]BPA. For (316Ala)-ERR $\gamma$  with the Arg316 $\rightarrow$ Ala substitution, [ $^3$ H]4-OHT exhibited only barely sufficient specific binding (~20% of the total binding), which was insufficient to perform the competitive binding assay. [ $^3$ H]BPA exhibited a sufficient specific binding (~50% of the total binding) for this (316Ala)-ERR $\gamma$ . It is clear that the same structural changes in ERR $\gamma$  differently affect the binding ability of 4-OHT and BPA, which is suggestive of their different receptor binding modes. BPA and 4-OHT share only a phenol group (Fig. 1), which has been demonstrated to occupy the same (Glu275, Arg316)-binding site in the ERR $\gamma$  receptor (12, 15, 29, 30). Thus, their different receptor binding modes are definitely due to structures other than the phenol group.

For the ERR $\gamma$  receptor, 4-OHT acts as an inverse agonist (1, 16), the binding of which dissociates the  $\alpha$ -helix 12 (H12) region from the LBD body (12, 15, 29). This discussion results in a deactivation

of the receptor. 4-OHT puts aside the H12 from an activation conformation to an inactivation conformation, as evidenced by the X-ray crystallography of the 4-OHT–ERR $\gamma$  complex (15). This process appears to be a highly energy-consuming option. In contrast, H12 of non-liganded ERR $\gamma$ –LBD is folded into the activation conformation (12, 29), and BPA is able to bind to this conformation without causing any structural changes in the receptor, as demonstrated by the X-ray crystallography of the BPA–ERR $\gamma$  complex (12). Thus, there is indeed a distinct difference in binding modes between 4-OHT and BPA for ERR $\gamma$ .

BPA suppressed the inverse agonist activity of 4-OHT, being a specific inhibitor against the inverse agonist 4-OHT. It should be noted that BPA's ability to antagonize 4-OHT is approximately one-order lower than its binding potency to ERR $\gamma$ , as reported previously by Okada *et al.* (11). This discrepancy is probably due to the BPA's difficulty in displacing 4-OHT (see above in the Results section), originating from the distinction in binding modes between BPA and 4-OHT for ERR $\gamma$ .

#### Different potentials of BPA and 4-OHT in binding to ERR $\gamma$

The difference in binding modes would induce novel differences in the binding and dissociation energies. For instance, with the wild-type ERR $\gamma$ , [<sup>3</sup>H]4-OHT exhibited an ~1.8-fold larger  $K_D$  value than that of [<sup>3</sup>H]BPA (Table I). Also, for a series of mutant receptors, [<sup>3</sup>H]4-OHT and [<sup>3</sup>H]BPA showed similar differences (Table I). [<sup>3</sup>H]BPA did bind to (316Ala)-ERR $\gamma$ , (Arg, Glu)-ERR $\gamma$  and (Arg, Ala)-ERR $\gamma$ , but [<sup>3</sup>H]4-OHT could not bind to any of them (Table I).

The difference between [<sup>3</sup>H]4-OHT and [<sup>3</sup>H]BPA is further revealed in their maximal receptor density  $B_{max}$  values. [<sup>3</sup>H]BPA afforded well-matched  $B_{max}$  values for the wild-type ERR $\gamma$ ; *i.e.* 18.4 nmol/mg protein as the observed value and 18.8 nmol/mg protein as the calculated value (Table III). However, the  $B_{max}$  value of [<sup>3</sup>H]4-OHT was diminished significantly, with only 1.26 nmol/mg protein (~7% compared with that of [<sup>3</sup>H]BPA) (Table III). This result must be due to 4-OHT repositioning H12 from an activation conformation to an inactivation conformation, making it difficult for [<sup>3</sup>H]4-OHT to bind to ERR $\gamma$ . BPA binds directly to ERR $\gamma$ –LBD folded in the activation conformation without any conformational changes in the receptor.

