J. Biochem. 2010;148(2):247–254 doi:10.1093/jb/mvq056

JB THE JOURNAL OF BIOCHEMISTRY

Distinction of the binding modes for human nuclear receptor ERRγ between bisphenol A and 4-hydroxytamoxifen

Received February 22, 2010; accepted May 26, 2010; published online June 11, 2010

Xiaohui Liu, Ayami Matsushima, Hiroyuki Okada and Yasuyuki Shimohigashi*

Laboratory of Structure-Function Biochemistry, Department of Chemistry, Research-Education Centre of Risk Science, Faculty and Graduate School of Sciences, Kyushu University, Fukuoka 812-8581, Japan

*Prof. Yasuyuki Shimohigashi, Laboratory of Structure-Function Biochemistry, Research Education Centre of Risk Science, Department of Chemistry, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan. Tel./Fax: +81-92-642-2584, email: shimo@chem.kyushu-univ.jp

Bisphenol A (BPA) strongly binds to human estrogen-related receptor γ (ERRγ). BPA is an oestrogenic endocrine disruptor that influences various physiological functions at very low doses. BPA functions as an inverse-type antagonist of ERRγ to retain its high basal constitutive activity by inhibiting the deactivating inverse agonist activity of 4-hydroxytamoxifen (4-OHT). We recently demonstrated that ERRγ 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↔Arg316 hydrogen bonding and the corroborative role of phenol-hydroxyl↔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 ERRγ and its mutants with amino acid alterations at positions 275 and 316. Both compounds exhibited a strong binding ability to wild-type ERRγ 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 ERRγ due to the strong ability of Glu275 and Arg316 to recruit phenol compounds.

Keywords: bisphenol A/estrogen-related receptor γ/4-hydroxytamoxifen/receptor binding mode/receptor binding assay.

Abbreviations: BPA, bisphenol A; DCC, dextran-coated charcoal; DES, diethylstilbestrol; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ERR, estrogen-related receptor; ERRE, ERR-response element; ERRγ, estrogen-related receptor γ; 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 γ (ERRγ) with high constitutive basal activity (*1*). ERRγ 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 ERRγ, it became an immediate and important requirement to evaluate whether the previously reported effects of BPA at low doses are mediated through ERRγ and its specific target gene(s) (*11*).

BPA has the chemical structure of HO–C_6H_4–$C(CH_3)_2$–C_6H_4–OH with two phenol groups (Fig. 1). We have recently reported the crystallization and structural analysis of the BPA/ERRγ-ligand binding domain (LBD) complex (*12*). In the complex, a single molecule of BPA stays at the ligand-binding pocket of each ERRγ-LBD protein molecule, whose α-helix 12 (H12) is stabilized in an activation conformation. The crystal structure of the complex suggests several essential interactions between the BPA and ERRγ-LBD molecules. For instance, BPA's phenol-hydroxyl group is tethered by hydrogen bonds to the Glu275 and Arg316 residues in the ERRγ-LBD (Fig. 2). These hydrogen bonds were also observed in the ERRγ–LBD complex with 4-α-cumylphenol (*13*). Since 4-α-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 ERRγ. Indeed, by examining the ERRγ–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 ERRγ–LBD (*15*). 4-OHT deactivates ERRγ 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 ERRγ (*1*). Although the phenol-hydroxyl group of 4-OHT shares the same site for its binding to ERRγ, 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↔Arg316 hydrogen bonding

and the corroborative role of phenol-hydroxyl ↔Glu275 hydrogen bonding (*14*). This result strongly suggested that the formation of these double hydrogen bonds is critical for phenol compounds to bind to ERRγ. In the present study, to shed light on the structural significance of these double hydrogen bonds, we carried out a detailed comparison between BPA and 4-OHT for binding to ERRγ–LBD in a site-directed point mutagenesis series. We here report that human NR ERRγ binds highly specifically to BPA, but barely binds to the inverse agonist 4-OHT.

