$G_{\alpha o}$-fused ORL1 mutant receptors in this study. We here describe that TM6 indeed possesses the amino acid residues important for receptor activation.

Results and Discussion

In the present study, we combined the two structural strategies important for the structure-function studies of GPCR. The one is Ala-scanning, a screening method that makes it possible to clarify the amino acid residues crucial for receptor activation. The other is $G_{\alpha o}$-fusion that affords a GPCR assay system to measure the receptor activation very efficiently. With these two strategies, we attempted to clarify the receptor activation mechanism of ORL1 receptor, especially focusing on the TM6 domain. The site-directed mutagenesis to achieve the Ala-substitution was carried out for all the 30 amino acid residues in TM6 (Fig. 1).

The mutant receptors are hereafter referred to as AxB, where A designates the original amino acid residue at position x of ORL1 and B denotes the amino acid to replace. Each mutant construct was prepared by two-step PCR method, and transiently expressed on the COS-7 cells. Firstly, we quantified the expression levels of receptors by measuring the saturation binding of [^{3}H]nociceptin. The Scatchard analysis was carried out for all the mutant receptors to estimate both the dissociation constant (K_d) and the receptor protein density (B_{max}). The latter shows the amount of receptors that were expressed soundly on the COS-7 cell membranes. As shown in Fig. 2, the P275A mutant receptor exhibited no detectable binding of [^{3}H]nociceptin. Although other mutant receptors showed the specific binding, the expression level calculated as B_{max} was found to be much lower than that of the wild-type ORL1. In particular, those of C272A and L281A were drastically decreased (Fig. 2). The reduction in expression level suggested that the mutated amino acid residue plays an important role in forming a structure proper to the receptor activation. Those

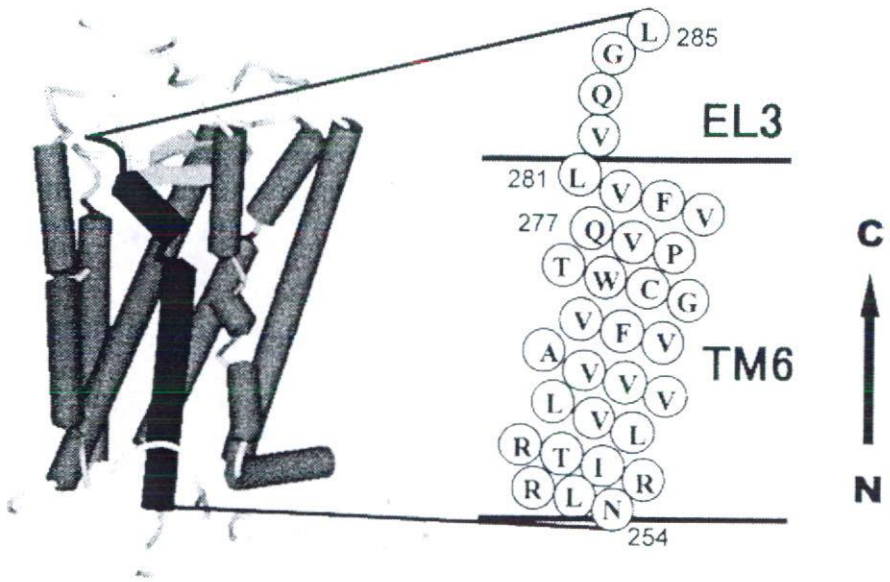

Fig. 1. Putative 3D structure of the rat ORL1 receptor (left) and the amino acid sequences in TM6 and EL3 (right). Amino acids of TM6, 30 in total from Asn254 to Leu285, were all mutated to Ala (except for Ala267). The allow indicates a direction of the peptide bond main chain.

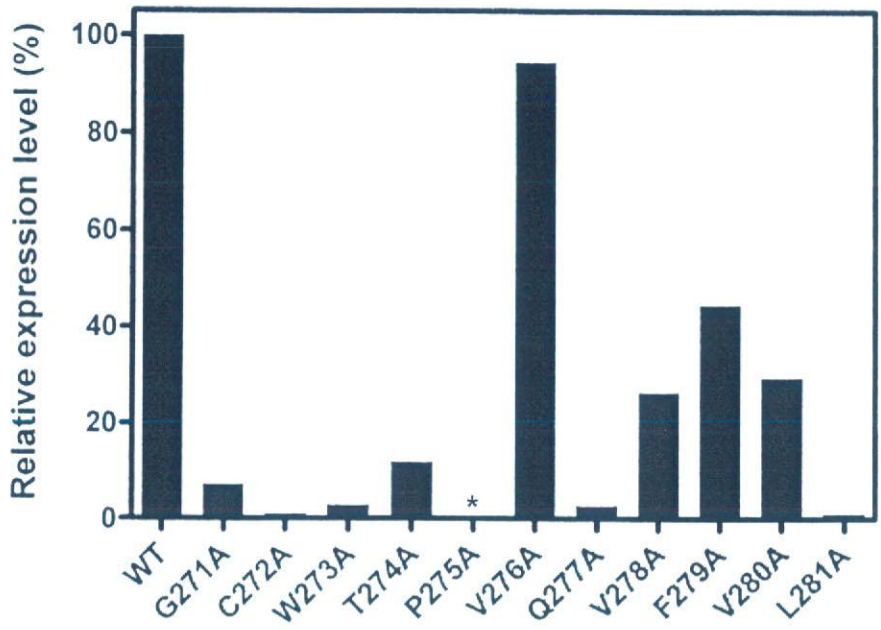

*Fig. 2. The relative expression level of the rat ORL1 receptors mutated. Membranes were prepared from COS-7 cells transiently transfected with each mutated receptor-$G_{\alpha o}$ fusion gene. The relative expression levels were caluculated by dividing the B_{max} value of mutant receptors with that of wild-type. *: Not determined its expression level because of no detectable [^{3}H]nociceptin binding.*

membrane preparations were used for the competitive binding assay and GTPγS functional assay, since the dissociation constant K_d of each mutant receptor was just as good as that of wild-type.

The competitive binding assay was carried out with mutant receptors to assess the binding ability of nociceptin. The binding potency of nociceptin was estimated by calculating the IC_{50} value. The IC_{50} values of the mutant receptors were found to be almost comparable to that of the wild-type ORL1. These results suggest that the Ala-substitution does not affect the overall ligand binding sites as observed for TM5 [2]. No assay was performed for P275A mutant receptor because of the lack of specific binding.

To further examine the individual amino acids in nociceptin-selective G protein activation, all mutant receptors were evaluated for their abilities to mediate the nociceptin stimulation of [^{35}S]GTPγS binding. To this end, we used the membrane preparations expressing ORL1-$G_{\alpha o}$ wild-type and mutant receptors. Although all mutant receptors were activated by nociceptin, each mutant receptor produced different maximum response, providing varying E_{max} and EC_{50} values. In particular, Q277A showed much lower receptor activation potency and maximal activation level (Fig. 3). This mutagenesis site Q (=Gln) at position 277 is seen only for ORL1 receptor. Other classic opioid receptors possess His instead of Gln at the same position of TM6, and thus it is likely that Gln277 in ORL1 plays a quite unique role in receptor activation. L281A also exhibited much decreased receptor activation (Fig. 3). These results indicate that the extracellular side of TM6 possesses amino acid residues functionally important for the activation (Fig. 1).

One of the purposes of Ala-scanning in this study is to identify the TM6's residue(s) interacting with that (or those) of TM5. In the previous study, we explored several key amino acid residues essential for receptor activation. It should be noted

that those residues (F212, F217, and F221) locate at the similar edge of TM5 α-helix [2]. Thus, we decided to exemplify such a putative interaction between the TM α-helices by focusing on the TM6 adjacent to TM5.

Using the crystal structure of bovine rhodopsin (PDB ID code: 1F88) as a template, we constructed initial 3D structure of the ORL1 receptor by the homology modeling procedure. In the modeling structure, two TM6 residues were assumed to interact with the key residues of TM5 at the extracellular side. One is Gln277 and the other is Leu281. These two residues are highly likely to interact with Phe221 and Phe217 in TM5, respectively, since the distance between two functional side-chains was calculated to be within 3.5 Å. When Gln277 was replaced to Ala, the binding potency to nociceptin was almost unchanged, but it decreased all the parameters related to receptor activation, such as the basal activity, activation efficacy, and maximal activation level. Similar results were also observed in the L281A mutation. From these results, we can hypothesize that Gln277 and Leu281 are interacting with Phe221 and Phe217 presumably by the NH/π and CH/π interaction, respectively. These interactions must be with relation, for example, to the change in receptor conformation from the inactive state to the active state.