As to the mutant receptors such as (275Ala)-, (275Gln)-, (275Asp)- and (316Lys)-ERR $\gamma$ , [<sup>3</sup>H]BPA gave fairly large  $B_{max}$  values: 6.72–12.4 nmol/mg protein (Table III). All of these contain Arg or Lys at position 316. However, the  $B_{max}$  values of [<sup>3</sup>H]4-OHT for these mutant receptors were drastically reduced, showing only 0.9–4.5% values as compared with that of [<sup>3</sup>H]BPA. Although it is not clear whether or not LBD in these mutant receptors is folded in the same activation conformation, at least 4-OHT is able to reposition H12 into an inactivation conformation. It is therefore obvious that 4-OHT barely binds to ERR $\gamma$  receptors.

Another important deduction from the present results is the crucial role of the Arg316 residue to bind BPA and 4-OHT. To the mutant receptor lacking Arg316 due to an Arg→Ala substitution, namely, (316Ala)-ERR $\gamma$ , [<sup>3</sup>H]BPA and [<sup>3</sup>H]4-OHT bound only weakly. For the ERR $\gamma$  mutant receptors containing Arg at position 275, namely (Arg, Glu)-ERR $\gamma$  and (Arg, Ala)-ERR $\gamma$ , [<sup>3</sup>H]BPA and [<sup>3</sup>H]4-OHT could bind, but with fairly small  $B_{max}$  values. By contrast, to the mutant ERR $\gamma$  receptors that are entirely lacking Arg at positions either 275 or 316, [<sup>3</sup>H]4-OHT and [<sup>3</sup>H]BPA did not reveal any specific binding. All of these results clearly indicate that the Arg316 residue plays a crucial role in the binding of BPA and 4-OHT to ERR $\gamma$ . Thus, this Arg316 residue would play a central role in capturing phenolic chemicals.

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#### Conflict of interest

None declared.

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## Functional Role of the C-terminal Helix 12 Peptide in the Receptor Activation Mechanism of Estrogen-related Receptor $\gamma$ (ERR $\gamma$ )

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*We have demonstrated that estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) binds strongly bisphenol A (BPA) with high basal constitutive activity. X-ray crystal structural analysis of the BPA/ERR $\gamma$  complex indicated that BPA sits in the pocket with 'active  $\alpha$ -helix (H12) peptide' in an activation. 4-hydroxytamoxifen (4-OHT) is an inverse agonist ligand of ERR $\gamma$ , it has no interaction with H12-peptide. In this study, we identified that H12-peptide is crucially important for the receptor activation and the selection of ligands.*

**Keywords:** bisphenol A, estrogen-related receptor  $\gamma$ , helix 12-peptide, ligand binding, receptor activation

### Introduction

Estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) is an orphan receptor that belongs to the 48 human nuclear receptors. ERR $\gamma$  is widely expressed in humans, especially strongly in the placenta and fetal brain. ERR $\gamma$  reveals a high constitutive activity, although its endogenous ligand and physiological roles has not been clarified yet. Almost all of the nuclear receptors have a unique ligand binding domain (LBD), in which the ligand binding pocket is constructed with 12  $\alpha$ -helices (H1-H12) peptide and 2  $\beta$ -strands. These helices play extremely important role to select a specific ligand for the receptor activation. Recently, we have demonstrated that bisphenol A (BPA), an estrogenic endocrine disruptor, binds very strongly to ERR $\gamma$  with high constitutive activity [1]. We have also successfully achieved in crystallization and structural analysis of the BPA/ERR $\gamma$ -LBD complex [2]. It was found that BPA sits in the pocket with 'active  $\alpha$ -helix (helix 12) peptide' in an activation conformation in accord with no-ligand bind of ERR $\gamma$ . However, when 4-hydroxytamoxifen (4-OHT) -- an inverse agonist ligand of ERR $\gamma$  -- binds to receptor, 4-OHT has no interaction with helix 12-peptide [3]. It may be that BPA/4-OHT binds ERR $\gamma$  with different mechanism by the H12-peptide.