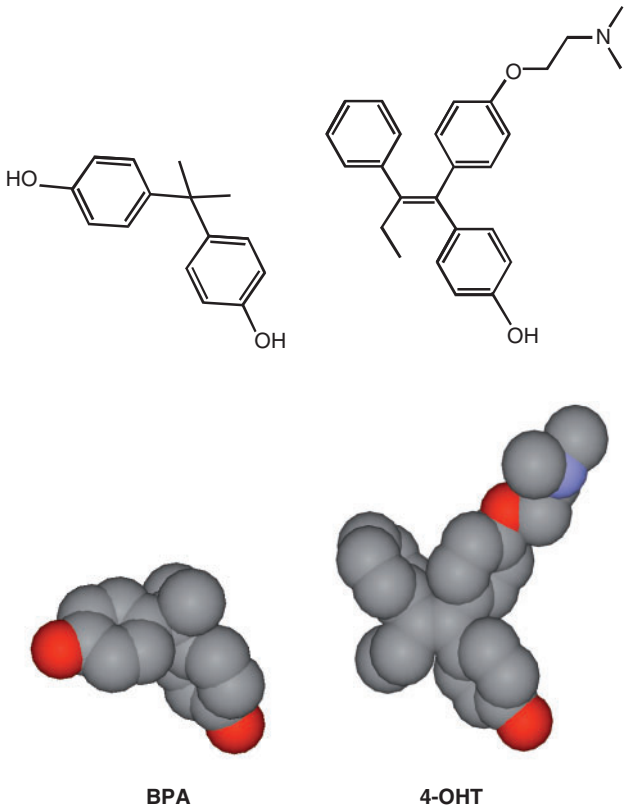

Fig. 1 Chemical structures of BPA and 4-OHT and their space-filling structures in the ligand-binding pocket of the ERRγ. The space-filling structure of BPA originated from the X-ray crystal structure [Protein Data Bank with accession code 2E2R (*12*)], and that of 4-OHT was also from the deposited structure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

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 ERRγ–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–ERRγ–LBD.

ERRγ mutants were generated using *PfuTurbo*® DNA Polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions using pGEX–ERRγ–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.

ERRγ–LBD protein expression

Two glutathione *S*-transferase (GST)-fused receptor proteins—the wild-type and mutant GST–ERRγ–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 × 100 mm, GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 8.0). The purity was confirmed by SDS–PAGE using 12.5% polyacrylamide

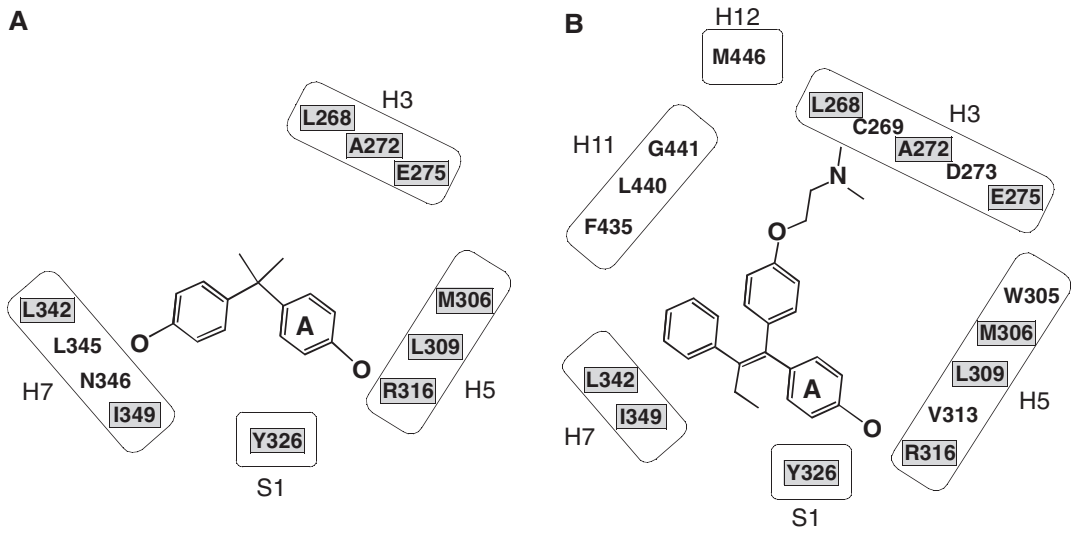

Fig. 2 Structural environments of BPA and 4-OHT in the ligand-binding pocket of the ERRγ. The proximity of each amino acid residue (within the distance 4 Å) to BPA (A) and to 4-OHT (B) is shown in the boxes depicting the α-helices. The amino acids in a grey backboard are the residues shared by both BPA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

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 ERRγ–LBD or its mutants—in 100 µl of binding buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM CHAPS and 2 mg/ml γ-globulin]. The assay was performed with or without the addition of unlabelled BPA or 4-OHT (final concentration of 1×10^{-5} M) to quantify the specific and nonspecific binding. After incubation with 100 µl 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 ERRγ–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 µl of 1% DCC in PBS (pH 7.4) for 10 min at 4°C. To estimate the binding affinity, 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 (*23*). Each assay was performed in duplicate and repeated at least three times.