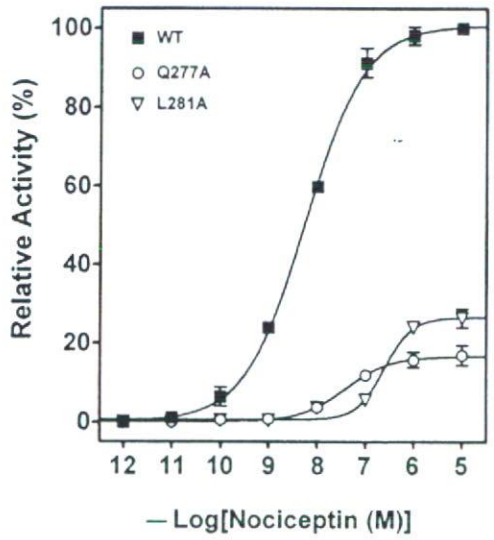

Fig. 3. Dose-response curves of nociceptin in the [^{35}S]GTPγS binding assay using the mutant receptors. The two typical mutated receptors of Q277A and L281A are shown. Responses are given as percentage changes from the wild-type (WT).

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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.

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 ERRγpossessing 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), and the charges were assigned by Biopolymer module in the neutral condition. CFF91 force field (Accerlys) was used in all molecular mechanics calculations. For

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calculation of the volume of the ligand-pocket, the 3D structure of apo-form ERRγ-LBD (1TFC: PDB code) 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**.

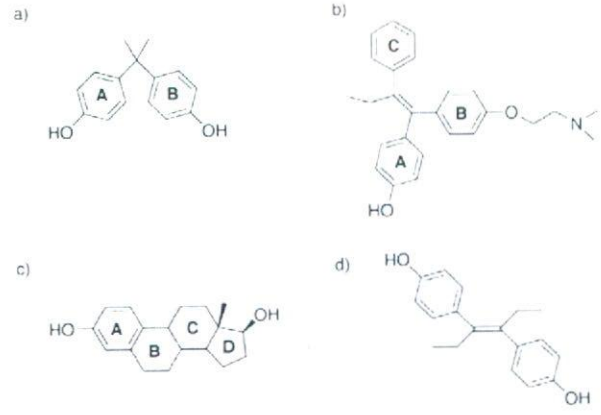

Figure 1. The structural formulas of bisphenol A (BPA), 4-hydroxytamoxifen (4-OHT), 17β-estradiol (E2), and diethylstilbestrol (DES). a: BPA, BPA possesses the two phenol groups A and B together with the two methyl groups. b: 4-OHT, 4-OHT possesses three benzene rings on the trans-ethylene double bond: 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 [37-29]. 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

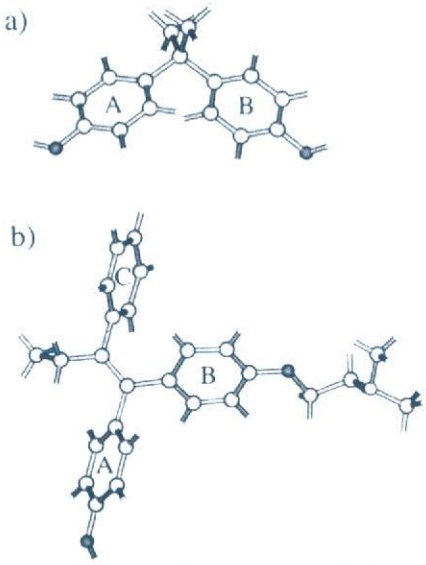

Figure 2. The three dimensional structures of bisphenol A (BPA) and 4-hydroxytamoxifen (4-OHT). a: BPA is shown by its minimum energy conformation. b: 4-OHT is shown by the structure pulled out from the 4-OHT/ERR γ-LBD complex (1S9Q). Characters (A and B in 'a' and A, B and C in 'b') indicated the same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

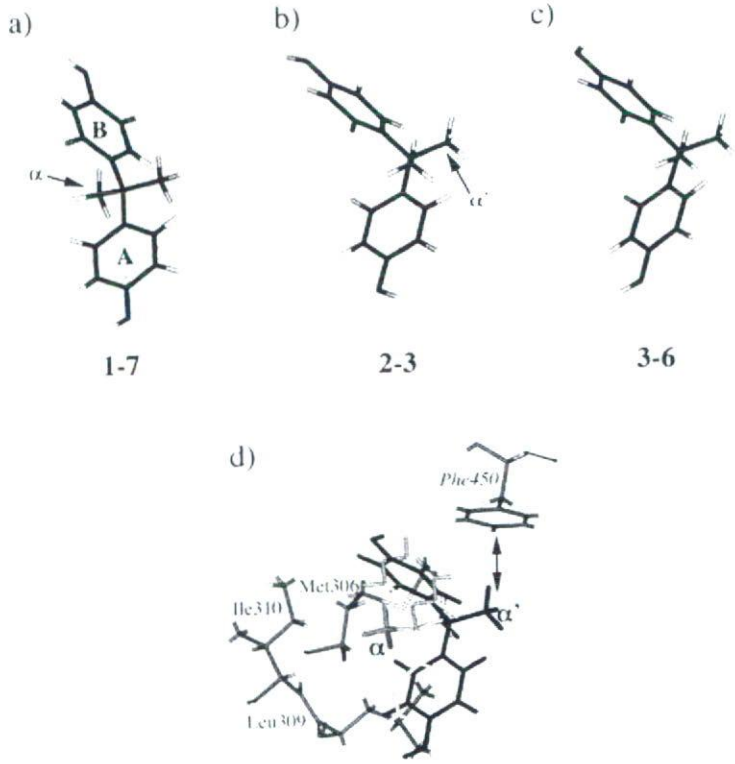

Figure 3. The three-dimensional structures of bisphenol A (BPA) docked in the apo-form ERRγ-LBD. Each structure (a)-(c) was obtained by calculations starting from different dockings with initial placements. The calculations were carried out by the computer program Affinity. **1-7** (a), **2-3** (b), 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

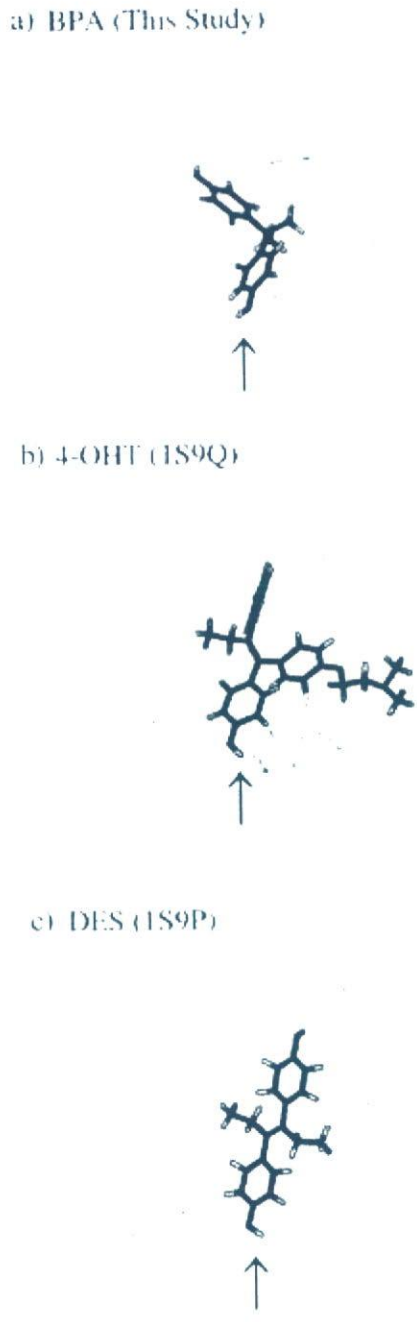

Figure 4. Complex structure of bisphenol A (BPA), 4-hydroxytamoxifen (4-OHT), and diethylstilbestrol (DES) bound to ERRγ-LBD. a) Calculated complex **2-3** in this study, b) 1S9Q, and c) 1S9P. Bold sticks display the ligand molecules BPA, 4-OHT, and DES, respectively. Arrows indicate the position of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