In the present study, in order to identify the structural elements important for ligand binding and constitutive receptor activation, we prepared a series of mutant ERR $\gamma$  receptor, in which ERR $\gamma$ 's helix 12-peptide was truncated every three amino acid residues (Fig. 1).

```

ERRγ-wild type: 420 430 440 450 458
---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLEMLEAK*
ERRγ-Δ(458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLEMLEAK*
ERRγ-Δ(457-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLEMLEA*
ERRγ-Δ(456-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLEMLE*
ERRγ-Δ(455-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLEML*
ERRγ-Δ(454-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLEM*
ERRγ-Δ(453-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKLFLE*
ERRγ-Δ(450-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPMHKL*
ERRγ-Δ(447-458): ---TLPLLRTSTKAVQHHFYNIKLEGKVPM*
ERRγ-Δ(444-458): ---TLPLLRTSTKAVQHHFYNIKLEGK*
ERRγ-Δ(441-458): ---TLPLLRTSTKAVQHHFYNIKL*
ERRγ-Δ(438-458): ---TLPLLRTSTKAVQHFYN*
ERRγ-Δ(435-458): ---TLPLLRTSTKAVQH*
ERRγ-Δ(441-458): ---TLPLLRTSTKA*

```

Fig. 1. Helix 12-peptide mutant receptors of ERR $\gamma$  design ("\_\_\_": H11 peptide part; "\_\_\_": H12 peptide part)

## Results and Discussion

The mutant receptors were prepared by the two-step PCR method using wild type ERR $\gamma$  as template, and cloned into the expression vector pGEX-6p-1 or pcDNA3.1. For the receptor binding assays, the wild-type and mutant LBD of ERR $\gamma$  were expressed in *E. coli* as a protein fused with glutathione *S*-transferase (GST) protein. Firstly, we used [<sup>3</sup>H]BPA as a tracer for the saturation binding assay. The Scatchard plot analysis was carried out for all the mutant receptor to estimate both the dissociation constant ( $K_d$ ) and the receptor protein density ( $B_{max}$ ). We found that the truncated one/two amino acid residues ERR $\gamma$  from C-terminal mutant receptors (ERR $\gamma$ - $\Delta$ (458) and ERR $\gamma$ - $\Delta$ (457-458)) were strongly bind to [<sup>3</sup>H]BPA the same as wild-type ERR $\gamma$ . However, the truncated three amino acid residues ERR $\gamma$  from C-terminal mutant receptor (ERR $\gamma$ - $\Delta$ (456-458)) was remarkable reduced the binding ability to [<sup>3</sup>H]BPA. All of the mutant receptors with helix 12-peptide truncated completely absent the binding ability to [<sup>3</sup>H]BPA. Furthermore, using the [<sup>3</sup>H]4-OHT as a tracer, all of the mutant receptors showed the specific binding with 50~100% binding affinity of wild-type ERR $\gamma$ .

For examine the constitutive receptor activation of the mutant receptors, luciferase reporter gene assay method was used. All mutant receptors and estrogen-related receptor response element (ERRE) transiently expressed in the HeLa cells and luciferase activity was measured after 24 hr for the cells treated with 1% BSA/PBS. We found that the mutant receptors of the C-terminal three amino acid residues truncated every one amino acid residue were kept the constitutive receptor activation as wild-type. The other mutant receptors with helix 12-peptide truncated partly or completely lost the constitutive receptor activity in the reporter gene assay.

The binding assay and reporter gene assay data indicated that helix 12-peptide of ERR $\gamma$  is crucially important for the constitutive activation and the selection of ligands.

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## The Agonist/Antagonist Differential-docking Screening (AADS) Method for Exploration of the Estrogen Receptor-binding Chemicals

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*We have established a novel method to screen endocrine-disrupting chemicals by in silico docking calculations, which utilized the both agonist-bound- and antagonist-bound-ligand binding domains as templates. This agonist/antagonist differential-docking screening (AADS) method predicted, for example, 4-(1-adamantyl) phenol as an agonist of the human estrogen receptor  $\alpha$ . The AADS method is an approach that appears to foresee both the binding potency and the agonist or antagonist activity of chemicals for the target nuclear receptors.*

**Keywords:** agonist/antagonist differential-docking screening (AADS), docking calculation, nuclear receptor

### Introduction

Nuclear receptors (NRs), which play a central role as transcription factors in various biological processes, consist of five domains: N-terminal domain, DNA-binding domain, hinge domain, ligand-binding domain (LBD), and C-terminal domain. In general, the LBD has a pocket to capture a chemical, which has agonist or antagonist activity [1]. Because the pocket, which was named ligand binding pocket (LBP), exists on the LBD, the LBD is considered to be the major binding site for the pharmacological agents and the endocrine disrupting chemicals (EDCs).

