Table 2-2
Contingency table based on the RBA giving a maximum concordance

		ER-binding assay		Total	Index	Rate(%)
		P	N			
Uterotrophic assay	P/P	*21*	0	21	Concordance	82
Estrogenic	P/N	*7*	3	10		
/Anti-estrogenic	N/P	*2*	2	4	False negative	14
activities	N/N	7	*23*	30		
Total		37	28	65	False positive	23

P: positives, N: negatives, P/N: positives in estrogenic and negatives in anti-estrogenic activities, P/P: positives in both estrogenic and anti-estrogenic activities, N/P: negatives in estrogenic and positives in anti estrogenic activities and N/N: negatives in both estrogenic and anti-estrogenic activities.

(Table 2-2). This cutoff achieved the best concordance and lowest false-negative ratios as shown in Fig. 2.

4. Discussion

After the potential of chemicals to disrupt the endocrine became apparent, numerous efforts have been made to test and assess chemicals for their endocrine disrupting potential. To detect ER mediated effects, the application of the *in vitro* ER binding assay and *in vivo* rodent uterotrophic assay have long been investigated since ER mediation has been considered as a major mechanism of endocrine disruption of exogenous chemicals.

In order to understand the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays and to investigate the biologically meaningful binding potency from an *in vitro* assay, we compared the results obtained from a receptor binding assay using hERα and the immature rat uterotrophic assay for 65 chemicals spanning a variety of chemicals classes.

For a quantitative comparison of logRBAs and logLEDs, the log RBA was found to be well correlated with both log LEDs of estrogenic and anti-estrogenic assay results at $r^2 = 0.67$ and 0.79, respectively (Fig. 1). These results strongly suggest that there was a positive relationship between the two assays and that both assays detect same biological mechanism, i.e., ER mediated biological responses. It also suggests that the result from the uterotrophic assay can be predicted, in some instances, from the results of the ER binding assay. However, care must be taken to extrapolate *in vitro* data because some important factors, such as the interaction of the ER with other endocrine related systems and metabolism of the test chemical in*in vivo* situation cannot be negligible.

The contingency table analysis of the results from the *in vitro* ER binding and the *in vivo* uterotrophic assays for all 65 chemicals revealed a relatively good concordance ratio (66%). In this comparison, androgens, phthalates and other classes of chemicals were identified as presenting conflicting results in the two assays under the test conditions. Two androgens, testosterone enanthate and 17α-methyltestosterone, were identified as non-ER binders that were estrogenic in the uterotrophic assay. The potential of androgens to stimulate uterine growth in immature female rat is known (Armstrongm et al., 1976). Armstrongm et al. (1976) investigated the effect of testosterone on uterine weight of immature female rat by subcutaneous administration, and clearly demonstrated the increase of uterine weight and the potential of aromatization to convert testosterone to E2. And the enzymatic activity of aromatase in immature female rat has been also observed (el-Maasarany et al., 1991). Testosterone enanthate could be converted to testosterone, i.e. the precursors of estrogens, by hydrolysis in the body. The aromatization of 17α-methyltestosterone to 17α-methylestradiol has been confirmed in *in vitro* assay using human aromatase (de Gooyer et al., 2003). Thus, both testosterone enanthate and 17α-methyltestosterone can be precursors of estrogens and can elevate the estrogen levels caused by aromatization of these administrated androgens, and this would be expected to result in an increase of the uterine weight. At this moment, the metabolic fate of test chemicals in the immature rat uterotrophic assay cannot be estimated precisely and therefore the impact of metabolic system on the inconsistency between these assays cannot be fully explained. Accordingly, the metabolic issue on their assay systems should be extensively explored in the future. *p*-Diethylaminobenzaldehyde that showed the same discrepancy as androgens has been reported as androgen receptor antagonist in the transcriptional activation assay (Araki et al., 2005). But its antiandrogenic effect on the uterotrophic assay is not known and the further investigation may be necessary. There were seventeen chemicals that had ER binding potency but neither estrogenic nor anti-estrogenic activities in the uterotrophic assay. Three benzothiazoles and six phthalates were included among these chemicals. Benzothiazoles seems to be readily metabolized and at least two benzothiazoles that had more than 0.002% of RBA would be metabolized to 2-mercaptobenzothiazole having 0.00165% of RBA (el Dareer et al., 1989; Elfarra and Hwang, 1990; Fukuoka and Tanaka, 1987). In this study, 9 phthalates were tested and 6 of them had ER binding affinity ranging from −3.49 to −1.15 as logRBA. However, none of phthalates elicited estrogenic or anti-estrogenic responses in the uterotrophic assay in this study. Some phthalates showed ER-mediated activities in *in vitro* assays but no estrogenic response in *in vivo* model as shown in this study (Hong et al., 2005; Zacharewski et al., 1998). These discrepancies

between *in vitro* and *in vivo* assays in phthalates are probably caused by the deactivation of phthalates to mono alkyl phthalates (Harris et al., 1997; Picard et al., 2001; Zacharewski et al., 1998). The other chemicals with inconsistent response outliers between the *in vitro* and *in vivo* assay comparison had relatively weak ER binding potencies.

The quantitative comparison found that the 0.00233% of RBA of *p-tert*-butylphenol was the lowest ER binding potency detected in the ER binding assay that elicited estrogenic or anti-estrogenic activities in the immature rat uterotrophic assay and this RBA is considered as the detection limit of estrogenic or anti-estrogenic activities observed in the uterotrophic assay. The use of this cutoff value considerably improved the concordance between the two assays without increasing the false negative rate by excluding the weak ER binders for which estrogenic or anti-estrogenic activities cannot be detected in the *in vivo* assay.

Our studies revealed that the quantitative relationship between the ER receptor binding assay and uterotrophic assay, and the application of cutoff based on meaningful ER binding affinity can provide the best concordance between two assays. These findings are useful in a tiered approach for identifying chemicals that have potential to induce ER-mediated effects in *in vivo*, though it is necessary to consider the metabolic capacity in *in vivo* situation.

Acknowledgement

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The Conformation Change-Sensing Antibodies for Retinoid-Related Orphan Receptor Family

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Rapid and efficient procedures for evaluation of endocrine disrupting chemicals are required extensively in the fields. We have established a novel assay procedure to evaluate simultaneously the binding ability and the biological activity of the particular compound for the target nuclear receptors (NRs). We designated this method as the conformation change-sensing assay. In order to expand this method to all NRs, we initiated the preparation of conformation sensing polyclonal antibodies for retinoid-related orphan receptors.

Keywords: conformation change-sensing antibody, endocrine disruptors, nuclear receptor, retinoid-related orphan receptor (ROR)

Introduction

Endocrine disruptors are thought to mediate some effects or influences through the transcriptional nuclear receptors (NRs). In order to evaluate the binding ability and biological activity of the particular compound for NRs, we have established a novel assay procedure, in which the conformation change along with the ligand binding is measured quantitationaly by antibodies. Antibodies allow for NRs to evaluate simultaneously the receptor binding and hormonal activity of chemicals. Such conformation sensing polyclonal antibodies can recognize the ligand-induced conformation change of helix-12 (H12) of NRs.

Recently, endocrine disruptors have acknowledged to influence on not only the estrogen receptor (ER) but also all other NRs. Thus, it is keen to develop the assay methodology applicable to all of 48 human NRs. In the present study, we prepared polyclonal antibodies specific for H12 of retinoid-related orphan receptor (ROR) family. This family consists of three members of subtypes, RORα, RORβ, and RORγ, which have a wide range of functions including the born metabolism, maintenance of circadian rhythm, and the gain of immunologic function [1, 2]. Therefore, it is important to establish an efficient assay method for RORs' disturbances. We describe here the design and preparation of antigen peptides and the specificity of antibodies prepared.

RORα: --PDI**VRLHFPPLYKELFTSEF**EPAM---
RORβ: --PEI**VNTLFPPLYKELFNPD**STGCK---
RORγ: --PIVVQ**AAFPPLYKELFSTET**ESPV---

Fig. 1. Amino acid sequences of C-terminal region of ligand binding domain of RORs. The sequences underlined indicate the helix 12, and the bold letters indicate the fragment used as antigen peptide raising specific antibodies anti-ROR-helix 12.

Results and Discussion

The C-terminal region of RORs is highly conserved. In particular, the amino acid sequences of H12 are completely identical. To obtain either specific or nonspecific antibodies for each ROR, H12-containing antigen peptides were designed as shown in Fig. 1. Peptides synthesized by the solid phase method were conjugated to a carrier protein, keyhole (KLH) for immunization. Obtained rabbit polyclonal antibodies were purified by KLH immunoprecipitation followed by affinity chromatography using H12 peptide-linked agarose gel.

To examine the ability of antibodies obtained to bind to the target peptides, competitive ELISA was performed (Fig. 2). RORα-antibody recognized RORβ and RORγ antigen peptides as well as the antigen peptide prepared for RORα itself, suggesting that it recognizes the sequence common to all these RORs. That is definitely H12. RORγ-antibody similarly recognized antigens for RORα and RORβ. On the other hand, RORβ-antibody recognized their own antigen very strongly. Thus, RORβ-antibody recognized the unique sequence of the RORβ-antigen peptide. A combination of these antibodies may afford the structural information to evaluate the receptor activities of chemicals.