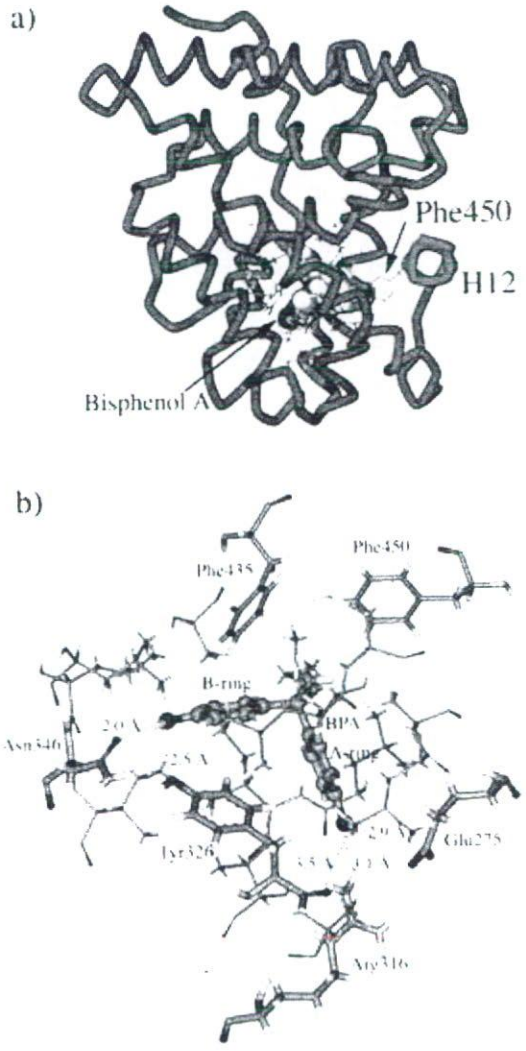

Figure 5. Calculated complex structure of bisphenol A (BPA)/ERRγ-LBD. a) Tube-model of the complex. H12 indicates the number 12 α-helix of ERRγ-LBD. b) The structure of BPA in the ligand-binding pocket. Broken lines (orange) indicate the hydrogen bonds in a reasonable distance. BPA represents a structure **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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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-(CH2)n-NH-; EDC.HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ER, estrogen receptor; ERR, estrogen-related receptor; ESI, electro spray 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 n-MeLi; step 2, the coupling of 17α-ethinylcarboxyestradiol with *N*-Cbz-α,ω-diaminoalkanes; step 3, deprotection of

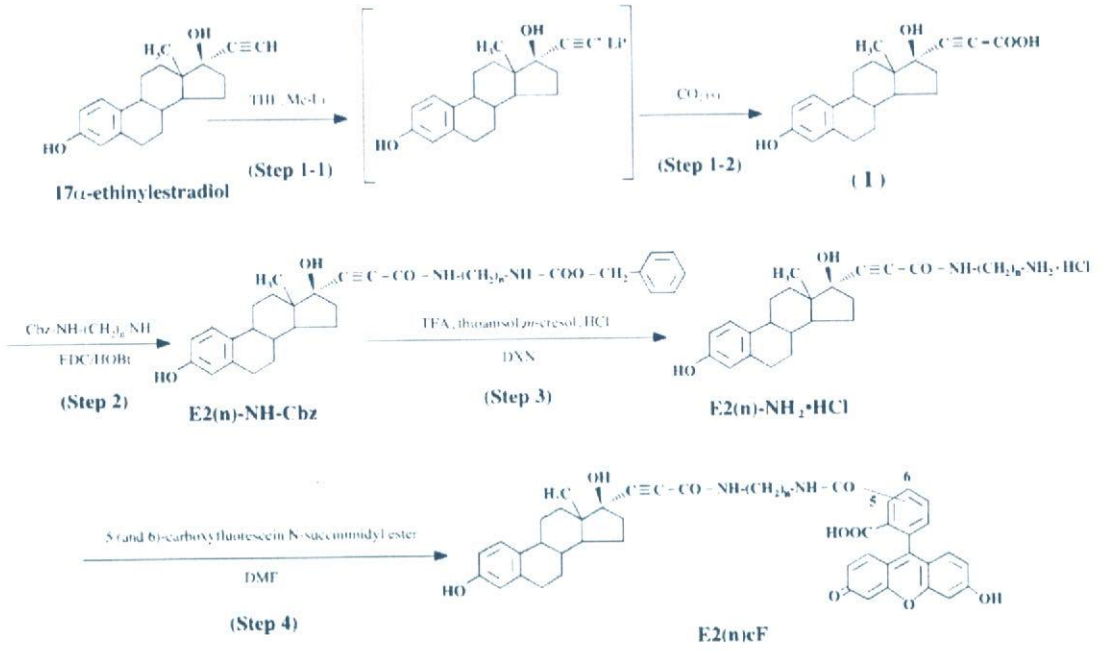

Fig. 1. **Synthetic scheme of the conjugates of 17α-ethinylestradiol and carboxyfluorescein via α,ω-diamino alkanes H_2N-$(CH_2)_n$-NH_2 ($n = 2$, 4, 6, 8, 10 and 12).**

the Cbz group by trifluoroacetic acid (TFA) in the presence of scavengers and step 4, the coupling with carboxyfluorescein.

17α-Ethinylcarboxyestradiol (I)—Under the condition of dry N_2 pressure, a solution of 17α-ethinylestradiol (4.60 g, 15.5 mmol) in THF (155 ml) was added to a solution of 1.14 M MeLi (46.5 mmol) in ether (40.8 ml) at approximately −70°C, and the solution was stirred for 30 min at the same temperature. Small pieces of dry ice (~300 g) were added to the reaction mixture in 30 min. During this addition, the solution was vigorously mixed with a magnetic stirrer, and the temperature was kept under −10°C. After removing the cooling bath, the reaction was continued for an additional 2 h at room temperature. The resulting white suspension was poured into 5.5 M ammonium chloride (20 ml), and the product, namely 17α-ethinylestradiol carboxylic acid denoted as 17α-ethinylcarboxyestradiol, was extracted with aq. NaOH. The alkaline solution was washed with ether to remove unreacted 17α-ethinylestradiol, and acidified with conc. HCl. The resulting solid was extracted with ethylacetate (EtOAc), and the solution was washed with saturated NaCl and dried over Na_2SO_4. After evaporation, the product 17α-ethinylcarboxyestradiol **I** was recrystallized from MeOH. Yield, 5.10 g (96%); HP-TLC, R_f 0.32; ^{1}H NMR (DMSO-d_6) δ 0.78 (s, 3H, CH_3), 5.76 (s, 1H, 17β-O*H*), 6.44–7.07 (m, 3H, Ar*H*), 8.98 (br, 1H, Ar O*H*); MS (FAB) m/z M^+ 340.24 (Calcd. for $C_{21}H_{24}O_4$, 340.4).

N-Cbz-α,ω-diaminoalkanes—A series of *N*-Cbz-α,ω-diaminoalkanes was prepared by the single carbobenzoxylation of α,ω-diaminoalkanes essentially as reported (*37*), but with some modifications. In this study, target compounds were obtained by careful purification of the reaction products. As a representative example, the synthesis of *N*-Cbz-1,6-diaminohexane (Cbz-NH-$(CH_2)_6$-NH_2) is described. To a solution of 1,6-diaminohexane (11.6 g, 100 mmol) in MeOH (100 ml) was added dropwise Cbz-Cl (14.3 ml, 100 mmol) at 0°C. The resulting precipitate (mostly *N,N*-diCbz-diaminohexane) was filtered off, and the filtrate was acidified with 3.6% HCl. The precipitate, mainly *N*-Cbz-1,6-diaminohexane, was collected by filtration. This precipitate was washed with ether and 4% $NaHCO_3$ and eventually treated with 4 M HCl/dioxane to afford the hydrochloride. *N*-Cbz-1,6-diaminohexane was also recovered from the filtrate. After extraction with ether to remove the dicarbobenzoxylated derivative, the filtrate was neutralized with 4% $NaHCO_3$ and extracted with ether. The organic solution was washed with saturated NaCl and dried over Na_2SO_4. After evaporation, 4 M HCl/dioxane was added dropwise to afford the product of Cbz-NH-$(CH_2)_6$-NH_2–HCl, namely, *N*-Cbz-1,6-diaminohexane hydrochloride. The combined yield was 7.00 g (24%). All other products ($n = 2$, 4, 8, 10 and 12) were prepared in a similar way.

17α-Ethinylcarboxyestradiol-N-Cbz-α,ω-diaminoalkanes E2(n)-NH-Cbz—17α- Ethinyl-carboxyestradiol I (340 mg, 1.0 mmol), 1-hydroxybenzotriazole (HOBt: 168 mg, 1.1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC–HCl: 211 mg, 1.1 mmol) were added to a solution of *N*-Cbz-1,6-diaminohexane hydrochloride (287 mg, 1.0 mmol) and Et_3N (140 μl, 1.0 mmol) in DMF (100 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C and overnight at room temperature, and then evaporated *in vacuo*. The residue was crushed into fine

Table 1. **Physicochemical properties of E2(n)-NH-Cbz, 17α-ethinylcarboxyestradiol-N- Cbz-α,ω-diaminoalkanes.**

Compounds E2(n)-NH-Cbz	Yield (%)	Mp (°C)	R_f[a]	*m/z* (M+H$^+$)	
				Found	Calcd.
E2(2)-NH-Cbz	82	106–108	0.23	517.3	517.6
E2(4)-NH-Cbz	86	93–94	0.19	545.2	545.7
E2(6)-NH-Cbz	81	84–85	0.26	573.3	573.7
E2(8)-NH-Cbz	98	70–71	0.37	601.3	601.8
E2(10)-NH-Cbz	96	91–92	0.44	629.4	629.8
E2(12)-NH-Cbz	98	79–80	0.46	657.4	657.8

[a]$CHCl_3$-MeOH-AcOH (50:10:2, v/v).