Currently, numerous industrial chemicals are suspected to be EDCs. If we could predict that the chemicals would bind to the LBP, it would definitely be advantageous for the design of agonists or antagonists of particular NR. It would also be very helpful to predict which chemicals would cause serious disruptions in the endocrine system. In this study, to establish a novel method that screens a huge number of chemicals to explore EDCs, the docking calculation procedure was newly developed by using one of NRs, the human estrogen receptor  $\alpha$  (ER $\alpha$ ). The ER $\alpha$ -LBD changes its conformation, depending upon the ligand docks as either agonist or antagonist. Such differences of the conformation were observed in 3D structures of ER $\alpha$ -LBD/ligand complexes, which determined by X-ray crystallography [2,3]. Therefore, we used two agonist-bound (PDB: 1ERE and 3ERD) and two another antagonist-bound (1ERR and 3ERT) ER $\alpha$ -LBD's crystal structures as templates for the docking calculation, and compared binding energies elucidated from

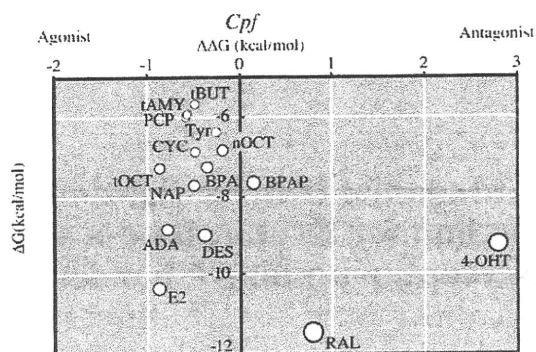


Fig. 1. Plotting analysis of the ADDS method.  $\Delta G$  versus  $\Delta\Delta G$  plotting analysis. The size of each plot corresponds to the relative molecular size of the test chemical. Abbreviations of chemicals are denoted in parentheses: pentachlorophenol (PCP), 4-tert-butylphenol (tBUT), 4-tert-amylphenol (tAMY), 4-cyclohexylphenol (CYC), 4-n-octylphenol (nOCT), 4-tert-octylphenol (tOCT), bisphenol A (BPA), 4-(2-naphthyl)phenol (NAP), 4-(1-adamantyl)phenol (ADA), bisphenol AP (BPAP), 17 $\beta$ -estradiol (E2), diethylstilbestrol (DES), raloxifene (RAL), and 4-hydroxytamoxifen (4-OHT).

the docking calculations by using each template. This calculation method was named as the agonist/antagonist differential-docking screening (AADS) [4].

### Results and Discussion

Docking calculations were carried out by the program Autodock 3.0 (The Scripps Research Institute) and tested 16 chemicals. The average binding energies ( $\Delta G$ ) of the chemicals were obtained from the results of the docking calculations using four LBDs. The agonist/antagonist factor ( $\Delta\Delta G=Cpf$ : conformation preference factor) was calculated as the difference between  $\Delta G$  from the docking calculations used by agonist-bound receptors and antagonist-bound receptors.

The calculated free energies of binding ( $\Delta G$ ) of the known ER-ligands, E2, DES, RAL and 4-OHT, ranged from  $-8.98$  to  $-11.47$  kcal/mol. Among test chemicals, there was only one putative antagonist (Fig. 1). Because of its small minus  $\Delta\Delta G$  value, it is possible that BPAP has weak antagonist-like activity. On the other hand, we identified 4-(1-adamantyl) phenol (ADA) as a relatively strong agonist for hER $\alpha$ . ADA attained a binding energy ( $-8.84$  kcal/mol) greater than that of the other test chemicals and it had a plus  $\Delta\Delta G$  value. The AADS method successfully predicted that ADA is almost as active as DES ( $\Delta G = -8.98$  kcal/mol).