Results

Critical structural elements to detain 4-OHT and BPA to ERRγ

For the receptor binding assays, the ERRγ–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–ERRγ–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 ERRγ receptor, the assay was carried out repeatedly for the specified times using varied concentrations of the receptor GST–ERRγ–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 ERRγ, 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 ERRγ were carried out for positions 275 and 316. When Glu275 and Arg316 were simultaneously mutated to Ala, the resulting (*Ala*, *Ala*)-ERRγ 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 (275*Ala*)-ERRγ 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 ERRγ) (Table I). [^{3}H]BPA's dissociation constant was 17.8 nM (32% of the binding affinity for the wild-type ERRγ). 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 ERRγ) 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 ERRγ–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 ERRγ, with IC_{50} values of 13.8 and 10.3 nM, respectively. For (275*Ala*)-ERRγ, the IC_{50} values were 365 nM (3.6% of the binding affinity for the wild-type ERRγ) and 356 nM (3.9%), respectively. However, for (316*Ala*)-ERRγ 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 ERRγ and its mutants by tritium-labelled BPA and 4-OHT.

Amino acid residues of ERRγ receptors[a]		Dissociation constant (K_D, nM)	
Position 275	Position 316	[^{3}H]BPA	[^{3}H]4-OHT
Glu (Wild-type)	Arg	5.70 ± 0.88	10.1 ± 0.29
Ala	Arg	17.8 ± 2.74	28.8 ± 3.28
Asp	Arg	22.0 ± 2.86	42.0 ± 6.73
Gln	Arg	23.4 ± 3.34	29.4 ± 3.74
Leu	Arg	NSB[b]	NSB
Glu	*Ala*	171 ± 39.5	210 ± 38.3
Glu	*Lys*	22.5 ± 4.26	29.4 ± 3.79
Glu	*Leu*	NSB	NSB
Ala	*Ala*	NSB	NSB
Arg	*Glu*	59.7 ± 6.79	77.3 ± 9.38
Ala	*Glu*	NSB	NSB
Arg	*Ala*	54.3 ± 6.82	74.8 ± 6.15

[a]Specifically mutated residues are designated in italics.
[b]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 ERRγ and its mutants by tritium-labelled 4-hydroxytamoxifen [^{3}H] 4-OHT.

Amino acid residues of ERRγ receptors[a]		Receptor binding potency IC_{50} (nM)	
Position 275	**Position 316**	**BPA**	**4-OHT**
Glu (Wild-type)	Arg	13.8 ± 2.55	10.3 ± 0.07
Ala	Arg	365 ± 16.1	356 ± 58.9
Asp	Arg	179 ± 24.1	184 ± 27.1
Gln	Arg	256 ± 21.2	222 ± 26.7
Leu	Arg	*Not carried out*[b]	
Glu	*Ala*	*Impossible to carry out*[c]	
Glu	*Lys*	228 ± 24.4	293 ± 36.7
Glu	*Leu*	*Not carried out*[b]	
Ala	*Ala*	*Not carried out*[b]	
Arg	*Glu*	*Impossible to carry out*[c]	
Ala	*Glu*	*Not carried out*[b]	
Arg	*Ala*	*Impossible to carry out*[c]	

[a]Specifically mutated residues are designated in italics.
[b]Because of 'no specific binding' in the saturation binding assay, the competitive binding assay was not carried out.
[c]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*)-ERRγ and (316*Leu*)-ERRγ 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*)-ERRγ mutant receptor exhibited a sufficient specific binding (~70% of the total binding) for [^{3}H]BPA, but just barely sufficient specific binding (~35% of the total binding) for [^{3}H]4-OHT. The K_D values were 23.4 nM (~25% of the binding affinity for the wild-type ERRγ) for [^{3}H]BPA and 29.4 nM (34%) for [^{3}H]4-OHT (Table I). The IC_{50} 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*)-ERRγ. It is therefore clear that Gln does not compensate for Glu, indicating that the carboxyl (COOH) group, but not the carboxyl amide ($CONH_2$) group, is crucial to capturing BPA and 4-OHT.