Table 2. **Physicochemical properties of E2(n)-NH_2·HCl, 17α-ethinylcarboxyestradiol-α,ω-diaminoalkanes hydrochloride.**

Compounds E2(n)-NH_2·HCl	Yield (%)	Mp (C)	R_f[a]	*m/z* (M+H$^+$)	
				Found	Calcd.
E2(2)-NH_2·HCl	61	108–109	0.24	382.1	382.5
E2(4)-NH_2·HCl	89	93–94	0.23	411.2	411.6
E2(6)-NH_2·HCl	92	83–84	0.20	439.3	439.6
E2(8)-NH_2·HCl	83	69–70	0.25	467.3	467.7
E2(10)-NH_2·HCl	59	89–90	0.24	495.4	495.7
E2(12)-NH_2·HCl	63	80–81	0.28	523.4	2523.8

[a]$CHCl_3$-MeOH-AcOH (50:10:2, v/v).

powder in water. This rather viscous solid was washed successively with 4% $NaHCO_3$, 5% $KHSO_4$ and water, and then dissolved in EtOAc. The solution was dried over Na_2SO_4. After evaporation, the residue was recrystallized from MeOH–water to obtain the final compound E2(6)-NH-Cbz. All other E2(n)-NH-Cbz ($n=2$, 4, 8, 10 and 12) products were prepared in a similar way. The yields and mass numbers of the compounds are shown in Table 1.

17α-Ethinylcarboxyestradiol-α,ω-diaminoalkanes hydrochloride E2(n)-NH_2·HCl—17α-Ethinylcarboxyestradiol-*N*-Cbz-1,6-diaminohexane E2(6)-NH-Cbz (1.15 g, 2.0 mmol) was dissolved in a mixture of TFA (80 ml), thioanisole (9.34 ml, 80 mmol), and *m*-cresol (8.37 ml, 80 mmol) and the solution was stirred for 4 h at room temperature. After evaporation, the residue was dissolved in ether, and the solution was treated with 4 M HCl/dioxane. The resulting precipitate was washed with pet. ether by decantation, and recrystallized from ether to obtain the final compound E2(6)-NH_2–HCl: yield, 0.89 g (92%). All other E2(n)-NH_2–HCl ($n=2$, 4, 8, 10 and 12) products were prepared in a similar way. The yield, melting point and mass number of the compounds are shown in Table 2.

17α-ethinylcarboxyestradiol-α,ω-diaminoalkane-carboxyfluorescein (E2(n)cF)—The solution of 17α-ethinylcarboxestradiol-α,ω-diaminohexane hydrochloride E2(6)-NH_2–HCl (48 mg, 0.1 mmol) in DMF (1 ml) was neutralized with Et_3N (14 μl, 0.1 mmol), and 5(and 6)-carboxyfluorescein *N*-succinimidyl ester (57 mg, 0.12 mmol) was added. The reaction mixture was stirred overnight at room temperature. After water (100 μl) was added to consume the unreacted *N*-succinimidyl ester, the solution was purified directly by gel filtration using a column (2×140 cm^2) of LH-20 eluted with DMF. Fractions containing the product were collected, and the solution was evaporated in vacuo. The residue was solidified with H_2O, and the obtained precipitate was finally purified by reverse-phase high-performance liquid chromatograph (RP-HPLC). The final product of 17α-ethinylcarboxylestradiol-α,ω-diaminoalkanes-5(and 6)-carboxyfluorescein are designated as E2(n)cF hereafter, in which the number 'n' denotes the chain length of the linker polymethylene. E2(6)cF was obtained as a white powder: yield, 12 mg (34%). All other E2(n)cF ($n=2$, 4, 8, 10 and 12) products were prepared in a similar way. The purity was verified by HP-TLC, analytical RP-HPLC and ESI-MS (Table 3).

Table 3. **Physicochemical properties of E2(n)cF, 17α-ethinylcarboxyestradiol-α,ω-diaminoalkane-carboxyfluorescein.**

Compounds E2(n)cF	Yield (%)	Mp (°C)	R_f[a]	*m/z* (M+H$^+$)	
				Found	Calcd.
E2(2)cF	42	248–250	0.67	741.3	740.8
E2(4)cF	42	208–210	0.67	769.2	768.8
E2(6)cF	34	198–200	0.64	797.2	796.9
E2(8)cF	29	189–191	0.74	825.5	824.9
E2(10)cF	61	154–156	0.86	852.7	853.0
E2(12)cF	52	142–144	0.87	881.1	881.0

[a]$CHCl_3$-MeOH-AcOH (50:10:2, v/v).

Radio-ligand Receptor-binding Assay—In order to assess the binding activity for the ER, a series of the conjugates E2(n)cF ($n=2$, 4, 6, 8, 10 and 12) were examined for their ability to inhibit the receptor binding of tritium-labelled estrogen. ER preparations used in this binding study were a full-length protein expressed in Sf9 cells (a kind gift from Sumitomo Chemical Co., Ltd) and a ligand-binding domain expressed in *E. coli* as a GST-fused protein. A solution (10 μl) of the recombinant estrogen receptor ERα-LBD (~1 nM) was added to Tris–HCl (pH 7.4, 70 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/ml BSA, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution (10 μl) of E2(n)cF and 10 nM [^{3}H]17β-estradiol (10 μl; 4.4–6.6 TBq/mmol, Amersham, Buckinghamshire, UK) was added, the reaction mixture (100 μl in total) was incubated for 1 h at room temperature. The compounds were dissolved in DMSO, the final concentration of which was adjusted to not exceed 3%.

Free radioligand was removed by centrifugation (10 min, 14,000 r.p.m.) after incubation with 0.4% dextran-coated charcoal (Sigma) in 0.2 M PBS (pH 7.4) for 10 min at 4°C. Scintillation counting of the supernatant was performed to measure [^{3}H]17β-estradiol bound to the receptor. 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 non-linear analysis program ALLFIT (*38*).

Optimization of Receptor Concentration for Change in Fluorescence Intensity—The fluorescence intensity of E2(n)cF ($n=2$, 4, 6, 8, 10 and 12) would be different under conditions with and without the ER, if the fluorescein moiety interacts non-specifically or specifically with the receptor. A solution (10 nM in final, 20 μl) of recombinant human ER α, which was expressed as

a ligand-binding domain protein (ERα-LBD) at a concentration of ~10 μM, was dissolved in Tris–HCl (pH 7.4, 160 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution dissolved in DMSO (20 μl) was added, the reaction mixture (200 μl in total) was incubated for 1 h at room temperature. Free estradiol derivatives were removed by incubating with 0.04% or 0.4% dextran-coated charcoal (Sigma) in 0.2 M PBS (pH 7.4, 200 μl) for 10 min at 4°C. All these experiments were performed on a 96-well polypropylene plate (Nunc A/S; Roskilde, Denmark), and the solution contained 0.5 mg/ml bovine γ-globulin to prevent adsorption of compounds to the plate wall. Plate centrifugation was carried out at 4°C for 10 min over 3,000*g* on a KUBOTA 6200 Centrifuge with a PF21 plate rotor (Kubota Co., Tokyo).

For measurement of fluorescence intensity, 200 μl of solution in each well was transferred into another 96-well FluoroNunc polystyrene plate (Nunc), a plate suitable for fluorometry measurements, by using an 8-channel dispenser (Nichiryo Co., Tokyo). The plate was placed on a microplate reader, the Wallac 1420 ARVOsx (Perkin Elmer, Turku, Finland), to measure the fluorescence intensity. Estimation of the emissions at 535 nm with excitation at 485 nm was carried out with the excitation energy around 10,000–40,000 for 1–3 s (see RESULTS section). Non-specific binding of fluorescein-linked estradiol derivatives was assessed by adding 10 μM of 17β-estradiol.

Fluorescence Measurement of E2(4)cF and E2(8)cF in the Presence of Estrogen Receptor—For E2(4)cF or E2(8)cF (500 nM), estrogen receptor ERα-LBD at 100, 250 and 500 nM concentrations was added at room temperature. Emission spectra were observed by the excitation at either 485 or 500 nm on a Spectrofluorometer FP-550A (Jasco, Tokyo).

Saturation Binding Assay of Fluorescein-ligand—The binding of fluorescent ligands to recombinant estrogen receptor ERα-LBD in the presence or absence of unlabelled estradiol was examined. A solution (100 nM, 20 μl) of ERα-LBD was dissolved in Tris–HCl (pH 7.4, 140 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 0.5 mg/ml bovine γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a fluorescent ligand (0.1–1 mM; 20 μl each) was added, the reaction mixture (200 μl) was incubated for 1 h at room temperature. Free estradiol derivatives of E2(4)cF or E2(8)cF were removed by incubating with 0.04% dextran-coated charcoal in 0.2 M PBS (pH 7.4, 200 μl) for 10 min at 4°C. Plate centrifugation was carried out at 4°C for 10 min as described above, and the fluorometry measurements were carried out with an excitation energy of 40,000 for 3 s. Non-specific binding was assessed by adding 10 μM 17β-estradiol.