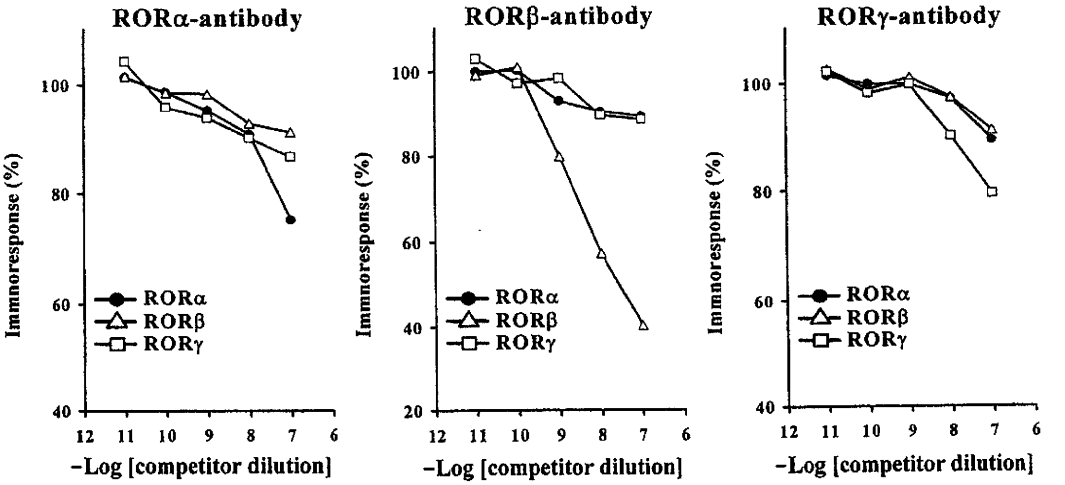

Fig. 2. The results of competitive ELISA. BThG-linked antigen peptides were coated at the varying concentration.

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A Docking Modelling Rationally Predicts Strong Binding of Bisphenol A to Estrogen-Related Receptor γ

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Abstract: A computer-aided docking study was carried out to quickly clarify the binding structure of the ligand-receptor complex between bisphenol A (BPA), a well-known endocrine disruptor, and estrogen-related receptor γ (ERRγ). The resulting complex indicated that BPA binds to the ligand-binding pocket of ERRγ without any disruptions of the activation conformation.

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Keywords: Estrogen-related receptor γ, bisphenol A, endocrine disruptors, docking calculation.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are chemicals that mimic the effects of hormones and thereby disrupt endocrine systems. Numerous common industrial chemicals are suspected of being EDCs. Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is a strong EDC candidate. BPA is now important as a raw material for epoxy resins and polycarbonate plastics. In 1993, Krishnan *et al.* reported that BPA leaked from a flask made of polycarbonates and caused abnormal growth of MCF-7 human breast cancer cells by mimicking the activity of the native estrogen 17β-estradiol (E2) [1]. Also, Gaido *et al.* described that BPA as well as E2 exhibited transactivation activity in a yeast-based estrogen receptor gene transcription assay [2]. Although the activities of these BPAs were much weaker than that of E2 (1/5,000 to 1/15,000 of the activity of E2), BPA was acknowledged as one of the EDCs that act upon estrogen receptor (ER).

Nuclear receptors are a family of 48 or more intracellular receptors in humans. Estrogen-related receptor (ERR) is a subfamily of human nuclear receptors closely related to ER [3–5]. In spite of their high homology to ER, ERR members do not respond to E2, and constitutively activate the transcription in eukaryotic cells. Meanwhile, vom Saal *et al.* have extensively documented numerous low-dose effects of BPA [6]. The low-dose effects of BPA have also been reported by many other groups (for review vom Saal *et al.* [7]); for example, Belcher *et al.* reported that BPA disrupts neural development in the rat fetus [8]. For these low-dose effects of BPA, it has been thought that ER is a target receptor. However, Takayanagi *et al.* reported recently that BPA strongly binds to ERRγ [9]. These results raise the possibility that BPA may be an EDC of ERRγ possessing unidentified activity. Thus, this unpredictable strong binding potency of BPA has underscored the need for development of a new rapid procedure to assess the risk posed to all nuclear receptors.

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As a strategy to screen a large number of chemicals with or without endocrine disruption potentials, studies on the quantitative structure-activity relationship (QSAR) have been carried out, especially for ER [10–12]. Recently, computational docking operation becomes a useful vehicle for investigating the molecular binding interactions [13-27]. Advances in three-dimensional (3D) modeling and docking strategies allow the application of *in silico* structure-based drug design studies (SBDD) to such assessments. These were originally designed to predict how small molecules such as ligands or drug candidates bind to a receptor whose 3D structure has been clarified. Indeed, if an *in silico* EDC screening system based on SBDD was available, such a system could perform a high-speed screening of chemicals against nuclear receptor-LBDs, thereby providing an effective risk assessment without the need for costly and time-consuming wet experiments.

The present study aims to examine the question of how BPA docks with the LBD, based on the fact that BPA shows strong binding activity to ERRγ. Heretofore, the attention has been paid to the sex steroid hormone receptors ER and AR as targets of EDCs. However, the binding of BPA to ERRγ invokes to involve all the nuclear receptors to investigate. In this report, we performed computer-aided docking studies on the BPA and ERRγ-LBD complexes to clarify the structural essentials by which they bind to each other. The complex structure of BPA/ERRγ-LBD, which was calculated in this study, successfully described its high constitutive activity. BPA bound to ERRγ has been found as a quite unique space-filler in the ligand-binding domain.

MATERIALS AND METHOD

3D structures of BPA and other ligands were constructed by the program Sketch, one of the modules of Insight II (Accerlys, San Diego, CA). In order to prepare the receptor molecule appropriately in the docking calculation, hydrogens were added onto heavy atoms identified by X-ray crystallography (1TFC: PDB code), and the charges were assigned by Biopolymer module in the neutral condition. CFF91 force field (Accerlys) was used in all molecular mechanics calcu-

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lations. For calculation of the volume of the ligand-pocket, the 3D structure of apo-form ERRγ-LBD (1TFC) was used [28]. The volume size of vacant ligand-pocket was estimated and determined by means of an active site finding tool called Binding Site Analysis (Accelrys). Using volume keyword, molecular volumes were computed by Gaussian 03 equipped with 6-31G basis set, following energy minimization step [29]. Structural formulas of all the ligands used in this study are shown in Fig. **1**.

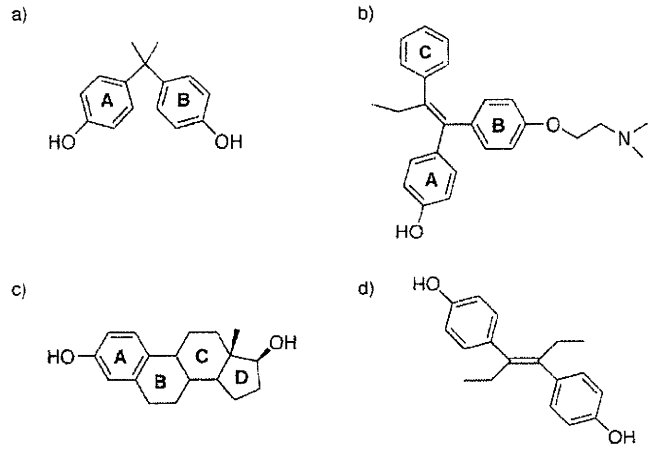

Figure 1. The structural formulas of bisphenol A (BPA), 4-hydroxytamoxifen (4-OHT), 17β-estradiol (E2), and diethylstilbestrol (DES). a: BPA, BPA possesses the two phenol groups A and B together with the two methyl groups. b: 4-OHT, 4-OHT possesses three benzene rings on the trans-ethylene double bond: i.e., A, phenol; B, *p*-β -dimethylethoxyphenyl; and C, phenyl rings. c: E2, and d: DES.

Docking calculations between BPA and ERRγ-LBD (1TFC) were carried out by using Affinity program (Accelrys) in grid docking methodology with CFF91 force field on SGI O2 workstation [30, 31]. The flexible region of the docking calculation includes the BPA molecule initially placed and all the residues in an 8 Å-surrounding distance in the ligand pocket of LBD. BPA was placed at three different positions by referring to the structure of 4-OHT in the 4-OHT/ERRγ-LBD complex (1S9Q) [28].

With the aim of binding energy calculation of BPA in each complex, 6-31G level *ab initio* FMO-MP2 calculations were performed by ABINIT-MP (Advanced Soft, Tokyo, Japan) with BPA and amino acid residues of ERRγ-LBD being within 6 Å from BPA [32-35]. FMO calculations were carried out on a parallel UNIX server, IBM eServer p5 model 595, at the computing and communications center of Kyushu university. The binding energies (ΔE) between BPA and ERRγ-LBD were calculated from the computed results of the FMO calculations by the method described by Fukuzawa *et al.* [36]. Binding energy (ΔE) between BPA and ERRγ-LBD can be expressed in the equation **1** as the difference in each energy value of the receptor ($E_{receptor}$), ligand (E_{ligand}), and complex ($E_{complex}$) [36].

$$\Delta E = E_{complex} - (E_{receptor} + E_{ligand}) \quad (1)$$

RESULTS AND DISCUSSION

ERRγ is a constitutively active and orphan receptor. Although no natural ligand is known, ERRγ is deactivated by DES and 4-OHT [37-39]. To date, the data on five 3D structures of ERRγ-LBD have been deposited in the RCSB-protein data bank. Two of the five structures explain the non-liganded apo-form [28, 40], and the other three structures show the holo-structures of ERRγ-LBD bound with either 4-OHT or DES [28]. In order to discuss the probability of BPA binding with ERRγ-LBD, we first calculated the volume of the ligand-pocket of ERRγ-LBD and the molecular volume of BPA. Since the binding sites of 4-OHT and DES in ERRγ-LBD have been determined to be the same by the X-ray crystal analysis, we first selected the one as a putative ligand binding pocket for BPA. The volume size of vacant ligand space in the apo-form ERRγ-LBD was calculated to be 293.6 $Å^3$. The molecular volume of BPA was computed precisely by using the volume keyword in Gaussian 03 and the calculated volume was 295.2 $Å^3$. Although the program Binding Site Analysis provided several other pockets, their volume sizes were much smaller than BPA's molecular size.

As a result, we could obtain compatible values for volumes of the ligand and the receptor. Based on this finding, BPA was judged to have a sufficient volume to bind to the vacant space of the ERRγ-LBD apo-form.