For (275*Asp*)-ERRγ with the Glu275→Asp substitution, [^{3}H]4-OHT exhibited just barely sufficient specific binding (~30% of the total binding) (data not shown). It was found that (275*Asp*)-ERRγ affords almost the same results obtained for (275*Ala*)-ERRγ and (275*Gln*)-ERRγ (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 ERRγ) (Table II). All these results indicate that the γ-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*)-ERRγ was found to be considerably weak for binding [^{3}H]4-OHT ($K_D = 29.4$ nM) and [^{3}H]BPA (22.5 nM) (Table I), since these activities are only ~25% that of the parent wild-type receptor ERRγ. In the competitive binding assay using (316*Lys*)-ERRγ and [^{3}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 ERRγ) (Table II). Furthermore, the inactivity of (316*Leu*)-ERRγ and the extremely weak activity of (316*Ala*)-ERRγ (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 ERRγ–LBD by hydrogen bonding.

Collectively, it is now clear that Glu275 and Arg316 are necessary for holding BPA and 4-OHT in ERRγ, but with different degrees of involvement in the hydrogen bonding. The results clearly indicate the major importance of phenol-hydroxyl↔Arg316 hydrogen bonding and the supportive role of the phenol-hydroxyl↔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}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*)-ERRγ 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}H]BPA was found to bind to this (*Arg*, *Glu*)-ERRγ 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}H]4-OHT, as shown in Table I. [^{3}H]4-OHT bound to (*Arg*, *Glu*)-ERRγ 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}H]BPA was ~65%. In contrast, [^{3}H]4-OHT exhibited only a very small specific binding (<20%) in relation to the total binding. Thus, since [^{3}H]4-OHT could only barely bind to (*Arg*, *Glu*)-ERRγ, it was impossible to carry out the competitive binding assay (Table II).

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

for [^{3}H]BPA and [^{3}H]4-OHT (Table I). In the saturation binding assay for (*Arg, Ala*)-ERRγ, these tritium-labelled compounds exhibited similar results to those obtained for (*Arg*, *Glu*)-ERRγ—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*)-ERRγ 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*)-ERRγ receptor by using [^{3}H]4-OHT as a tracer (Table II).

The fact that the Arg-containing (*Arg, Glu*)-ERRγ and (*Arg, Ala*)-ERRγ mutant receptors were still active, but (*Ala, Glu*)-ERRγ with no Arg was completely inactive, led us to conclude that phenol-hydroxyl↔275*Arg* hydrogen bonding plays a primary role, while the role of phenol-hydroxyl↔316*Glu* hydrogen bonding is only supportive. Furthermore, our finding that (*Arg*, *Glu*)-ERRγ and (*Arg, Ala*)-ERRγ are almost equipotent (Table I) indicates that the supportive role of the phenol-hydroxyl↔316*Glu* 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*)–ERRγ complex (under submission). Although Glu275 and Arg316 in ERRγ are interchangeable for keeping up the interaction with BPA and 4-OHT, ERRγ 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 ERRγ 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, (275*Ala*)-, (275*Asp*)-, (275*Gln*)-, (316*Lys*)- and (*Arg*, *Glu*)-ERRγ, 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 (275*Ala*)-ERRγ 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 ERRγ 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