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食品安全委員会に評価を諮問した。環境ホルモン問題は、今なお終わっていない。

### 3. ビスフェノール A の受容体を発見

我々の生活環境中には、膨大な数の化学物質が存在し、それらの一部は生体に悪影響を及ぼす「内分泌かく乱物質（環境ホルモン）」である。その代表的なものが、ポリカーボネート製プラスチックやエポキシ樹脂の原料・ビスフェノール A である。ビスフェノール A は、女性ホルモン（エストロゲン）様の作用を示すと指摘され、プラスチック製品からの溶出基準が各国で定められている。しかしながら、2000 年前後より、溶出基準を大きく下回る低濃度において、ラット乳幼仔の行動などに悪影響を与えるという報告が相次ぎ<sup>3)</sup>、こうした「低用量問題」が大きくクローズアップされるようになった。ビスフェノール A の内分泌かく乱作用については、これまで学界と産業界の論争という形で、問題の有無から議論されてきたが、ここに来て「作用あり」の観点から再評価する気配になってきた。そこで問題となるのがビスフェノール A が結合する受容体である。ビスフェノール A は「エストロゲン様」と形容されてきたにもかかわらず、実際にはエストロゲン受容体 (ER) への結合性がきわめて低い。筆者らは「ビスフェノール A の標的受容体が ER 以外に存在する」可能性を考え、ヒト核内受容体の 48 種類すべてについて、トリチウム標識したリガンドをトレーサに用いた競争結合試験によるスクリーニングの取り組みを開始した。その結果、ER とよく似た構造を持つものの、全く別の核内受容体・エストロゲン関連受容体  $\gamma$  型 (ERR $\gamma$ ) がビスフェノール A の標的受容体であることを発見した<sup>4), 5)</sup>。

### 4. ERR $\gamma$ は自発活性化型受容体

ERR $\gamma$  はヒト核内受容体スーパーファミリーに属し、ステロイド受容体グループに分類される。このグループは一般に、ステロイドホルモ

ンが結合すると活性化されるが、ERR $\gamma$  はリガンドなしで最初から 100% フルに活性化の状態になっている。筆者らは、こうした受容体を「自発活性化型核内受容体」と呼んでいる。ERR $\gamma$  の機能を制御する内因性リガンドは見つかっていないが、4-hydroxytamoxifen (4-OHT) は強く結合し、活性を低下させる。つまり、4-OHT は ERR $\gamma$  のインバーサゴニスト（自発活性を抑制するリガンド）である<sup>6)</sup>。この報告をもとに、当初、筆者らは、トリチウム標識した 4-OHT をトレーサとして ERR $\gamma$  に結合する化学物質をスクリーニングしていたが、驚くほど強く結合する化合物があることに気づき、これがビスフェノール A であった<sup>4)</sup>。このため、すぐさま、トリチウム標識したビスフェノール A (<sup>3</sup>H]BPA) をトレーサとして結合試験系（飽和結合試験及び競争結合試験）を構築した。そして、ERR $\gamma$  へのビスフェノール A の非常に強い結合特性を精密に解析することに成功した<sup>7)</sup>。ビスフェノール A の結合定数  $K_D$  値は、5.5 nM である（図 1）。この値は、生体内の一般的な天然ホルモンと受容体の結合親和性に匹敵するほど非常に高い結合親和性であることを意味している。

### 5. ビスフェノール A 類似体の結合性と活性

ビスフェノール A を原材料にしたポリカーボネートで製造される製品は数限りない。携帯電話、コンピュータ、眼鏡レンズ、DVD、医療用チューブ。これらは、我々の生活に欠かせない。高性能ポリカーボネートとして、ビスフェノール A の類似化合物の開発も急ピッチで進められている。果たしてビスフェノール A だけが問題だろうか？ きっと他にも ERR $\gamma$  に結合する化合物があるはずである。そこで、ERR $\gamma$  に結合するために必須な分子構造を明らかにするために、<sup>3</sup>H]BPA とビスフェノール A の構造類似体を用いて競争結合試験を行った。例えば、ビスフェノール A の OH 基の重要性を解析する場合、化合物①と化合物②の結合能をビ