To examine how BPA binds to ERRγ-LBD, flexible docking calculations were carried out using the program Affinity (Accelrys) with the apo-form ERRγ-LBD (1TFC) as a template [30, 31]. In this study, BPA was manually placed at three different positions by referring to the structure of 4-OHT in the 4-OHT/ERRγ-LBD complex (1S9Q) before the docking calculation [28]. As shown in Fig. **1** and **2**, 4-OHT possesses 3 different aromatic rings, namely, the phenol (A-ring), the *p*-β -dimethylethoxyphenyl (B-ring) and the phenyl (C-ring) on the trans-ethylene double bond. BPA has two phenol groups (A and B) on the sp^3-carbon atom. Placing the A-ring of BPA at the point where the A-ring of 4-OHT is located, we attempted to place the B-ring of BPA at the points corresponding to the point where the B- or C-ring of 4-OHT is located. In addition to these two arrangements, we further attempted to place BPA to take the initial positioning with the B- and C-rings of 4-OHT. From each docking calculation, 5-7 different structures of the BPA/ERRγ-LBD complex were obtained, and their affinity scores are listed in Table **1**.

Complexes **1-7**, **2-3**, and **3-6** gave the best affinity score in each calculation, and we selected these as the representative complexes. When BPA was placed at random in the LBD, the Affinity docking calculations resulted in the structures similar to **1-7** and **2-3**. Since these structures never gave the Affinity scores greater than **1-7** and **2-3**, we just selected the complexes **1-7**, **2-3**, and **3-6** as the structures for further examinations.

Fig. **3** illustrates these selected docked structures of BPA. It should be noted that BPA has almost the same position in each of these docked structures, even though the calculations were initiated from completely different placements of BPA. The binding structure of **2-3** is almost completely compatible to that of **3-6**, although BPA in **1-7** is in a different orientation. In particular, one of the methyl groups of **1-7**, the left

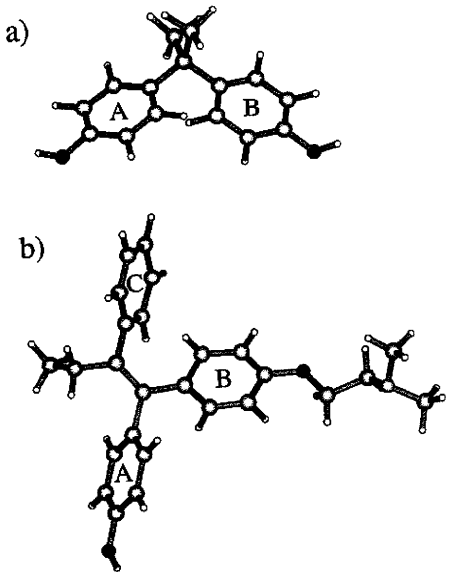

Figure 2. The three dimensional structures of bisphenol A (BPA) and 4-hydroxytamoxifen (4-OHT). a: BPA is shown by its minimum energy conformation. b: 4-OHT is shown by the structure pulled out from the 4-OHT/ERR γ-LBD complex (1S9Q). Characters (A and B in 'a' and A, B and C in 'b') indicated the same ring structures as shown in Fig. **1**.

side methyl group (α in Fig. **3a**), was found to be apart from that of **2-3** (Fig. **3**). As a result, the methyl group α of **1-7** is located in the hydrophobic pocket constructed by Met306, Leu309, and Ile310. On the other hand, the methyl group α' of **2-3** (Fig. **3b**) is in close proximity to the benzene ring of Phe450 (< 3.1 Å), which may be responsible for the CH/π interaction. These results clearly indicate that the pocket or vacant space available for BPA is uniform and there are only a limited number of attachment positions by which BPA can occupy it.

We preliminary carried out the Affinity docking calculations to complex BPA into other templates derived from ERRγ-LBD/4-OHT or DES (1S9Q or 1S9P). However, the resulting BPA-binding structures were found to leave a considerably large empty space, with the activation function (AF)-containing H12 being in deactivation conformation. Apparently, this is inappropriate to explain BPA's high binding affinity and high basal constitutive activity.

For detailed comparison of the binding energies of BPA in these three BPA/ERRγ-LBD complexes, we carried out *ab initio* (HF and MP2 level) calculations by the fragment molecular orbital (FMO) method [32-36]. As shown in Table **2**, HF and MP2 calculations afforded the results of negative ΔE values for BPAs, indicating a structural stabilization due to the ligand binding. Such negative ΔE values reveal that BPA is a favorable binder of ERRγ, as 4-OHT and DES are.

Table 1. Results of the Flexible Docking Calculations of Bisphenol A (BPA) to the ERRγ-LBD apo-form by the Computer Program Affinity

Complex No.[a]	Number of Appearances[b]	Energy (kcal/mol)	Ranking
1-1	9	–864.683	7
1-2	16	–887.407	6
1-3	15	–887.565	5
1-4	13	–890.651	3
1-5	33	–890.461	4
1-6	15	–898.950	2
1-7	13	–898.996	1
2-1	7	–921.715	5
2-2	20	–928.930	4
2-3	18	–930.596	1
2-4	14	–930.595	2
2-5	30	–928.931	3
3-1	6	–842.793	5
3-2	4	–848.302	4
3-3	1	–840.223	6
3-4	1	–880.767	3
3-5	26	–882.895	2
3-6	33	–882.896	1

[a]Complex number **1**, **2**, and **3** represent the calculations started from different initial positionings, respectively. In complex **1**, the phenol rings of BPA are placed at the positions of the A- and B-rings of 4-OHT (see Fig. **2**). Complex **2** is placed in the positions of the A- and C-rings, and complex **3** is placed in the positions of the B- and C-rings.
[b]It means the times appeared as the result in each affinity calculation.

To compare the binding energy of BPA with that of a weak binder, we selected E2, an endogenous ligand of ER. Flexible docking calculations between E2 and ERRγ-LBD (1TFC) followed by FMO calculations were carried out. It was found that E2 exhibits ΔE value of +19.8 kcal/mol, which is much larger than those of binders 4-OHT, DES and BPA in the HF calculation (Table **2**). This is a demonstration that E2 is indeed a weak binder of ERRγ. This notably large ΔE value obtained by the HF calculation indicates that there was an unfavorable spatial contact and conformation change along with a complex formation between E2 and ERRγ-LBD. In the calculated E2/ERRγ-LBD complex, the steroid structure of E2 in a planer configuration warped almost 45 degree at the B-ring. The ligand binding pocket of ERRγ-LBD (1TFC) was too small to bind E2.

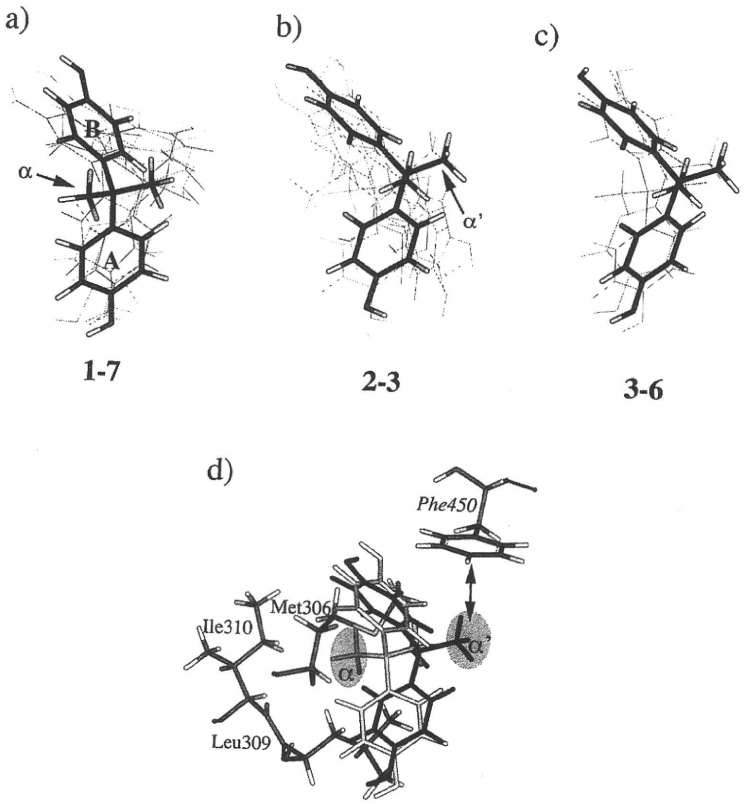

Figure 3. The three-dimensional structures of bisphenol A (BPA) docked in the apo-form ERRγ-LBD. Each structure (a)-(c) was obtained by calculations starting from different dockings with initial placements. The calculations were carried out by the computer program Affinity. **1-7** (a), **2-3** (b), and **3-6** (c) show the structure of BPA (bold sticks) obtained with the best Affinity score (see Table **1**). In each calculation, the other structures of BPA are shown by thin stick lines. A and B are labeled on the two phenol rings of BPA. α: The methyl group on the left side of **1-7**; and α': Another methyl group on the right side of **2-3**. (d) Structural comparison of **1-7** (white molecule) and **2-3** (black molecule). All amino acid residues were from the results of **1-7**, with the only exception being *Phe450* from the results of **2-3**. α and α' are described above.

Table 2. Calculated Binding Energies (ΔE) of the ERRγ Complexes with BPA, 4-OHT, DES and E2 by *ab initio* Calculations

Complex (No.)	ΔE (HF)	ΔE (MP2)
1TFC+BPA (**1-7**)	–7.90	–57.6
1TFC+BPA (**2-3**)	–14.4	–68.8
1TFC+BPA (**3-6**)	–0.14	–48.1
1S9Q (4-OHT)[a,b]	–10.6	–82.4
1S9P (DES)[a]	–4.03	–64.5
1TFC+E2	19.8	–49.3

Energies are in kcal/mol.
[a]Crystal Structure, in which water molecule(s) are ignored. [b]A cholic acid, closely existed with 4-OHT in 1S9Q, regarded as a part of the receptor molecule in the calculations.

The differences between the HF and MP2 calculations may be due to the exclusion and inclusion of the weak hydrogen bonding, especially the π interaction in calculating the BPA-binding to ERRγ-LBD [41]. The MP2 method practically corrects the electron correlation energy, which was ignored by the HF method. It is clear that, among the three different BPA complexes, **2-3** is judged to be the most stable with the smallest energy values. As shown in Fig. **3**, **2-3** and **3-6** are in almost the same binding conformation, although their apparent binding energies calculated are considerably different (Table **2**). This difference in binding energies is probably due to the difference in the energies of intermolecular interactions involving the phenol-hydroxyl groups. The hydroxyl groups in **2-3** and **3-6** direct towards different receptor sites, and as a result **2-3** won the largest energy stabilization.