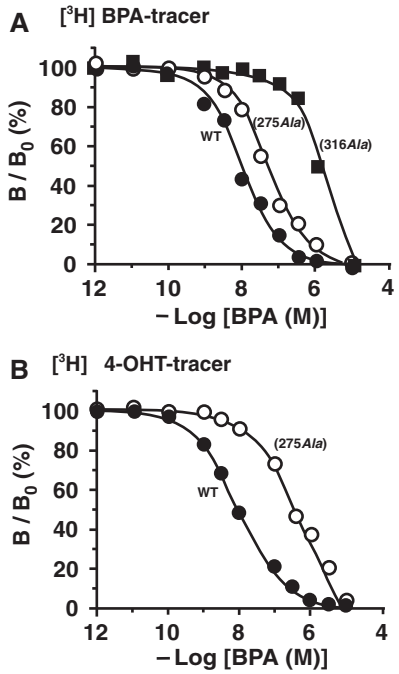

Fig. 3 Concentration-dependent curves of BPA for ERRγ–LBD and its site-directed mutant derivatives by using tritium-labelled BPA (A) and 4-OHT (B). The receptor competitive binding assays were carried out to measure the ability to displace [^{3}H]BPA (A) and [^{3}H]4-OHT (B). Used receptors are wild-type ERRγ (filled circle), (275*Ala*)-ERRγ with the Glu275→Ala substitution (open circle), and (316*Ala*)-ERRγ with the Arg316→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 ERRγ and its mutant receptors. The IC_{50} values of BPA in the [^{3}H]BPA binding assay (A) were 9.70 ± 0.59 nM for wild-type ERRγ, 35.7 ± 5.45 nM for (275*Ala*)-ERRγ and 990 ± 78.4 nM for (316*Ala*)-ERRγ (*14*). Those in the [^{3}H]4-OHT binding assay (B) were 10.3 ± 0.07 nM for wild-type ERRγ and 365 ± 16.1 nM for (275*Ala*)-ERRγ, as shown in Table II. The assay for (316*Ala*)-ERRγ 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*).

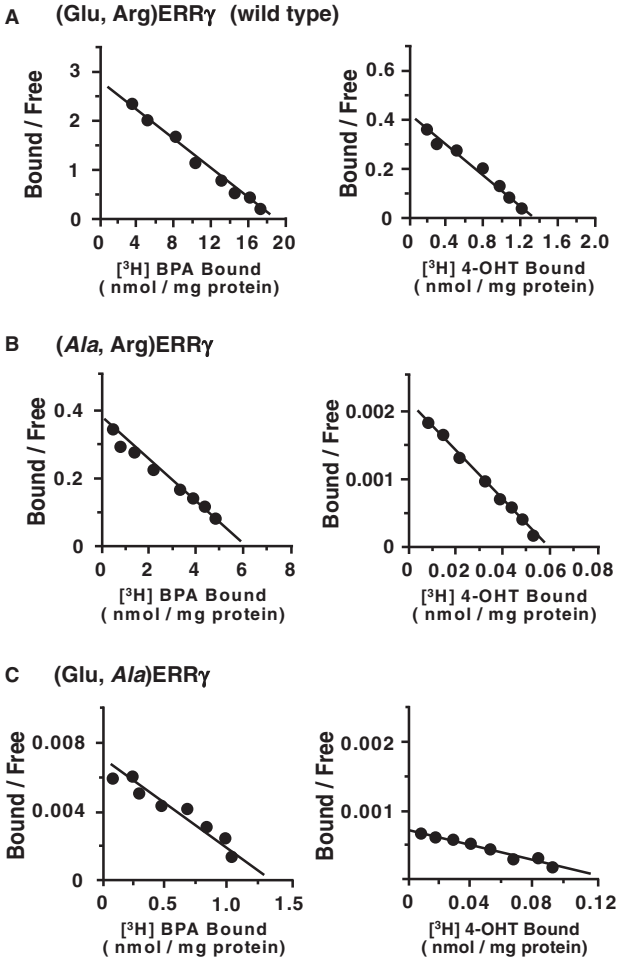

Fig. 4 Scatchard plot analyses showing a single binding mode with a binding affinity constant (K_d) and receptor density (B_{max}). Analyses were carried out from the radioligand receptor saturation binding curves of [^{3}H]BPA (left-side figures) and [^{3}H]4-OHT (right-side figures) for the human ERRγ ligand-binding domain (LBD) and its site-directed mutant derivatives. Those include the wild-type ERRγ (A), (275*Ala*)-ERRγ with the Glu275→Ala substitution (B), and (316*Ala*)-ERRγ with the Arg316→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 ERRγ 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 ERRγ 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 ERRγ is relatively small, as is also true for other mutant receptors.

Discussion

Structural requirements of ERRγ to bind BPA and 4-OHT

Among the total 48 human NRs, 26 NRs have Arg at the position corresponding to ERRγ's 316 (*14*). In contrast to this Arg316, Glu275 is conserved among only five NRs: ERα, ERβ, ERRα, ERRβ and ERRγ.