Competitive Receptor-binding Assay by Using a Fluorescence Probe—To assess the ability of a compound to bind to the ER, the competitive binding assay was constructed by using the fluorescent ligand developed in the present study. A solution (10 nM, 20 μl) of the recombinant ERα-LBD was dissolved in Tris–HCl (pH 7.4, 140 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 0.5 mg/ml bovine γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution (20 μl) and fluorescent ligand E2(4)cF or E2(8)cF (5 nM, 20 μl) were added, the reaction mixture (200 μl) was incubated for 1 h at room temperature. After B/F separation with 0.04% dextran-coated charcoal followed by plate centrifugation at 4°C, the supernatant was used for fluorometry measurements carried out with an excitation energy of 40,000 for 3 s. Dose–response curves were assessed by the computer program ALLFIT. The binding affinity of the compounds was estimated as IC_{50} values, which exhibit the concentration for the half-maximal inhibition.

RESULTS

Chemical Synthesis of Fluorescein-linked 17α-Ethinylcarboxylestradiols—Figure 1 shows the scheme for a four-step synthesis of conjugates of 17α-ethinylcarboxylestradiol and CF, starting from 17α-ethinylestradiol. Cross-linking was performed by α,ω-diaminoalkanes ($n = 2$, 4, 6, 8, 10 and 12) in order to obtain a conjugate with an optimized methylene chain length for enhanced specific binding to the ER. Thus, '*n*' indicates eventually the number of methylene chains between estradiol moiety and fluorescein in the conjugates. The use of α,ω-diaminoalkanes with varying methylene chain lengths was eventually judged to be an important and critical issue to obtain the best fluorescent tracer for the receptor-binding assay.

The most difficult synthesis was carboxylation of 17α-ethinylestradiol, namely, the step 1. According to the method described by Carlson *et al.* (*34*), carbon dioxide (CO_2) gas was used first. However, almost no reaction occurred, and we therefore used dry ice in small pieces instead of CO_2 gas. This made the reaction in THF proceed very smoothly, but only under strict control of the reaction temperature. We kept the reaction temperature strictly under −10°C during the initial 30 min, which resulted in a considerably high-reaction yield (81–98%).

17α-Ethinylcarboxyestraol-*N*-Cbz-α,ω-diaminohexane E2(*n*)-NH-Cbz ($n = 2$, 4, 6, 8, 10 and 12) was prepared from 17α-ethinylcarboxylestradiol **I** and *N*-Cbz-α,ω-diaminoalkanes ($n = 2$, 4, 6, 8, 10 and 12). Although removal of the Cbz group was carried out by TFA containing thioanisole and *m*-cresol, the TFA salt could not be crystallized. Instead, the HCl salt was obtained successfully by treatment with 4 M HCl/dioxane. For purification of the final compounds, namely, carboxyfluorescein-linked 17α-ethinylcarboxyestradiol E2(*n*)cF, there were several problems to be solved. Because of their extremely high hydrophobic nature and thus their serious adsorption nature to the gel, the compounds could not be recovered very well from the column. For RP-HPLC, the sampling solution could be prepared only by using 75% acetic acid. Although E2(*n*)cF exists as a mixture of isomers, the cross-linker of which is attached to either position 5 or 6 of fluorescein, we could not separate these isomers, even on HPLC.

Receptor-binding Affinity of Fluorescein-linked 17α-Ethinylcarboxyestradiols—The binding affinity of synthesized conjugates E2(*n*)cF for the ER was evaluated

Table 4. **Receptor-binding affinity of the conjugates of 17α-ethinylcarboxylestradiol with carboxyfluorescein cross-linked by α,ω-diaminoalkanes and their intermediate precursors.**

Chain length (n) of polymethylene	Receptor binding affinity, IC_{50} (μM)		
	Cbz-protected derivatives E2(n)-NH-Cbz	Amino-free derivative E2(n)-NH_2·HCl	Fluorescent ligands E2(n)cF
2	0.29 ± 0.12	1.60 ± 1.2	12.0 ± 8.0
4	0.16 ± 0.15	0.72 ± 0.30	2.0 ± 2.0
6	0.27 ± 0.20	0.89 ± 0.10	1.8 ± 1.2
8	0.71 ± 0.11	0.44 ± 0.11	0.19 ± 0.15
10	0.75 ± 0.10	0.48 ± 0.13	0.90 ± 0.62
12	0.74 ± 0.42	0.10 ± 0.12	0.53 ± 0.23

The IC_{50} values for derivatives are the averages ± SEM of at least three separated experiments. In this assay, using 10 nM [^{3}H]17β-estradiol (4.4–6.6 TBq/mmol) and GST-fused ER-LBD, B/F separation was performed with 0.4% dextran-coated charcoal. 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 non-linear analysis program ALLFIT.

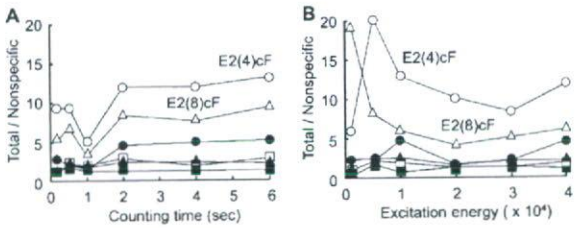

Fig. 2. **Effects of the fluorescence excitation energy and counting time on the specific and non-specific bindings of fluorescent ligands E2(*n*)cF.** Each 100 nM fluorescent ligand E2(*n*)cF, namely, E2(2)cF (filled circle), E2(4)cF (open circle), E2(6)cF (filled triangle), E2(8)cF (open triangle), E2(10)cF (filled square), E2(12)cF (open square), was incubated with ER (10 nM) at room temperature for 1 h either with (total binding) or without (non-specific binding) 17β-estradiol (E2: 10 μM). Specific binding to estrogen receptor was estimated by the subtraction of non-specific binding from total binding. Fluorescence intensity was measured after removal of unbound free fluorescent ligand by using 0.4% (w/w) dextran-coated charcoal. Fluorescent ligand was individually excited at 485 nm, and the fluorescence intensity was monitored at 535 nm. (A) The intensity ratio of total binding to non-specific binding was analysed under different counting times (seconds). (B) The intensity ratio of total binding to non-specific binding was analysed under different excitation energies.

first by the conventional binding assay using [^{3}H]17β-estradiol as a tracer. It was found that 17α-ethinylestradiol (IC_{50} = 0.87 nM) was able to bind to the ER as strongly as 17β-estradiol (IC_{50} = 1.20 nM). However, its carboxylic acid derivative drastically reduced the affinity (IC_{50} = 25 μM, approximately four orders of magnitudes weaker than 17β-estradiol). This extremely diminished activity may have been due to the steric hindrance or the electrostatic effects of the attached carboxyl group, apparently being unfavourable in binding to the receptor. It was reported that the receptor-binding affinity of 17α-substituted estradiol derivatives is reasonably low (*39–41*). Indeed, it was found that the receptor-binding affinities of 17α-ethinylestradiol cross-linked with α,ω-diaminoalkanes and its *N*-Cbz derivatives are only moderately high, although they are apparently more potent than their parent carboxylic acid derivatives (Table 4). These results suggest that the hydrophobic interaction between the methylene chain and the receptor is superior to that of free carboxylic acid. The diaminoalkane derivatives lacking a fluorophore did not show a binding affinity dependent upon methylene chain lengths (Table 4).

When the final compounds of carboxyfluorescein-linked 17α-ethinylcarboxyestradiol derivatives E2(*n*)cF were assayed, it was found that they bind quite strongly to the ER in a dose-dependent manner. The receptor-binding affinity of a series of compounds was found to be dependent upon the lengths of the polymethylene chains–$(CH_2)_n$– of the linkers. Since carboxyfluorescein itself was absolutely inactive in this binding assay, it is unlikely that carboxyfluorescein binds to the binding pocket of 17β-estradiol. In a series of E2(*n*)cF, E2(8)cF with 1,8-diaminooctane (*n* = 8) exhibited the highest affinity to the receptor (IC_{50} = 186 nM, Table 4). This relatively enhanced binding activity is probably due to the moderately enhanced receptor interaction of carboxyfluorescein at a site other than the E2 binding pocket. Octamethylene–$(CH_2)_8$–is to occupy an optimum distance between E2 and cF, which must be positioned somewhere near the binding pocket of E2. Pseudo-specific binding of cF to the receptor would be substantiated with hydrophobic and electrostatic interactions, since the cF moiety has a few functional groups. Nonetheless, it should be noted that E2(*n*)cF is considerably potent to bind to the ER.