These results indicate that, when performing the energy calculations, it is important to compute the electron correlation by involving the π interaction. Since even the E2-ERRγ complex afforded a considerably lower energy value (–49.8 kcal/mol) in the MP2 calculation, the usage of both HF and MP2 methods appears necessary for a correct prediction.

Fig. **4** illustrates the ligand binding site of ERRγ complexed with ligands BPA, 4-OHT and DES. When the most stable complex **2-3** was compared with the 4-OHT and DES complexes, one of the two phenol-benzene rings of BPA, namely, the A-ring, was found to be placed at the same position where the phenol-benzene group (A-ring) of 4-OHT and DES are placed (Fig. **4**). However, another phenol-benzene ring of BPA, the B-ring, is not sitting on the position where the second benzene group of 4-OHT or DES is placed. As seen in (Fig. **5a**), the calculated structure of the BPA/ERRγ-LBD complex, namely **2-3**, well defined to discuss the binding manner of BPA with the ligand binding pocket of ERRγ-LBD. It should be noted that the BPA locates in the activation conformation of ERRγ-LBD. Phe450 present on the inside surface of helix 12 is placed to direct towards the ligand-binding pocket, with the result that the helix is held in a position in which the cofactors can bind correctly. Although Greschik *et al.* reported that antagonism induced by DES and 4-OHT is ascribed to the rotation of the side chain of Phe-435 [39], such a rotation of Phe435, defined as antagonist binding, was not observed in this BPA/ERRγ-LBD complex (Fig. **5b**). Consequently, the characteristics of the binding mode of BPA became prominent, since BPA binds to the ERRγ-LBD apo-form without any disruption to its activation conformation.

In a calculated complex **2-3**, three hydrogen-bondings are present between BPA and LBD. One of the phenol-hydroxyl groups (B-ring) of BPA forms a hydrogen-bond with the side-chain amide group of Asn346 (2.0 Å) and also with the side-chain hydroxy group of Tyr326 (2.5 Å). On the other side, another phenol group (A-ring) forms a hydrogen bond with the side-chain carboxyl group of Glu275 (2.9 Å) and with the side-chain guanidino group of Arg316 (3.1 Å) (Fig. **5b**). It is also identified that another hydrogen bond between the hydroxy group of A-ring and α-carbonyl group of Tyr326 exists (3.5 Å). In the 4-OHT or DES/ERRγ-LBD complex, a water molecule is present near the space of Glu275, and this H_2O participates in the hydrogen bonding

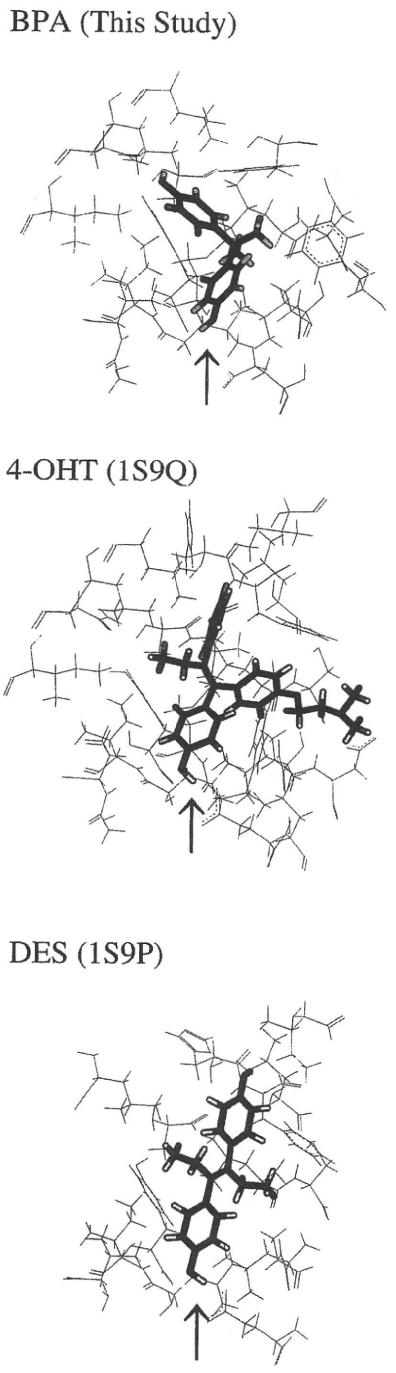

Figure 4. Complex structure of bisphenol A (BPA), 4-hydroxytamoxifen (4-OHT), and diethylstilbestrol (DES) bound to ERRγ-LBD. a) Calculated complex **2-3** in this study, b) 1S9Q, and c) 1S9P. Bold sticks display the ligand molecules BPA, 4-OHT, and DES, respectively. Arrows indicate the position of the phenol-benzene A ring in each ligand.

with the 4-OHT- and DES-phenol-hydroxyl group. In this study, the docking program utilized ignores or excludes the water molecule while docking calculations occur. If we could simulate water molecules in the calculations, it would

be possible to predict the binding energies and structures of complexes more precisely.

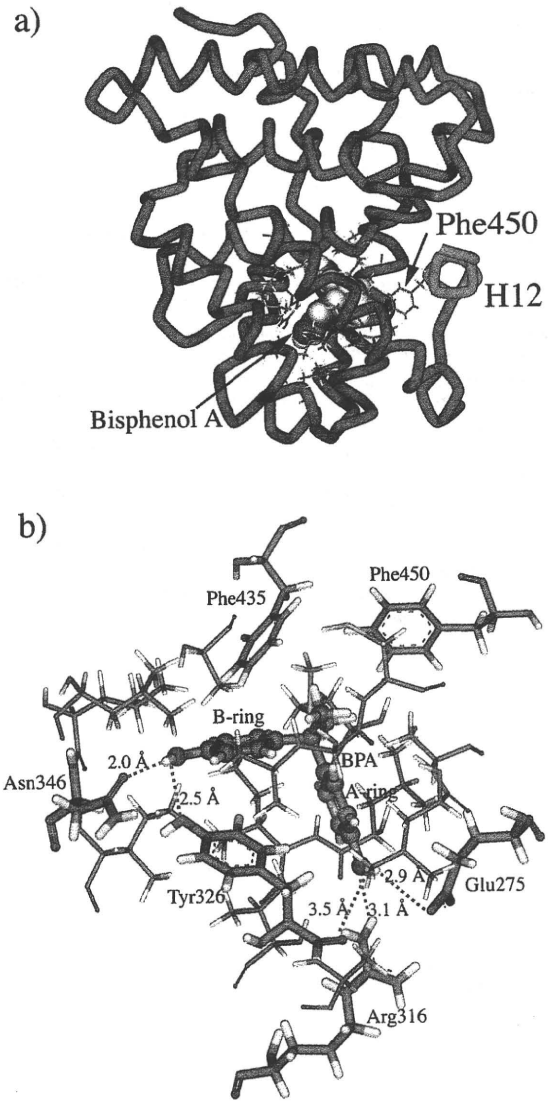

Figure 5. Calculated complex structure of bisphenol A (BPA)/ERRγ-LBD. a) Tube-model of the complex. H12 indicates the number 12 α-helix of ERRγ-LBD. b) The structure of BPA in the ligand-binding pocket. Broken lines (orange) indicate the hydrogen bonds in a reasonable distance. BPA represents a structure **2-3** calculated in Table **1** and Fig. **3**.

In the present study, we described the flexible docking calculation of BPA with ERRγ-LBD, and the results revealed that BPA is a strong binder of ERRγ with high spontaneous constitutive activity. This agrees well with the results reported [6]. Furthermore, we provided evidence that BPA in the complex is indeed a space-filler of ERRγ-LBD. This conformation is characterized by one of the BPA-phenol-benzene rings, which is placed at the vacant space, but not by the placement of the benzene rings of 4-OHT and DES. Consequently, BPA has a unique binding site in ERRγ-LBD. In the future, in order to better understand ERRγ it will be necessary to clarify the roles of BPA binding or of the BPA/ERRγ complex, which sustains a high constitutive activity.

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ABBREVIATIONS

BPA = Bisphenol A
DES = Diethylstilbestrol
EDC = Endocrine disrupting chemical
E2 = 17β-estradiol
ER = Estrogen receptor
ERR = Estrogen-related receptor
HF = Hartree-Fock
LBD = Ligand binding domain
MP2 = Second order Møller-Plesset perturbation theory
4-OHT = 4-Hydroxytamoxifen
QSAR = Quantitative structure-activity relationship
SBDD = Structure-based drug design studies

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ERRc tethers strongly bisphenol A and 4-a-cumylphenol in an induced-fit manner

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abstract

A receptor-binding assay and X-ray crystal structure analysis demonstrated that the endocrine disruptor bisphenol A (BPA) strongly binds to human estrogen-related receptor c (ERRc). BPA is well anchored to the ligand-binding pocket, forming hydrogen bonds with its two phenol-hydroxyl groups. In this study, we found that 4-a-cumylphenol lacking one of its phenol-hydroxyl groups also binds to ERRc very strongly. The 2.0 Å crystal structure of the 4-a-cumylphenol/ERRc complex clearly revealed that ERRc's Leu345-b-isopropyl plays a role in the tight binding of 4-a-cumylphenol and BPA, rotating in a back-and-forth induced-fit manner.

Bisphenol A (BPA) has been recognized as one of the most potent endocrine disruptors, functioning even at very low doses. Although it is anticipated that estrogen receptor (ER) would mediate these effects of low-dose BPA, studies revealed that BPA bound to ER very weakly [1–5]. On the other hand, we have recently discovered that a human nuclear receptor, estrogen-related receptor c (ERRc), acts as a specific receptor for BPA (K_D = ca. 5.5 nM) [6,7]. ERRc elicits a high basal constitutive activity with no ligand, and BPA was found to fully retain this activity. These findings necessitate that we reevaluate the low-dose effects of BPA in relation to the high-binding ability of BPA to ERRc.