Table III. Receptor density of ERRγ and its mutants by tritium-labelled BPA and 4-OHT.

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

[a]Specifically mutated residues are designated in italics.
[b]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 ERRγ 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/ERRγ complexes (unpublished data). Although the natural hormone of ERα and ERβ is 17β-estradiol, ERRα, ERRβ and ERRγ are all orphan receptors whose endogenous ligand is not identified (*25–28*). It is highly likely that if the ERRs have any endogenous ligands, they must be phenol compounds.

When the Glu275→Ala substitution was accomplished, the resulting mutant receptor (275*Ala*)-ERRγ 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 (316*Ala*)-ERRγ with the Arg316→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 (316*Ala*)-ERRγ. It is clear that the same structural changes in ERRγ 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 ERRγ receptor (*12*, *15*, *29*, *30*). Thus, their different receptor binding modes are definitely due to structures other than the phenol group.

For the ERRγ receptor, 4-OHT acts as an inverse agonist (*1*, *16*), the binding of which dissociates the α-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–ERRγ complex (*15*). This process appears to be a highly energy-consuming option. In contrast, H12 of non-liganded ERRγ–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–ERRγ complex (*12*). Thus, there is indeed a distinct difference in binding modes between 4-OHT and BPA for ERRγ.

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 ERRγ, 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 ERRγ.

Different potentials of BPA and 4-OHT in binding to ERRγ

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

The difference between [^{3}H]4-OHT and [^{3}H]BPA is further revealed in their maximal receptor density B_{max} values. [^{3}H]BPA afforded well-matched B_{max} values for the wild-type ERRγ; *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 [^{3}H]4-OHT was diminished significantly, with only 1.26 nmol/mg protein (~7% compared with that of [^{3}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 [^{3}H]4-OHT to bind to ERRγ. BPA binds directly to ERRγ–LBD folded in the activation conformation without any conformational changes in the receptor.

As to the mutant receptors such as (275*Ala*)-, (275*Gln*)-, (275*Asp*)- and (316*Lys*)-ERRγ, [^{3}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 [^{3}H]4-OHT for these mutant receptors were drastically reduced, showing only 0.9–4.5% values as compared with that of [^{3}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 ERRγ 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, (316*Ala*)-ERRγ, [^{3}H]BPA and [^{3}H]4-OHT bound only weakly. For the ERRγ mutant receptors containing Arg at position 275, namely (*Arg*, *Glu*)-ERRγ and (*Arg*, *Ala*)-ERRγ, [^{3}H]BPA and [^{3}H]4-OHT could bind, but with fairly small B_{max} values. By contrast, to the mutant ERRγ receptors that are entirely lacking Arg at positions either 275 or 316, [^{3}H]4-OHT and [^{3}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 ERRγ. Thus, this Arg316 residue would play a central role in capturing phenolic chemicals.

Funding

This study was supported by Health and Labour Sciences Research Grants for Research on the Risk of Chemical Substances from the Ministry of Health, Labor and Welfare of Japan. This work was also supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture in Japan to Y.S.

Conflict of interest

None declared.

References

1. Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., and Shimohigashi, Y. (2006) Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. *Toxicol. Lett*. **167**, 95–105
2. Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839
3. Robinson-Rechavi, M., Carpentier, A.S., Duffraisse, M., and Laudet, V. (2001) How many nuclear hormone receptors are there in the human genome? *Trends Genet*. **17**, 554–556
4. Dodds, E.C. and Lawson, W. (1938) Molecular structure in relation to oestrogenic activity. Compounds without a phenanthrene nucleus. *Proc. Royal Soc. Lon. B* **125**, 222–232
5. Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., and Feldman, D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279–2286
6. Olea, N., Pulgar, R., Perez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A.M., and Sonnenschein, C. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect*. **104**, 298–305
7. vom Saal, F.S., Cooke, P.S., Buchanan, D.L., Palanza, P., Thayer, K.A., Nagel, S.C., Parmigiani, S., and Welshons, W.V. (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol. Ind. Health* **14**, 239–260
8. Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R., and Aou, S. (2003) Low dose effects of bisphenol A

