

Fig. 1. Synthetic scheme of the conjugates of 17 α -ethinylestradiol and carboxyfluorescein via α,ω -diaminoalkanes H₂N-(CH₂)_n-NH₂ ($n = 2, 4, 6, 8, 10$ and 12).

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

17 α -Ethinylcarboxyestradiol (I)—Under the condition of dry N₂ pressure, a solution of 17 α -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 17 α -ethinylestradiol carboxylic acid denoted as 17 α -ethinylcarboxyestradiol, was extracted with aq. NaOH. The alkaline solution was washed with ether to remove unreacted 17 α -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 Na₂SO₄. After evaporation, the product 17 α -ethinylcarboxyestradiol I was recrystallized from MeOH. Yield, 5.10 g (96%); HP-TLC, R_f 0.32; ¹H NMR (DMSO-*d*₆) δ 0.78 (s, 3H, CH₃), 5.76 (s, 1H, 17 β -OH), 6.44–7.07 (m, 3H, ArH), 8.98 (br, 1H, Ar OH); MS (FAB) m/z M⁺ 340.24 (Calcd. for C₂₁H₂₄O₄, 340.4).

***N*-Cbz- α,ω -diaminoalkanes**—A series of *N*-Cbz- α,ω -diaminoalkanes was prepared by the single carbobenzylation 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-(CH₂)₆-NH₂) 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 $^{\circ}\text{C}$. 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% NaHCO₃ 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 dicarbonylated derivative, the filtrate was neutralized with 4% NaHCO₃ and extracted with ether. The organic solution was washed with saturated NaCl and dried over Na₂SO₄. After evaporation, 4 M HCl/dioxane was added dropwise to afford the product of Cbz-NH-(CH₂)₆-NH₂·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.

17 α -Ethinylcarboxyestradiol-*N*-Cbz- α,ω -diaminoalkanes (E2(*n*)-NH-Cbz)—17 α -Ethinylcarboxyestradiol 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 Et₃N (140 μ l, 1.0 mmol) in DMF (100 ml) at 0 $^{\circ}\text{C}$. The reaction mixture was stirred for 2 h at 0 $^{\circ}\text{C}$ 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, 17 α -ethinylcarboxyestradiol-*N*-Cbz- α,ω -diaminoalkanes.

Compounds	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

^aCHCl₃-MeOH-AcOH (50:10:2, v/v).

Table 2. Physicochemical properties of E2(n)-NH₂·HCl, 17 α -ethinylcarboxyestradiol- α,ω -diaminoalkanes hydrochloride.

Compounds	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	523.8

^aCHCl₃-MeOH-AcOH (50:10:2, v/v).

powder in water. This rather viscous solid was washed successively with 4% NaHCO₃, 5% KHSO₄ and water, and then dissolved in EtOAc. The solution was dried over Na₂SO₄. 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.

17 α -Ethinylcarboxyestradiol- α,ω -diaminoalkanes hydrochloride (E2(n)-NH₂·HCl)—17 α -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)-NH₂·HCl: yield, 0.89 g (92%). All other E2(n)-NH₂·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.

17 α -ethinylcarboxyestradiol- α,ω -diaminoalkane-carboxy-fluorescein (E2(n)cF)—The solution of 17 α -ethinylcarboxyestradiol- α,ω -diaminohexane hydrochloride E2(6)-NH₂·HCl (48 mg, 0.1 mmol) in DMF (1 ml) was neutralized with Et₃N (14 μ l, 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 μ l) was added to consume the unreacted *N*-succinimidyl ester, the solution was purified directly by gel filtration using a column (2 \times 140 cm) of LH-20 eluted with DMF. Fractions

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

Compounds	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

^aCHCl₃-MeOH-AcOH (50:10:2, v/v).

containing the product were collected, and the solution was evaporated in vacuo. The residue was solidified with H₂O, and the obtained precipitate was finally purified by reverse-phase high-performance liquid chromatograph (RP-HPLC). The final product of 17 α -ethinylcarboxyestradiol- 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).