Recently, we successfully performed an X-ray analysis of the crystal structure of the complex formed between BPA and the ligand-binding domain (LBD) of ERRc [8]. BPA, 2,2-bis(4-hydroxyphenyl)propane, was found in the binding pocket of ERRc-LBD just like a tightly bound natural ligand. BPA-bound ERRc maintains the activation conformation of authentic ERRc, which helps to explain why the BPA-ERRc complex retains a high basal constitutive activity.

In this study, we preliminarily examined the binding ability of 4-a-cumylphenol, which lacks one of the two phenol-hydroxyl groups of BPA (Fig. 1). Surprisingly, 4-a-cumylphenol was found to bind to ERRc as potently as BPA. Since diphenylpropane with no phenol-hydroxyl group was completely inactive, the phenol-hydroxyl group of 4-a-cumylphenol is essential for the binding to ERRc. This means that any compounds having the phenol group are potential candidates for strong binders of ERRc.

One of the two phenol-hydroxyl groups of BPA simultaneously forms hydrogen bonds with Glu275 and Arg316, while the other makes a hydrogen bond with Asn346. The question of which hydrogen bond holds 4-a-cumylphenol in the ligand-binding pocket of ERRc is crucial for predicting the binding potential of the phenol compounds. In addition, answering this question is crucial for elucidating the structural reason why 4-a-cumylphenol is as potent as BPA. In the present study, to answer to these questions, we analyzed the X-ray crystal structure of the complex of 4-a-cumylphenol/ERRc. We succeeded in crystallizing the complex, and the X-ray analysis revealed a strong hydrogen bonding of the phenol-hydroxyl group with Glu275/Arg316 of ERRc and a strong hydrophobic interaction between the phenyl group and the isopropyl group of Leu345.

Abbreviations: BPA, bisphenol A; CBB, Coomassie Brilliant Blue; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DES, diethylstilbestrol; ER, estrogen receptor; ERR, estrogen-related receptor; LBD, ligand-binding domain; 4-OHT, 4-hydroxytamoxifen; PCR, polymerase chain reaction.

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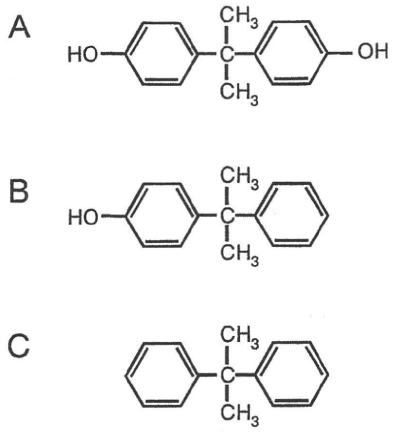

Fig. 1. Chemical structure of bisphenol A (BPA) and its derivatives. (A) BPA. (B) 4-a-cumylphenol, and (C) 2,2-diphenylpropane.

Materials and methods

Receptor protein expression and purification. Preparation of the receptor protein was carried out essentially as reported previously [8]. The cDNA fragment encoding human ERRc-LBD (corresponding to amino acid residues 222–458) was generated by PCR, and the amplified product was cloned into the expression vector pGEX 6P-1 (Amersham Biosciences, Piscataway, NJ, USA) to express the product as a glutathione-S-transferase (GST) fusion protein by using Escherichia coli BL21 [6]. GST was cleaved by PreScission Protease (Amersham Biosciences), and the protein concentration was determined by the Bradford method [9].

Radio-ligand-binding assays. The receptor-binding assay was conducted essentially as reported previously [10] using [^{3}H]4-hydroxytamoxifen (4-OHT) (80 Ci/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). To estimate the binding affinity, the IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves by using the nonlinear analysis program ALLFIT [11].

Luciferase reporter gene assay. A luciferase reporter gene assay using HeLa cells was carried out by the method of transient transfection as reported previously [7]. Luciferase activity was measured by using luciferase assay reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Crystallization of protein complex followed by X-ray data collection and processing. Purified ERRc-LBD was concentrated by ultrafiltration. Co-crystallization with a threefold molar excess of 4-a-cumylphenol was carried out with the hanging drop vapor diffusion method as described [8]. X-ray diffraction data were collected at SPring-8 (Hyogo, Japan). The data were integrated and scaled using the HKL2000 package [12]. ERRc-LBD with no ligand (apo-ERRc-LBD) was obtained using essentially the same method as for the 4-a-cumylphenol/ERRc-LBD complex. For data collection, crystals were transferred into a cryoprotectant solution containing 30% sucrose.

Structure determination and refinement. A monomer model of BPA/ERRc-LBD (2E2R) was used as a search molecule for molecular replacement using MOLREP [13] in CCP4 [14]. The position of the 4-a-cumylphenol/ERRc-LBD complex and apo-ERRc-LBD in each asymmetric unit was located and the structure was refined at 2.0 and 1.8 Å, respectively, using REFMAC5 [15] in CCP4. Manual adjustment and rebuilding of the model including 4-a-cumylphenol and water molecules were performed using the program Coot [16].

The final 4-a-cumylphenol/ERRc-LBD complex model contained residues 232-458 of ERRc, one 4-a-cumylphenol, one glycerol, and 149 water molecules. The final apo-ERRc-LBD model also contained residues 232–458 of ERRc and 219 water molecules. The final models were validated with PROCHECK [17]. Atomic coordinates for the structure has been deposited in the Protein Data Bank with accession code 2ZAS for the 4-a-cumylphenol/ERRc-LBD complex and 2ZBS for the apo form of ERRc-LBD.

Results and discussion

The binding site of phenol-hydroxyl group of 4-a-cumylphenol in ERRc

The competitive receptor-binding assay was performed using [^{3}H]4-OHT for GST-ERRc-LBD. As shown in Table 1, it was found that BPA, 4-a-cumylphenol, and 4-OHT are almost equally potent, having similar IC_{50} values of approximately 10 nM. In contrast, 2,2-diphenylpropane was extremely weak (IC_{50} > 10,000 nM). Since 2,2-diphenylpropane has no phenol group (Fig. 1), it is clear that one of the phenol-hydroxyl groups of BPA is indispensable for the interaction with a binding pocket of ERRc. These results were confirmed in a separate assay using [^{3}H]BPA [18]. It should be noted that 4-a-cumylphenol is as potent as BPA, although 4-a-cumylphenol lacks one of the two phenol-hydroxyl groups of BPA.

A major goal of the present study was to elucidate the phenol-hydroxyl groups shared by both BPA and 4-a-cumylphenol. In the case of BPA, we designated the phenol group bridged by hydrogen bonds to Glu275/Arg316 as the A ring, and the phenol group hydrogen-bonded to Asn346 as the B ring. Thus, one of the purposes of the present study was to determine whether 4-a-cumylphenol possesses the A ring or the B ring. We solved the crystal structure of ERRc-LBD in complex with 4-a-cumylphenol at a resolution of 2.0 Å (space group $P4_12_12$) (Supplementary Table). The complex formed was crystallized in a homodimeric form using crystallographic 2-fold symmetry (Fig. 2A). A 4-a-cumylphenol molecule was defined very well in the complex (Fig. 2B).

For 4-a-cumylphenol, we simply found the A ring in the 4-a-cumylphenol/ERRc- LBD complex. The solo phenol-hydroxyl group of 4-a-cumylphenol was involved in the hydrogen bonding with both Glu275 and Arg316. Superimposition of 4-a-cumylphenol and BPA (2E2R) [8] in the ERRc-LBD complexes showed a conformational identity that could readily account for the similarity in binding modes to the binding pocket. The phenol A ring of BPA superimposed almost completely with the corresponding A ring of 4-a-cumylphenol.

We have recently reported the binding potentials between the phenol-hydroxyl group of BPA and ERRc receptor residues Glu275 and Arg316 in the LBD [7]. Wild-type ERRc-LBD showed a strong-binding ability (K_D = ca. 5.5 nM) for [^{3}H]BPA, but the simultaneous mutation to Ala at positions 275 and 316 resulted in an absolute inability to capture BPA. The ERRc receptor appears to form an appropriate structure with the Glu275 and Arg316 residues, presumably to arrest the phenolic compound as an endogenous ligand.

Table 1
The receptor-binding affinity of chemicals in the assay using [^{3}H]4-OHT as a tracer for the human estrogen-related receptor c (ERRc)

Chemicals	Binding affinity (IC_{50}, nM)
Bisphenol A (BPA)	13.1 ± 2.34
4-Hydroxytamoxifen (4-OHT)	10.3 ± 0.80
4-a-Cumylphenol	13.9 ± 1.98
2,2-Diphenylpropane	Inactive

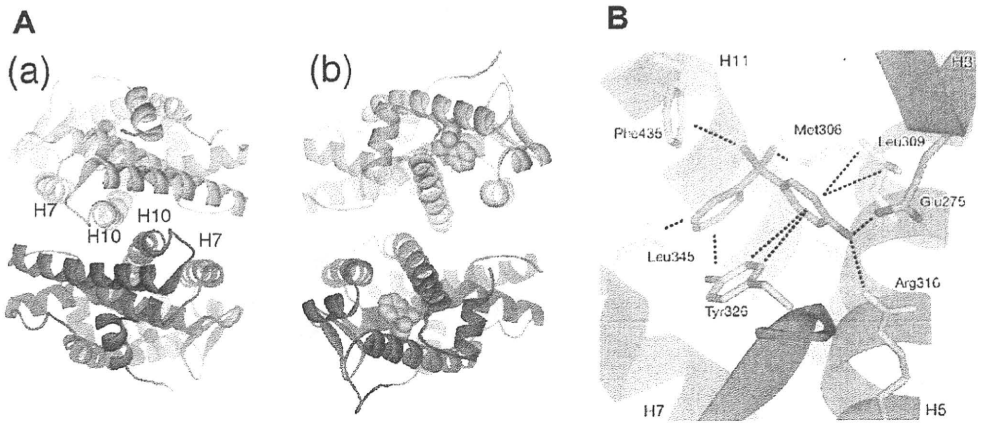

Fig. 2. Overall structure of the dimer of the 4-a-cumylphenol/ERRc-LBD complex (A) and the complex structure between 4-a-cumylphenol and ERRc-LBD (B). (A) The panoramic view of the whole-sphere 4-a-cumylphenol/ERRc-LBD complex homodimer. Each panel shows the 3D-structure pictured from the top and the bottom with 180 rotation. One molecule of 4-a-cumylphenol (space-filling) is in a ERRc-LBD molecule (ribbon). (B) The complex structure in which 4-a-cumylphenol is placed in the ligand-binding pocket of the ERRc-LBD. The dotted lines show the hydrogen and the hydrophobic bonds.