Determination of Fluorescent Probe for Receptor-binding Assay—In order to use the conjugates of E2(*n*)cF as a fluorescent probe of the receptor-binding assay for ER, each fluorescent ligand was carefully evaluated for their fluorescence characteristics and receptor-binding characteristics. First of all, in order to optimize the measurement conditions of the fluorometry, the excitation energy and counting time were examined for all E2(*n*)cF (*n* = 2, 4, 6, 8, 10 and 12) (Fig. 2). When the intensity ratio of the total binding versus non-specific binding was plotted against either the counting time or the excitation energy, it was found that E2(4)cF and E2(8)cF provide specific-receptor binding that is clearly higher than others. For stable fluorometry measurements, we selected three different instrumentation conditions; i.e. a combination of excitation energy and counting time, 10,000/1 s, 20,000/2 s and 40,000/3 s, respectively.

In the fluorometry measurements, the fluorescence intensity decreased gradually to reach the equilibrium constant. This was prominent when the time-course of change in the specific binding of E2(4)cF to the ER was plotted in the presence or absence of 17β-estradiol (Fig. 3). During the initial several minutes after centrifugation for B/F separation, the solutions exhibited a rapid decline in fluorescence intensity. It takes ~10 min to reach a steady state. Consequently, the sample solutions were measured for their fluorescence intensity at more than ~10 min after the B/F separation. In this B/F separation, we found that dextran-coated charcoal (dry preparation) should be

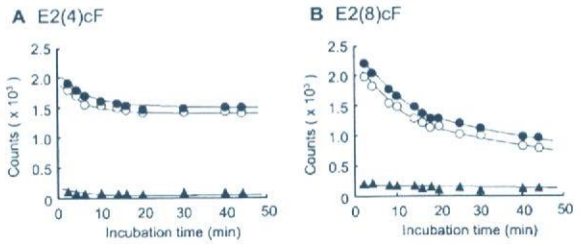

Fig. 3. **Change in the fluorescence intensity of fluorescent ligands E2(4)cF and E2(8)cF in the presence and absence of unlabelled estrogen.** E2(4)cF (A) and E2(8)cF (B) (100 nM) were incubated with ER (10 nM) at room temperature for 1 h. Total binding (filled circle) and non-specific binding (filled triangle) were obtained by the incubation with and without 17β-estradiol (E2: 10 μM), respectively. Specific binding (open circle) was estimated by subtraction of non-specific binding from total binding. The reaction mixture was treated with the same volume of 0.4% (w/w) dextran-coated charcoal in ice-cold 0.2 M PBS in order to remove unbound free fluorescent ligand. After incubation for 10 min, the mixture was centrifuged at 3,000*g* for 10 min at 4°C. The fluorescence intensity was measured for 200 μl supernatant aliquots transferred into 96-well polystyrene plates. After excitation at 485 nm (excitation energy: 20,000), the fluorescence intensity was monitored at 535 nm (counting time: 2 s) on a plate reader.

Table 5. **Binding characteristics of fluorescent ligands E2(n)cF to the estrogen receptor.**

E2(n)cF	Fluorescence intensities		
	Total binding	Non-specific binding	Specific binding
E2(2)cF	338 ± 41	101 ± 38	338 ± 14
E2(4)cF	1450 ± 77	178 ± 86	1280 ± 45
E2(6)cF	1020 ± 16	309 ± 88	713 ± 74
E2(8)cF	1980 ± 110	476 ± 18	1500 ± 97
E2(10)cF	522 ± 57	374 ± 32	148 ± 36
E2(12)cF	657 ± 120	155 ± 20	502 ± 99

The fluorescence intensity of E2(*n*)cF was measured in the presence or absence of the estrogen receptor ERα-LBD (10 nM in final). Receptor-unbound free ligand molecules were absorbed and removed by incubating with 0.4% dextran-coated charcoal. All experiments were performed on a 96-well polypropylene plate, and the solution contained 0.5 mg/ml bovine γ-globulin to prevent adsorption of compounds to the plate wall. Plate centrifugation was carried out at 4°C for 10 min over 3,000*g* on a plate rotor.

stirred for at least 1 h in PBS to get a well-swelled preparation. If not, it was not possible to obtain reproducible assay results, probably due to the insufficient and insecure adsorption capabilities of charcoal.

One of the essential conditions for the tracer in the receptor-binding assay is that it exhibit specific binding. Thus, all E2(*n*)cF were tested to determine their specific binding under the best assay conditions. Each E2(*n*)cF (100 nM in final) was incubated with the ER in the presence and absence of 17β-estradiol (10 μM) to ascertain the amounts of non-specific binding and total binding, respectively. Subtraction of the non-specific binding from the total binding affords the specific binding, and the results are summarized in Table 5. It is evident that E2(4)cF and E2(8)cF reveal sufficiently large specific

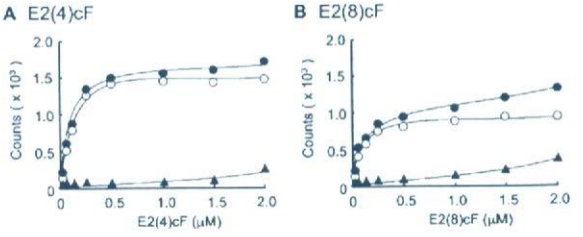

Fig. 4. **Saturation binding of fluorescent ligands E2(4)cF and E2(8)cF to ER.** Fluorescent ligands E2(4)cF (A) and E2(8)cF (B) were incubated with ER (10 nM) at room temperature for 1 h. Total binding (filled circle) and non-specific binding (filled triangle) were obtained by the incubation with and without 17β-estradiol (E2: 10 μM), respectively. Specific binding (open circle) was estimated by subtraction of non-specific binding from total binding. The reaction mixture was treated with the same volume of 0.4% (w/w) dextran-coated charcoal in ice-cold 0.2 M PBS in order to remove unbound free fluorescent ligand. After incubation for 10 min, the mixture was centrifuged at 3,000*g* for 10 min at 4°C. The fluorescence intensity was measured for 200 μl supernatant aliquots transferred into 96-well polystyrene plates. After excitation at 485 nm (excitation energy: 40,000), the fluorescence intensity was monitored at 535 nm (counting time: 3 s) on a plate reader.

bindings, which are 2–10 times higher than those of other fluorescent derivatives of E2(*n*)cF. We therefore decided to use both E2(4)cF and E2(8)cF as tracers in a novel fluorescence receptor-binding assay.

The specific binding of E2(4)cF and E2(8)cF to the estrogen receptor ERα-LBD in the presence of unlabelled 17β-estradiol (10 μM) was investigated by saturation binding. Figure 4 shows the saturation binding analysis of the binding of these fluorescent ligands to ERα-LBD. Under the same assay conditions, E2(4)cF and E2(8)cF exhibited binding profiles specific for each binding characteristic. It should be noted that the fluorescence intensity in saturation of E2(4)cF is considerably higher (about 2-fold) than that of E2(8)cF, suggesting that E2(4)cF is in a condition more restricted than E2(8)cF (Fig. 4). The cF moiety in E2(4)cF appears to be in firm and rigid surroundings that fix its molecular motion. It was concluded that the specific bindings of both E2(4)cF and E2(8)cF are sufficiently to construct a binding assay.

Scatchard plot analyses have demonstrated that the recombinant ERα-LBD shows a single binding mode (Fig. 5). Estimated B_{max} for E2(4)cF and E2(8)cF are 15.1 nmol/mg protein and 16.3 nmol/mg protein, respectively. These values are very compatible with the calculated value (16 nmol/mg protein) for the GST-fused ERα-LBD (molecular weight, 61,000).

Specific Emission Spectra of E2(4 or 8)cF with Estrogen Receptor—In the fluorescence spectra, we see two peaks at different wavelengths, namely, the excitation wavelength and the emission wavelength. At these peaks or spectra, we observed the increment of fluorescence intensity of E2(4)cF. Figure 6A shows the emission spectra of E2(4)cF in the presence and absence of ER in an aqueous buffer. The fluorescence intensity at the excitation wavelength (498 nm) by binding to the receptor increased only slightly (12%) over the initial value with a 1/5-fold amount of receptor. The increase became greater

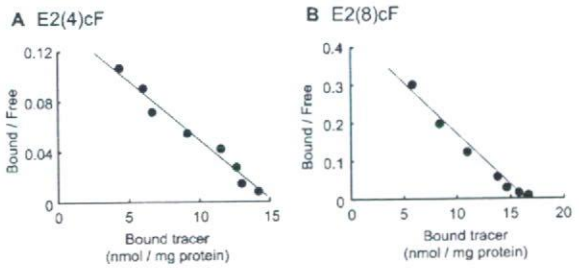

Fig. 5. **Scatchard analysis of saturation binding of fluorescent ligands E2(4)cF and E2(8)cF to ER.** The horizontal axis is the concentration (nmol/mg protein) of bound fluorescent ligands E2(4)cF (A) and E2(8)cF (B) to ER. The vertical axis is the ratio of the concentration of bound fluorescent ligand against free ligand to ER. (A) E2(4)cF; B_{max} (15.1 nmol/mg protein), K_d (104 nM). (B) E2(8)cF; B_{max} (16.3 nmol/mg protein), K_d (37.2 nM).