on sexual differentiation of the brain and behavior in rats. *Neurosci Res*. **45**, 345–356
9. vom Saal, F.S. and Hughes, C. (2005) An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ. Health Perspect*. **113**, 926–933
10. Kamrin, M.A. (2007) The 'low dose' hypothesis: validity and implications for human risk. *Int. J. Toxicol*. **26**, 13–23
11. Okada, H., Tokunaga, T., Liu, X., Matsushima, A., and Shimohigashi, Y. (2008) Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor γ (ERRγ). *Environ. Health Perspect*. **116**, 32–38
12. Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M., and Shimohigashi, Y. (2007) Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERRγ. *J. Biochem*. **142**, 517–524
13. Matsushima, A., Teramoto, T., Okada, H., Liu, X., Tokunaga, T., Kakuta, Y., and Shimohigashi, Y. (2008) ERRγ tethers strongly bisphenol A and 4-α-cumylphenol in an induced-fit manner. *Biochem. Biophys. Res. Commun*. **373**, 408–413
14. Liu, X., Matsushima, A., Okada, H., Tokunaga, T., Isozaki, K., and Shimohigashi, Y. (2007) Receptor binding characteristics of endocrine disruptor bisphenol A for the human nuclear receptor of estrogen-related receptor γ (ERRγ): chief and corroborative hydrogen bonds of bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues. *FEBS J*. **274**, 6340–6351
15. Greschik, H., Flaig, R., Renaud, J.P., and Moras, D. (2004) Structural basis for the deactivation of the estrogen-related receptor gamma by diethylstilbestrol or 4-hydroxytamoxifen and determinants of selectivity. *J. Biol. Chem*. **279**, 33639–33646
16. Coward, P., Lee, D., Hull, M.V., and Lehmann, J.M. (2001) 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor gamma. *Proc. Natl Acad. Sci. USA* **98**, 8880–8884
17. Tremblay, G.B., Bergeron, D., and Giguere, V. (2001) 4-Hydroxytamoxifen is and isoform-specific inhibitor of orphan estrogen-receptor-related (ERR) nuclear receptors beta and gamma. *Endocrinology* **142**, 4572–4575
18. Nelson, R.M. and Long, G.L. (1989) A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction. *Anal. Biochem*. **180**, 47–51
19. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**, 248–254
20. Nakai, M., Tabira, Y., Asai, D., Yakabe, Y., Shimyozu, T., Noguchi, M., Takatsuki, M., and Shimohigashi, Y. (1999) Binding characteristics of dialkyl phthalates for the estrogen receptor. *Biochem. Biophys. Res. Commun*. **254**, 311–314
21. Pichon, M.F. and Milgrom, E. (1973) Competitive protein binding assay of progesterone without chromatography. *Steroids* **21**, 335–346
22. Scatchard, G. (1948) The attractions of proteins for small molecules and ions. *Ann. NY. Acad. Sci*. **51**, 660–672
23. DeLean, A., Munson, P.J., and Rodbard, D. (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol*. **235**, E97–E102
24. Heard, D.J., Norby, P.L., Holloway, J., and Vissing, H. (2000) Human ERRγ, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue-specific isoforms are expressed during development and in the adult. *Mol. Endocrinol*. **14**, 382–392
25. Brown, M. and Sharp, P.A. (1990) Human estrogen receptor forms multiple protein-DNA complexes. *J. Biol. Chem*. **265**, 11238–11243
26. Katzenellenbogen, B.S. (1996) Estrogen receptors: bioactivities and interaction with cell signaling pathway. *Biol. Reprod*. **54**, 287–293
27. Giguere, V. (2002) To ERR in the estrogen pathway. *Trends Endocrinol. Metab*. **13**, 220–225
28. Horard, B. and Vanacker, J.M. (2003) Estrogen receptor-related receptors: orphan receptors desperately seeking a ligand. *J. Mol. Endocrinol*. **31**, 349–357
29. Greschik, H., Wurtz, J.M., Sanglier, S., Bourguet, W., van Dorsselaer, A., Moras, D., and Renaud, J.P. (2002) Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. *Mol. Cell* **9**, 303–313
30. Wang, L., Zuercher, W.J., Consler, T.G., Lambert, M.H., Miller, A.B., Orband-Miller, L.A., Mckee, D.D., Willson, T.M., and Nolte, R.T. (2006) X-ray crystal structures of the estrogen-related receptor-γ ligand binding domain in three functional states reveal the molecular basis of small molecule regulation. *J. Biol. Chem*. **281**, 37773–37781