Structural elements holding 4-a-cumylphenol in the ERRc-LBD

4-a-Cumylphenol was found to be in a very prominent-binding site constructed of a series of amino acid residues in the ERRc-LBD. The receptor residues within a range of 5 Å include Leu268, Cys269, Leu271, Ala272, and Glu275 from H3; Trp305, Met306, Leu309, Ile310, Val313, and Arg316 from H5; Tyr326 from b-strand 1 (S1); Leu342, Leu345, Asn346, and Ile349 from H7; Ala431 from H10; Phe435 from H11; and Phe450 from H12.

Tyr326 in S1 has been reported to interact with Asn346 (H7) through a hydrogen bond [19,20]. This hydrogen bond is also maintained in the 4-a-cumylphenol/ERRc-LBD complex. It should be noted that Tyr326 is the major amino acid residue necessary for the placement of 4-a-cumylphenol in the ERRc-LBD complex. As seen for BPA [8], the A ring of 4-a-cumylphenol is sandwiched by hydrophobic interactions between Tyr326 and Leu309 (Fig. 2B).

Tyr326 also keeps the cumyl-benzene ring of 4-a-cumylphenol by the OH/p interaction [21,22] (Fig. 2B). This cumyl-benzene ring also interacts with the isobutyl group of Leu345. Thus, the cumyl-benzene ring is in a binding site constructed by Tyr326 and Leu345. As to the two methyl groups on the sp^3-C atom, the one faces Phe435 (H11), and the other faces the Met306 sulfur atom with a non-covalent electron pair.

Conformation changes of ERRc-Leu345-b-isobutyl by the back-and-forth rotation to receive either phenol or phenyl

Another purpose of this study was to determine why 4-a-cumylphenol exhibits very strong-binding activity in the manner of BPA. The reason was found be the back-and-forth rotation of the Leu345 residue (Fig. 3A), which causes the residue either to interact with the phenyl group of 4-a-cumylphenol or to avoid the phenol group (B ring) of BPA.

The phenol B ring of BPA is directed towards H7 to capture Asn346 by its hydrogen bond. Also, the benzene ring of the cumyl group ($-C(CH_3)_2-C_6H_5$), namely, the phenyl group of 4-a-cumylphenol, is directed towards Asn346. However, this benzene ring does not have a hydroxyl group, and thus there is no hydrogen bond between the benzene ring and Asn346. Usually, the lack of such a hydrogen bond greatly reduces the ability of the compound to bind to a receptor molecule, thereby weakening the receptor-binding affinity. However, 4-a-cumylphenol consistently exhibited the same strong binding to ERRc. It was thus assumed that there must be a certain structural element of the ERRc-LBD-binding pocket that stabilizes the binding of the cumyl-benzene ring. When we superimposed the 4-a-cumylphenol/ERRc-LBD complex with the BPA/ERRc-LBD complex, we found a very clear conformation difference of the isobutyl group of Leu345, as shown in Fig. 3A.

This Leu345-isobutyl group ($-_bCH_2-_cCH(_dCH_3)_2$) was found to face the benzene ring of 4-a-cumylphenol in the 4-a-cumylphenol/ERRc-LBD complex (Fig. 3A and B(a)). In this conformation, the distance between the carbon atom of either $_cCH$ or $(_dCH_3)_2$ and the para-carbon atom of the benzene ring is approximately 3.7 Å, and thus $_cCH$ and $(_dCH_3)_2$ undergo a so-called CH/p-type hydrophobic interaction with the benzene p-electrons. This interaction stabilizes the receptor binding of the cumyl-benzene ring.

In the BPA/ERRc-LBD complex, however, the Leu345-isobutyl group was found to turn its back against the B ring of BPA (Fig. 3A and B(b)). The isobutyl group, $-_bCH_2-_cCH(_dCH_3)_2$, attaches to the a carbon ($_aC$) of Leu345, and this group can rotate freely around the $_aC-_bC$ bond. It is clear that the isopropyl group rotates about 180 between these two complexes (Fig. 3A). In the BPA/ERRc-LBD complex, Leu345–$_bCH_2-_cCH(_dCH_3)_2$ faces its two methyl $(_dCH_3)_2$ groups ('back'-face) toward the phenol B ring of BPA. This creates an appropriate space for the phenol-hydroxyl group (Fig. 3B(b)), giving the hydroxyl group a chance to form a hydrogen bond with the adjacent Asn346 amide group, $-CH_2-CONH_2$.

The finding that Leu345–$_cCH(_dCH_3)_2$ directly or indirectly contributes to the stable binding of 4-a-cumylphenol and BPA is of great interest. ERRc-LBD appears to make the most of the small space by the back-and-forth rotations of the isobutyl group of Leu345, in order to place the phenol group (BPA) and the benzene ring (4-a-cumylphenol). Given that such a back-and-forth rotation was not feasible, BPA could not be held in this pocket. As shown in Fig. 3B(b^0), when the Leu345-isobutyl group keeps its conformation, as in the 4-a-cumylphenol/ERRc-LBD complex, the phenol B ring of BPA comes into complete collision with Leu345–$_cCH(_dCH_3)_2$. Also, in the event that the Leu345-isobutyl group maintains its conformation, as in the case of the BPA/ERRc-LBD complex, there is too large a space for the benzene ring of 4-a-cumylphenol and Leu345–$_cCH(_dCH_3)_2$ to interact with each other, as shown in Fig. 3B(a').

Induced-fit binding of BPA and 4-a-cumylphenol to ERRc

We also succeeded in analyzing the crystal structure of the apo form of ERRc-LBD (PDB id code 2ZBS) as reported by Greschik et al. (1TFC and 1KV6) [19,20], and also by Wang et al. (2GP7) [23]. When the superimposition between ERRc-LBD from the 4-a-cumylphenol/ERRc-LBD complex and apo-ERRc-LBD was