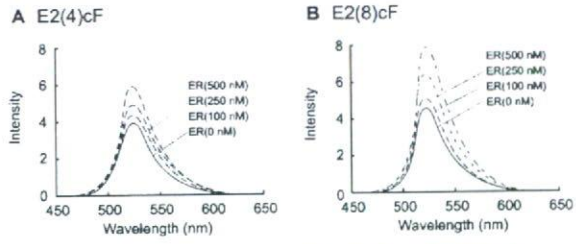

Fig. 6. **Fluorescence spectra of E2(4)cF and E2(8)cF in the presence or absence of ER.** For E2(4)cF (A) or E2(8)cF (B) (500 nM each), ER of 100, 250 and 500 nM concentrations were added at room temperature. Emission spectra were observed by excitation at 498 nm. Excitation at 485 nm afforded almost the same spectra. The results of the emission spectra are shown for solo 500 nM E2(4)cF (straight line), 500 nM E2(4)cF with 100, 250 and 500 nM ER (dashed line).

with increasing amounts of receptor; i.e. 25% with 1- to 2-fold, 67% with equivalent amounts of receptor (data not shown). The peak maximum remained unchanged (λ_{ex} = 498 nm). When excited at 498 nm, the fluorescence intensity at the emission wavelength also increased to 16% of the initial value with a 1- to 5-fold amount of receptor. The increment was enhanced by increasing the amount of receptor; i.e. 37% with 1- to 2-fold, 62% with equivalent amounts of receptor. No shift in the peak maximum was observed (λ_{em} = 520 nm). When excited at 485 nm, which is the wavelength set on a plate reader, the fluorescence intensity at the emission wavelength (λ_{em} = 520 nm) increased to 16% of the initial value with a 1- to 5-fold amount of receptor. The increment was enhanced by increasing the amount of receptor; i.e. 26% with 1- to 2-fold, 48% with equivalent amounts of receptor. When E2(8)cF was examined for its emission spectra in the presence of the ER, it exhibited very enhanced increments in the fluorescence intensities (Fig. 6B). For instance, with the excitation at 485 nm, E2(8)cF showed a 96% increment with an equivalent amount of receptor, which is twice as large as that of E2(4)cF.

Receptor-binding Assay Using Fluorescent Tracers—Using E2(4)cF as a tracer, the receptor-binding assay was

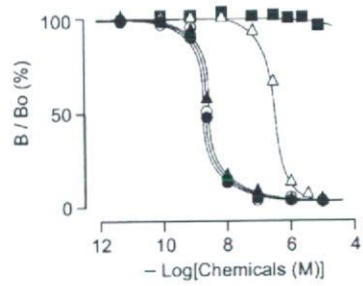

Fig. 7. **Concentration-dependent curves in a competition binding assay using fluorescent ligand E2(4)cF as a tracer for ER.** To a solution of ER (10 nM) was added fluorescent tracer E2(4)cF (5 nM) and compounds of 17β-estradiol (filled circle), estriol (open circle), estrone (filled triangle), 4-nonylphanol (open triangle) and fluorescein (filled square), respectively. The reaction mixture was incubated at room temperature for 1 h. The reaction mixture was treated with the same volume of 0.04% (w/w) dextran-coated charcoal in ice-cold 0.2 M PBS in order to remove unbound free fluorescent ligand. After incubation for 10 min, the mixture was centrifuged at 3,000*g* for 10 min at 4°C. The fluorescence intensity was measured for 200 µl supernatant aliquots transferred into 96-well polystyrene plates. After excitation at 485 nm (excitation energy: 40,000), the fluorescence intensity was monitored at 535 nm (counting time: 3 s) on a plate reader.

carried out essentially as performed in the assay using radiolabelled [^{3}H]17β-estradiol. The concentrations of estrogen receptor ERα-LBD (10 nM) and dextran-coated charcoal (0.04%) for the B/F separation were eventually determined to optimize the assay conditions to elicit a reasonable binding efficacy of fluorescent ligand E2(4)cF (5 nM). As shown in Fig. 7, standard estrogens such as estrone (E1), 17β-estradiol (E2) and estriol (E3) exhibited ideal dose-response curves with a high ability to displace E2(4)cF. Their IC_{50} values are compatible with those obtained from the binding assay using [^{3}H]17β-estradiol (Table 6).

As shown in the radiolabel binding assay, cF, a fluorophore of E2(4)cF and E2(8)cF, was completely inactive in both receptor-binding assays, indicating that fluorescein cannot displace estradiol in the estrogen-binding site. This result also implies that the putative interaction of the fluorophore of E2(4)cF and E2(8)cF with the receptor is extremely weak as compared with the specific interaction of the estrogen moiety. All these results clearly reveal that fluorescent ligands E2(4)cF and E2(8)cF possess essential structural and kinetic characteristics as a probe or tracer of the ER. This was also proven by the assay for a series of naturally occurring steroid hormones and the derivatives of estradiol (Table 6).

Diethylstilbestrol (so-called DES), a highly active alternative of 17β-estradiol, was also equipotent in both assays; i.e. 1.5–1.9 nM in the fluorescent binding assay and 1.8 nM in the radiolabel-binding assay (Table 7). Also, 4-hydroxytamoxifen, an antagonist of 17β-estradiol, was highly potent; i.e. 3.1–3.3 nM in the fluorescent binding assay and 3.1 nM in the radiolabelled binding assay. These results clearly indicate that ordinary aromatic organic compounds are able to displace fluorescent ligands E2(4)cF and E2(8)cF as well as steroids.

Table 6. **Binding activity of naturally occurring steroid hormones and derivatives of estradiol to estrogen receptor with fluorescence and radiolabeled tracers.**

Chemicals	IC_{50} (nM)		
	E2(4)cF	E2(8)cF	[^{3}H]17β-estradiol
Estrone (E1)	2.61 ± 0.23	2.57 ± 0.15	2.98 ± 0.23
17β-estradiol (E2)	1.13 ± 0.11	1.13 ± 0.13	1.18 ± 0.11
Estriol (E3)	3.67 ± 0.39	3.72 ± 0.69	3.44 ± 0.13
17α-ethinylestradiol	1.63 ± 0.10	1.72 ± 0.10	1.25 ± 0.11
17α-estradiol	4.09 ± 0.30	4.07 ± 0.13	4.02 ± 0.13
Testosterone	NB	NB	NB
Progesterone	NB	NB	NB
Carboxyfluorescein	NB	NB	NB

NB, not bound, implies that the chemical is not bound to the receptor at its 10 μM concentration.

Table 7. **Binding activity of various chemicals to estrogen receptor with fluorescence and radiolabeled tracers.**

Chemicals	IC_{50} (nM)		
	E2(4)cF	E2(8)cF	[^{3}H]17β-estradiol
17β-estradiol (E2)	1.13 ± 0.11	1.13 ± 0.13	1.18 ± 0.11
Diethylstilbestrol	1.53 ± 0.13	1.89 ± 0.13	1.80 ± 0.72
4-Hydroxytamoxifen	2.14 ± 0.27	2.58 ± 0.56	2.29 ± 0.49
4-Nonylphenol	1380 ± 191	1350 ± 185	1440 ± 176
n-Nonylphenol	2510 ± 566	2240 ± 730	ND
Bisphenol A	1050 ± 243	1090 ± 225	1160 ± 110
4-Octylphenol	2290 ± 166	2120 ± 427	ND
t-Octylphenol	4040 ± 524	4760 ± 647	ND
4-Cyclohexylphenol	700 ± 165	642 ± 234	ND
Diethylphthalate	NB	NB	NB
Di-*n*-butylphthalate	NB	NB	NB
Tributyltin chloride	NB	NB	NB
Triphenyltin Chloride	NB	NB	NB

ND not determined, implies that the IC_{50} (nM) value was not determined because of very weak receptor-binding affinity at its 10 μM concentration. NB not bound, implies that the chemical was almost completely inactive with no binding at its 10 μM concentration.

On the other hand, 4-nonylphenol, a putative endocrine disruptor for the ER, was only moderately active (IC_{50} = 1.35–1.38 μM). This result is also comparable to that obtained from the radiolabel-binding assay (1.44 μM) (Table 7). Similarly, bisphenol A, currently the most notable endocrine disruptor claimed to have the low-dose effects, was almost equipotent in both assays; i.e. 1.04–1.09 μM in the fluorescent-binding assay and 1.05 μM in the radiolabelled binding assay. Here, it is definite that bisphenol A is very weak to interact with ERα. Various candidate chemicals as endocrine disrupting chemicals were also examined in these three assay systems (Table 7). Tri-*n*-butyltin chloride, triphenyltin hydroxide and all phthalates were confirmed to be inactive for ERα.

It should be noted that the assay using E2(8)cF as a tracer afforded almost the same binding results as observed in the assay with E2(4)cF (Tables 6 and 7). The assay *per se* was performed under the same experimental conditions as those for E2(4)cF (Fig. 6), producing ideal dose–response curves, as seen in Fig. 6 for standard estrogens E1, E2 and E3. In conclusion, it is definitely worthwhile to use E2(4)cF and E2(8)cF as fluorescence labels in the binding assay for the ER.