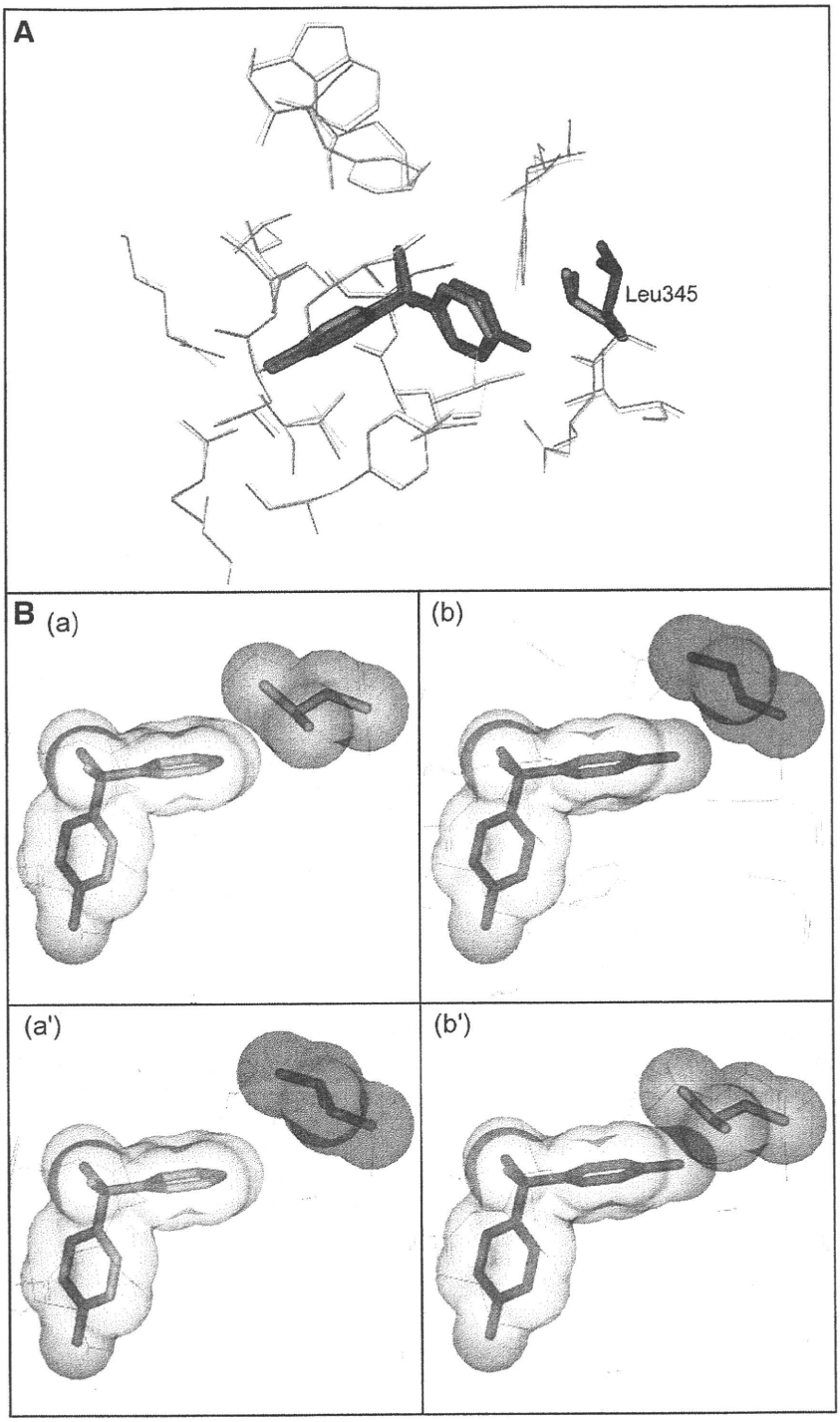

Fig. 3. Superimposition between the 4-a-cumylphenol/ERRc-LBD and the BPA/ERRc-LBD complexes. (A) The 4-a-cumylphenol/ERRc-LBD complex (blue) (2ZAS) is superimposed with the BPA/ERRc-LBD (green) (2ZBS). The amino acid residues shown are in close proximity to ligands within 5 Å. The isopropyl group ($-CH(CH_3)_2$) of Leu345 is in the shape of the letter Y. The back-face of Leu345-isopropyl of the 4-a-cumylphenol/ERRc-LBD complex (blue) is directed towards the phenyl group of 4-a-cumylphenol (blue), whereas the forth-face of the BPA/ERRc-LBD complex (green) is directed towards the phenyl group of BPA (green). (B) The back-and-forth rotation of Leu345 ensures the appropriate binding pocket for 4-a-cumylphenol (a and a^0) and BPA (b and b^0). The surfaces based on the van der Waals radius of ligands and Leu345 are shown in each panel. (a) 4-a-Cumylphenol (blue) and Leu345 in the 4-a-cumylphenol/ERRc-LBD complex (blue) maintain an appropriate space at the nearest C-C distance of approximately 3.7 Å. (a^0) The virtual complex between 4-a-cumylphenol (blue) and Leu345 in BPA/ERRc-LBD (green) shows too large a space for mutual interaction. (b) BPA (green) and Leu345 in the BPA/ERRc-LBD complex (green) maintain an appropriate distance of about 4.0 Å. (b^0) In the virtual complex between BPA (green) and Leu345 in 4-a-cumylphenol/ERRc-LBD (blue), Leu345 comes into collision with the phenol-hydroxyl group of BPA.

carefully checked, the Leu345-isobutyl group was found to be in almost the same position (Fig. 4A). This implies that ERRc originally keeps the Leu345 residue in the complex just as in apo-ERRc-LBD, and thus 4-a-cumylphenol binds to a natural ERRc. Turning to the binding of BPA to ERRc, it is evident that Leu345 does make a rotation of about 180 to adopt the phenol group of BPA. If this rotation were not feasible, BPA should not have bound to ERRc.

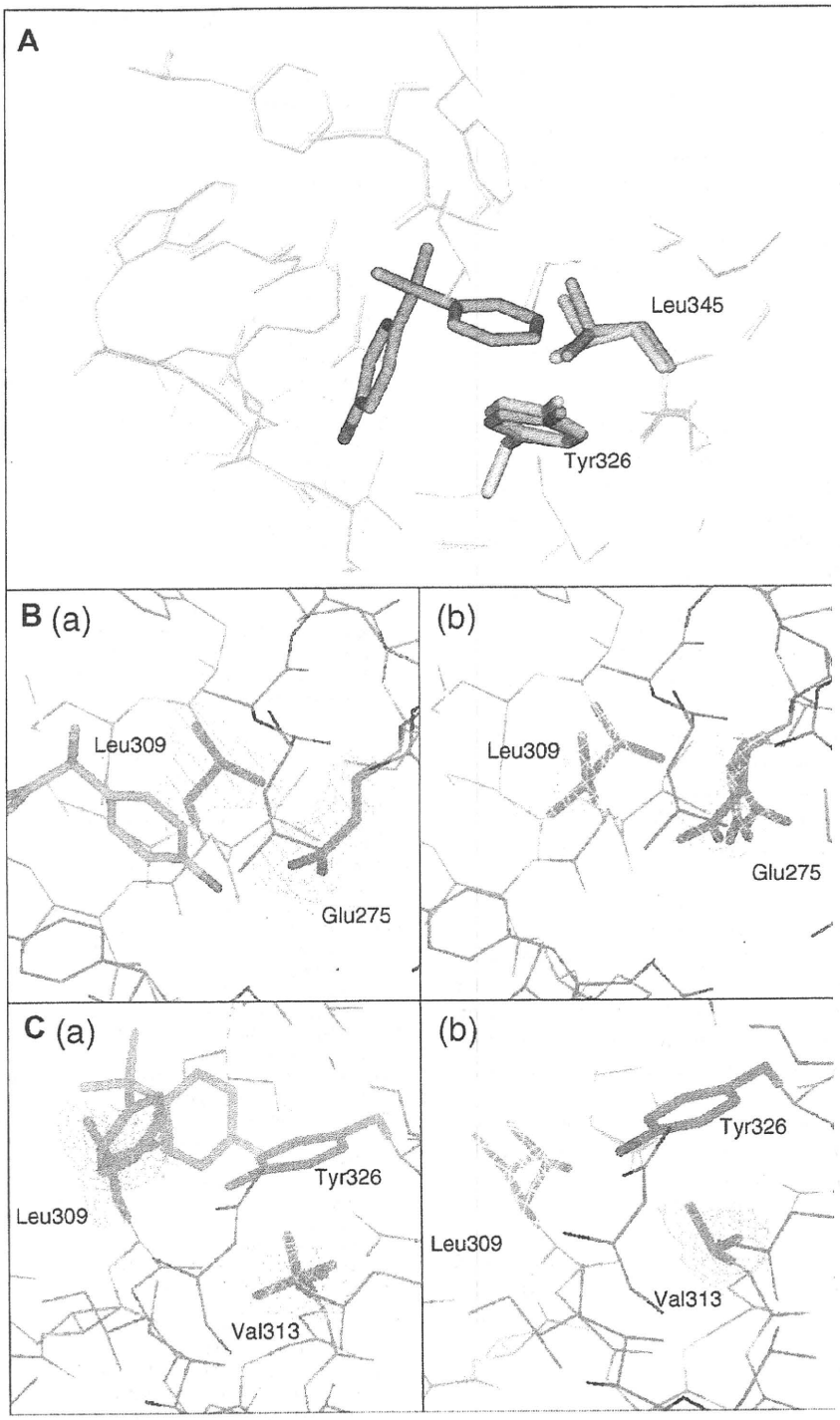

Fig. 4. Induced-fit binding of 4-a-cumylphenol to the ERRc-LBD apo form. Superimposition of the 4-a-cumylphenol/ERRc-LBD complex and apo-ERRc-LBD. (A) The residues shown are in close proximity (within 5 Å) to 4-a-cumylphenol. The Leu345-isobutyl group and Tyr326-para-hydroxyl benzyl group are highlighted. (B) (a) Induced repositioning of Glu275 and Leu309 by the binding of 4-a-cumylphenol and (b) their conformationally released forms in the apo form. (C. a) In conjunction with the binding of 4-a-cumylphenol. Val313 is conformationally in the two different conformations, one of which is seen in the apo form (C. b). In the apo form, the Val313-isopropyl group is in the tight interaction with the Tyr326-phenol phenyl group. However, in the complex structure, the Tyr326-phenol ring tilts at the angle of about 15 degree (see Fig. 4A), reorienting Val313-isopropyl in the second position as seen in (C. a). The residues netted in blue are defined in a certain conformation, while those in magenta are in the mobile positions undefined or in a multiple conformations.

The side chain of Glu275 is not well defined in apo-ERRc-LBD owing to its high mobility (Fig. 4B(b)). However, in the 4-a-cumylphenol complex, it reorients towards the hydroxyl group of the phenol A ring and is tightly positioned by the hydrogen bonding (Fig. 4B(a)). Similarly, the isobutyl side chain of Leu309 is first in the highly mobile position undefined in apo-ERRc-LBD (Fig. 4B(b) and C(b)), while it reorients towards the phenol A benzene ring of 4-a-cumylphenol (Fig. 4B(a) and C(a)). By contrast, the side chain of Val313 became defined in the two different conformations in the 4-a-cumylphenol complexes (Fig. 4C(a)). This reorientation of Val313-isopropyl in the two different positions appears to be due to the tilt of Tyr326-phenol ring at the angle of about 15 de-

gree. This small tilt was induced by the binding of Tyr326-phenol ring to the phenol A ring of 4-a-cumylphenol (Fig. 4A). The Val313-isopropyl group is in the tight interaction with the Tyr326-phenol phenyl group in apo-ERRc-LBD (Fig. 4C(b)).

All these results imply that the binding of chemicals, particularly BPA to ERRc-LBD, is just an induced-fit-type binding at the Glu275, Leu309, Tyr326, and Leu345 residues. Due to these conformation changes, BPA and 4-a-cumylphenol are able to bind to ERRc-LBD in a space-filling manner. Such tolerable flexibility of the ligand-binding pocket must be to adopt an authentic ligand probably with a BPA-like structure.

4-a-Cumylphenol as a phenol ligand of ERRc in an activation conformation

Both H12 in the BPA/ERRc-LBD complex and H12 in apo-ERRc-LBD are in the transcriptionally active conformation [8]. H12 is placed rigidly on the LBD body, where the coactivator binds. ERRc per se elicits a very high basal activity in the luciferase reporter gene assay, and BPA sustains this high spontaneous constitutive activity of ERRc [6]. These findings were reproduced for the 4-a-cumylphenol/ERRc-LBD complex, H12 in the complex being in the activation conformation. 4-a-Cumylphenol has also been shown to retain ERRc's high spontaneous constitutive activity [18].

4-Hydroxytamoxifen (4-OHT) deactivates the ERRc receptor, dissociating the H12 region from the LBD body [19]. It should be noted that BPA and 4-a-cumylphenol reverse this deactivation activity of 4-OHT, displacing 4-OHT to reposition the H12 from the transcriptionally inactive conformation to the active conformation. The compounds that deactivate the receptor are termed 'inverse agonists,' whereas those that inhibit such inverse agonists are to be defined as 'inverse antagonists.'

In the present study, we found that ERRc-LBD adopts both BPA and 4-a-cumylphenol, but BPA requires the back-and-forth rotation of the Leu345-isobutyl group. Thus, we should define 4-a-cumylphenol as a genuine space-filler of ERRc. This implies that the phenol compounds can become a potent ligand of ERRc. In fact, it was found that a number of phenols bind to ERRc very strongly [18]. These results suggest that ERRc may have a phenol-containing endogenous ligand.