DISCUSSION

Fluorescent Tracer with Cross-linker Between Estrogen and Fluorophore—In this study, we established a novel binding assay method, in which the specific binding was determined by a direct measure of fluorescence intensities. This is the first example of a binding assay utilizing a fluorescent tracer to measure the fluorescence intensity for the NRs. The results clearly indicate that an assay system using either E2(4)cF or E2(8)cF is adequate for assessing the ability of compounds to displace these tracers. Assay data are compatible with those obtained from the ordinary radiolabel binding assay using [^{3}H]17β-estradiol.

A receptor-binding assay for measuring the fluorescence intensity has been reported for G protein-coupled receptors such as seletonin $5HT_3$ receptor. However, selection or optimization of tracer ligands to deduce the maximal condition for measurement of fluorescence intensity was not carried out in this case. There is a similar case for assays utilizing fluorescence polarization. The pharmacophores were cross-linked to the fluorophores with just a single cross-linker. The present results clearly show that the fluorescein characteristics of E2(*n*)cF are dependent upon the varying cross-linking methylene chain lengths, and are able to be optimized.

The fact that changes in the fluorescence intensity upon specific binding to the receptor depend upon the methylene lengths of cross-linkers between 17α-ethinylestradiol and cF indicates that the interaction of fluorescein with the receptor can be substantiated only at acceptable sites at a proper distance. This was clearly shown by the ordinary receptor-binding assay, in which E2(8)cF afforded the best binding affinity to the receptor among a series of E2(*n*)cF. Although E2(8)cF is still ~100 times less potent than 17β-estradiol itself, its binding specificity is sufficient to estimate the ability of compounds to displace it at the binding site. E2(4)cF, which is ~10 times less potent than E2(8)cF in displacing [^{3}H]17β-estradiol, exhibited somewhat larger changes in the fluorescence intensity upon specific binding to the receptor. This result may imply that the cF moiety is in a structurally restricted circumstance due to its shorter (by approximately half) cross-linker.

In the assay methods to measure the fluorescence polarization, two different types of fluorescent ligands were used. One of these fluorophores was fluormone ES1, a structurally modified diethylstilbestrol (DES). ES1 is an intrinsically fluorescent non-steroid estrogen and exhibits a short excitation wavelength (360 nm), providing a weak fluorescence at the emission wavelength (530 nm). Since many samples of interest such as biological fluids often contain adventitious fluorescence, a short excitation wavelength seriously raises the background against which the measurement must be made. Another fluorophore reported is FITC, fluorescein isothiocyanate, in which fluorescein exhibits a rather long excitation (490 nm) and emission (520 nm). However, synthesized 17α-substituted E2 derivative has a much shorter cross-linker that corresponds to E2(0)cF. A short spacer might cause steric hindrance between estradiol and fluorescein to interact with the receptor-binding site, although their binding affinity was not evaluated in the regular

radioligand-binding assay. The present study clearly shows that fluorescent tracers should be optimized for their cross-linker between the pharmacophore and the fluorophore,

Specific Interaction of Fluorescein with Estrogen Receptor—Changes in the fluorescence intensity depend upon specific binding to the receptor. The increase in fluorescence intensity of E2(4)cF at the emission wavelength in the presence of estrogen receptor ER-LBD is due to its binding to the receptor. Since the increase is dependent upon the concentration of receptor, the characteristic changes in the fluorescence intensity are due to the interaction between the fluorophore and the receptor protein. This specific interaction may result in a freeze in movement of the fluorophore, cF.

E2(8)cF exhibited much enhanced increments in fluorescence intensities in its emission spectra in the presence of the ER. As indicated, it showed a 96% increment with an equivalent amount of receptor, which is twice as large as that of E2(4)cF. This result appears to disagree with the result obtained from the saturation binding analysis. The fluorescence intensity of E2(8)cF in saturation analysis was definitely smaller than that of E2(4)cF (Fig. 4). As shown in Table 4, the receptor-binding affinity of E2(8)cF is almost 10 times higher than that of E2(4)cF. Thus, if we add the same concentration of the receptor ER, ER would bind much more abundantly E2(8)cF than E2(4)cF. This would make the fluorescent intensity of E2(8)cF stronger than that of E2(4)cF as in Fig. 6. It should be noted that the saturation binding analysis was performed under the condition of the charcoal treatment for B/F separation, while the examination was carried out with no charcoal. Charcoal may remove receptor-unbound ligands and affect the receptor–ligand equilibrium. Such a removal would be more effective for E2(8)cF than for E2(4)cF, because E2(8)cF has a hydrophobic methylene chain twice as long as that of E2(4)cF.

Both E2(4)cF and E2(8)cF are very unique in having a rather long cross-linking spacer between estrogen and fluorescein. Flexibility due to attachment of the fluorophore may cause a so-called propeller effect, resulting in depolarization to bring out no changes in the fluorescence intensity. To minimize this propeller effect, the molecular flexibility should be diminished by the 'specific' interaction of the fluorophore with the receptor. Based on this rationale, fluorophore cF should stay at this certain 'specific' receptor site, presumably a highly hydrophobic location. This 'specific' binding would also diminish the flexibility of the methylene chain. To identify such a 'specific' binding site for the fluorophore, it is essential to determine the optimal chain length. To make such a determination, we prepared candidate compounds with a series of aliphatic polymethylene cross-linkers of varying chain lengths. In the preparation of tracers, it is now evident that optimization of the spacer structure, namely, the structure of a cross-linker between the fluorophore and the pharmacophore, is extremely important.

It was eventually found that E2(n)cF with tetramethylene $(CH_2)_4$ and octamethylene $(CH_2)_8$ exhibit much larger changes in fluorescence intensity. As such, cF, attached to 17α-ethinylcarboxyestradiol via polymethylene $(CH_2)_4$ in E2(4)cF and $(CH_2)_8$ in E2(8)cF, is captured at different sites of the ER-LBD. These sites are ~6 Å apart from each other, corresponding to the $(CH_2)_4$ chain length, the structural circumstances of which must be different, especially with regard to its fluorescent characteristics.

Optimization of Assay Conditions and Advantages of Assay—To analyse the precise interactions of cF in E2(n)cF, its adsorption to the assay plates should be prevented. In a preliminary stage of the experiment to set the assay conditions, we used BSA as a blocker that protects the tracer to adsorb to the polypropylene microwell plate surface. However, unstable and irreproducible results were obtained from the successive assays. When the fluorometry measurement was carried out for plates of exactly the same concentration, the fluorescence intensity was found to vary from plate to plate. These results suggest that the synthesized fluorescein-linked estrogen derivatives had a strong interaction with BSA, perturbing the fluorometry measurements. Indeed, several reports have shown the nonspecific adsorption of fluorescein to BSA (*42*, *43*). This issue is resolved only when BSA is replaced by bovine γ-globulin (*44*). After all, bovine γ-globulin has been found to afford stable and reproducible results in fluorometry measurements.

The present study provides for the first time a fluorescence receptor-binding assay that measures the changes in the fluorescence intensity. Although this method requires B/F separation of the tracer, treatment with dextran-coated charcoal gives a full separation. One of the greatest advantages of this method is the direct use of microwell plates of 96-holes for centrifugation followed by plate fluorometry measurements. Another advantage is that experimentation can be performed in an ordinary laboratory, with no special regulations, unlike the RI laboratory required for the radiolabel receptor-binding assay. The present method would afford a universal procedure to evaluate the binding affinity of NRs.

Bisphenol A is a Weak Binder of Estrogen Receptor—Bisphenol A is one of the highest volume chemicals produced worldwide as a starting material for polycarbonate plastics and epoxy resins. Long known as an estrogenic chemical, bisphenol A is suspected of interacting with human ER (*45*, *46*) or acting as an antagonist for a human androgen receptor (AR) (*47*). However, it has been notified that its binding to ER and AR and its hormonal activity are extremely weak. Indeed, in the present study, we demonstrated that the binding activity of bisphenol A is 700–800 times weaker than with natural hormone 17β-estradiol (Table 7). Based on the idea that bisphenol A may interact with NRs other than ER and AR, we screened a series of NRs and eventually explored ERRγ as the target receptor of bisphenol A (*9*, *11*).

Bisphenol A was found to bind strongly to ERRγ, one of 48 human NRs, with high constitutive basal activity (*9*). Bisphenol A's binding to ERRγ was further demonstrated by X-ray crystallographic analysis of the complex between bisphenol A and ERRγ (*10*). Whether or not bisphenol A is an endocrine disruptor that exhibits 'low-dose effects' has long been controversial, and there is a scientific debate over whether or not low BPA doses have reproductive and developmental effects in humans (*48–50*). To evaluate correctly the receptor-binding affinity is crucially important for appropriate interpretation of receptor responses