It is now important to examine whether or not the phenol compounds including 4-a-cumylphenol cause low-dose effects similar to those reported for BPA. At the same time, it is necessary to clarify what the physiological roles of ERRc are, and to examine the extent of, and direction in which, BPA and phenols may influence these. This is particularly important because ERRc is expressed very strongly in the mammalian fetal brain and also in the placenta, at sites that could have important outcomes for newborns.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.050.

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Direct Measure of Fluorescence Intensity for Efficient Receptor-binding Assay: Conjugates of Ethinylcarboxyestradiol and 5(and 6)-Carboxyfluorescein via α,ω-Diaminoalkanes as a Tracer for Estrogen Receptor

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Steroidal nuclear receptors (NRs) have been acknowledged as a target binding protein of so-called endocrine disruptors. It is therefore necessary to develop an efficient assay system for screening these endocrine-disrupting chemicals. We here describe the first exemplification of a direct measure of fluorescence intensity for a binding assay of NRs. We designed and synthesized a series of conjugates of 17α-ethinylcarboxyestradiol with carboxyfluorescein, both carboxyl groups of which were cross-linked with α,ω-diaminoalkanes. The resulting fluorescein-linked estradiol derivatives E2(*n*)cF (*n* = 2, 4, 6, 8, 10 and 12) were evaluated for their fluorescence and receptor-binding characteristics. E2(4)cF and E2(8)cF exhibited the sufficient binding affinity to the recombinant estrogen receptor (ER) in the radiolabel binding assay using [^{3}H]17β-estradiol, and showed excellent fluorescent characteristics in the fluorescence measurements with and without ER. They exhibited sufficiently large specific binding characteristics with adequate K_d- and B_{max}-values. When these fluorescent ligands were used as a tracer for the binding assay against the ER, assay data of various compounds were shown to be compatible with those obtained from the ordinary binding assay using [^{3}H]17β-estradiol. The present study clearly shows that measurement of fluorescence intensity, instead of fluorescence polarization, affords an adequate receptor-binding assay system.

Key words: endocrine disruptors, estrogen receptor, fluorescence intensity, fluorescent tracer, receptor-binding assay.

Abbreviations: Cbz, carbobenzoxy; DMF, *N,N*-dimethylformamide; DMSO, *N,N*-dimethyl sulfoxide; E2(n)cF, the conjugates between 17α-ethinylcarboxyestradiol (E2) and caryboxyfluorescein (cF) via α,ω-diaminoalkanes -NH-$(CH_2)_n$-NH-; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ER, estrogen receptor; ERR, estrogen-related receptor; ESI, electrospray ionization; EtOAc, ethyl acetate; FAB, fast atom bombardment; GST, glutathione-*S*-transferase; HOBt, 1-hydroxybenzotriazole; HP-TLC, high-performance thin-layer chromatography; LBD, ligand binding domain; MS, mass spectrometry; PBS, phosphate buffer saline; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid and THF, tetrahydrofuran.

INTRODUCTION

With the accomplishment of the human genome project in 2001, it became evident that the nuclear receptors (NRs) form a superfamily of proteins that includes 48 different receptor proteins (*1*, *2*). The NRs were first discovered as a binding protein for steroids, thyroid hormones and retinoic acids. The NR elicits a transcriptional activity that is modulated by binding of the agonist or antagonist ligand. This activity affects cell growth and cell differentiation. The estrogen receptors (ERα and ERβ) are a member of the steroid hormone receptor protein family (*3–6*), which includes such receptors as estrogen-related receptors (ERRα, ERRβ and ERRγ), glucocorticoid receptor, mineral corticoid receptor, progesterone receptor and androgen receptor.

ER has been acknowledged as a target binding protein of a number of environmental chemicals called endocrine disruptors. Endocrine disruptors are suspected to cause interference or disorder in the endocrine system, producing undesirable effects on the reproductive system related to fetal development in animals and humans (*7*, *8*). These chemicals have such a damaging influence

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upon not only ERs, but also other NRs. Most recently, we have reported that bisphenol A, one of the endocrine disruptor candidates, binds strongly to ERRγ (*9–11*). This finding was confirmed only after many difficulties in establishing a specific receptor-binding assay. It is imperative that an efficient binding assay system be developed to screen for these endocrine disruptors.

Assays using the high specificity of NRs make it possible to quantify many chemicals present in only minute traces in environmental substances or complex biological materials. Efforts have continued toward increasing the sensitivity, specificity and convenience of such assays. The methods depend upon labelling of the ligand being quantified. The general types of label that afford the requisite sensitivity include primarily radiolabels and fluorescence labels. The readout of an assay with radiolabels then finally depends upon a determination of the amount of label present, usually ^{3}H or ^{125}I, by counting the radioactivity. This line of radiolabel receptor-binding assays for ER has been extensively developed during the last 15 years (*12–15*).

In the assays with fluorescence labels, determination of the amount can be given by the fluorescence polarization. The method for measuring fluorescence polarization has been applied to a binding assay for ER (*16–18*). However, this method often faces central problems. For example, the measurement should be dictated by special instrumentation, in which any fluorometer must be equipped with polarization capability to determine binding by steady-state fluorescence polarization measurements. This instrumentation is usually quite expensive, and parts replacement to improve sensitivity or to optimize the emission signals relative to background is often necessary.

Fluorescence polarization is determined principally based on fluorescence intensities polarized either parallel or perpendicular to the direction on the electric vector of the exciting wave (*16*, *17*). It should be noted that the results of an assay with fluorescence labels are to be obtained also by measurement of fluorescence intensity, instead of fluorescence polarization (*19*). To date, no systemic and complete investigations have been carried out to establish the receptor-binding assay based on fluorescence intensity *per se*. This is presumably due to the lack of proper fluorescence labels that can afford sufficient specific binding.

Fluorescent probes or tracers should retain high-receptor specificity in addition to essential fluorescent characteristics. Two different types of fluorescent estrogens have been reported: one is a group of estrogen derivatives in which the fluorophore is connected chemically to the estrogen (*20–27*). However, most of these ligands generally show low-specific binding affinity for the receptor, but high non-specific binding. A group of inherent fluorescent ligands possesses fluorochrome built within the structure of the ligand (*28–34*). This type of ligand usually suffers from suboptimal fluorescence or binding characteristics, and the molecular design is extremely difficult. In the present study, we attempted to identify the best fluorescence label from very common fluorescent estrogens.

Recent X-ray structural analyses have revealed the important structural essentials for the interaction between estrogen ligand (17β-estradiol) and receptor (*35*, *36*). For the design of fluorescence labels, all these structural requirements are to be satisfactorily retained, and we therefore selected 17β-estradiol (E2) as a pharmacophore. Choosing carboxyfluorescein (cF) as a fluorophore, we decided to prepare the E2-cF conjugates by cross-linking with a series of α,ω-diaminoalkanes with varying methylene chain lengths (Fig. 1). We here describe the synthesis and characterization of these fluorescence ligands and their usage in a binding assay for the ER.

MATERIALS AND METHODS

Materials—17α-Ethinylestradiol and *m*-cresol were purchased from Wako (Osaka) and methyllithium in diethyl ether was from Kanto Chemicals (Tokyo). The 5(and 6)-Carboxyfluorescein *N*-succinimidyl ester was obtained from Molecular Probes (Leiden, The Netherlands) and thioanisole and α,ω-diaminoalkanes ($n=2$, 4, 6, 8, 10 and 12) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Carbobenzoxy chloride (Cbz-Cl) and *N,N*-dimethyl sulfoxide (DMSO) were purchased also from Tokyo Kasei. All other chemicals of the best grade available were obtained from several different sources. Tetrahydrofuran (THF) was purified by distillation from sodium benzophenone, and *N,N*-dimethylformamide (DMF) was of the grade suitable for peptide synthesis (Kanto Chemicals).

Recombinant Human Estrogen Receptor α Ligand-binding Domain—The recombinant human estrogen receptor α ligand-binding domain (ERα-LBD) was expressed as a glutathione-*S*-transferase (GST)-fused protein. The LBD was ligated into a prokaryotic expression vector pGEX-4T1 (Amersham Pharmacia Biotech, Amersham, Bucks, UK) at the *Bam*HI and *Not*I sites. *Escherichia coli* BL21α transformed with the expression plasmid was cultured in 1 l of L-broth containing 0.16 mg/ml of ampicillin, and protein expression was induced by isopropyl 1-thio-β-D-galactoside. The cells were harvested by centrifugation (3,000*g*, 10 min, 4°C) and resuspended in 4 ml of 50 mM Tris–HCl (pH 8.0) containing 50 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol. After sonication and centrifugation (17,800*g*, 30 min, 4 C), a soluble fraction was loaded to the affinity column of Glutathione-Sepharose 4B (Amersham Pharmacia Biotech). After incubation for 60 min at 4°C, the column was washed three times with phosphate buffered saline containing 0.2% (v/v) Triton X-100 (PBST), and the fusion protein was eluted with PBST containing 20 mM reduced glutathione.

Chemical Synthesis and Characterization—Methods—High-performance thin-layer chromatography (HP-TLC) was carried out on silica gel 60 (Merck, Frankfurt, Germany) with the following solvent systems (v/v): R_f; $CHCl_3$-MeOH-AcOH (50:10:2). For structural verification, ^{1}H-NMR spectra, ESI mass spectrometry (MS) (Micro Mass Quatro-2 spectrometer) and/or FAB MS spectra (JEOL SX/SX 102A tandem mass spectrometer) were measured.

Figure 1 shows the synthetic scheme of fluorescein-labelled estrogens. The synthesis includes four different reaction steps, as follows: i.e. step 1, the carboxylation of 17α-ethinylestradiol by CO_2 under the catalytic MeLi; step 2, the coupling of 17α-ethinylcarboxyestradiol with *N*-Cbz-α,ω-diaminoalkanes; step 3, deprotection of