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 μ l) of the recombinant estrogen receptor ER α -LBD (~1 nM) was added to Tris-HCl (pH 7.4, 70 μ l) containing 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₃, 10% glycerol, 10 mg/ml BSA, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin. After a sample solution (10 μ l) of E2(n)cF and 10 nM [³H]17 β -estradiol (10 μ l; 4.4–6.6 TBq/mmol, Amersham, Buckinghamshire, UK) was added, the reaction mixture (100 μ l 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, 17,500g) 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 [³H]17 β -estradiol bound to the receptor. To estimate the binding affinity, the IC₅₀ 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 (100 nM, 20 μ l) of recombinant human ER α , which was expressed as

a ligand-binding domain protein (ER α -LBD) at a concentration of $\sim 10 \mu\text{M}$, was dissolved in Tris-HCl (pH 7.4, 160 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₃, 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 3,000g 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 ARVox (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 17 β -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 ER α -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 ER α -LBD in the presence or absence of unlabelled estradiol was examined. A solution (100 nM, 20 μl) of ER α -LBD was dissolved in Tris-HCl (pH 7.4, 140 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₃, 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 0.4% 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 17 β -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 (100 nM, 20 μl) of the recombinant ER α -LBD was dissolved in Tris-HCl (pH 7.4, 140 μl) containing 1 mM EDTA, 1 mM EGTA,

1 mM NaVO₃, 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 (50 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 IC₅₀ values, which exhibit the concentration for the half-maximal inhibition.

RESULTS

Chemical Synthesis of Fluorescein-linked 17 α -Ethinylcarboxyestradiols—Figure 1 shows the scheme for a four-step synthesis of conjugates of 17 α -ethinylcarboxyestradiol and CF, starting from 17 α -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 17 α -ethinylestradiol, namely, the step 1. According to the method described by Carlson *et al.* (34), carbon dioxide (CO₂) gas was used first. However, almost no reaction occurred, and we therefore used dry ice in small pieces instead of CO₂ 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%).

17 α -Ethinylcarboxyestradiol-*N*-Cbz- α,ω -diaminohexane E2(n)-NH-Cbz ($n=2, 4, 6, 8, 10$ and 12) was prepared from 17 α -ethinylcarboxyestradiol 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 17 α -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 17 α -Ethinylcarboxyestradiols—The binding affinity of synthesized conjugates E2(n)cF for the ER was evaluated

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

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

The IC₅₀ values for derivatives are the averages \pm SEM of at least three separated experiments. In this assay, using 10 nM [³H]17 β -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 IC₅₀ values, the concentrations for the half maximal inhibition, were calculated from the dose-response curves evaluated by the non-linear analysis program ALLFIT.

first by the conventional binding assay using [³H]17 β -estradiol as a tracer. It was found that 17 α -ethinylestradiol (IC₅₀ = 0.87 nM) was able to bind to the ER as strongly as 17 β -estradiol (IC₅₀ = 1.20 nM). However, its carboxylic acid derivative drastically reduced the affinity (IC₅₀ = 25 μ M, approximately four orders of magnitudes weaker than 17 β -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 17 α -substituted estradiol derivatives is reasonably low (39–41). Indeed, it was found that the receptor-binding affinities of 17 α -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 17 α -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—(CH₂)_{*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 17 β -estradiol. In a series of E2(*n*)cF, E2(8)cF with 1,8-diaminooctane (*n* = 8) exhibited the highest affinity to the receptor (IC₅₀ = 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—(CH₂)₈—is to occupy an optimum distance between E2 and cF, which must be positioned somewhere

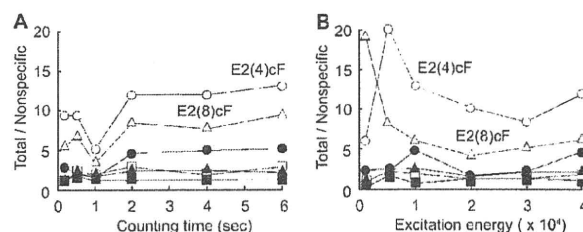


Fig. 2. Effects of the fluorescence excitation energy and counting time on the specific and non-specific bindings of fluorescent ligands E2(*n*)cF. Each 100 nM fluorescent ligand E2(*n*)cF, namely, E2(2)cF (filled circle), E2(4)cF (open circle), E2(6)cF (filled triangle), E2(8)cF (open triangle), E2(10)cF (filled square), E2(12)cF (open square), was incubated with ER (10 nM) at room temperature for 1 h either with (non-specific binding) or without (total binding) 17 β -estradiol (E2: 10 μ M). 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.

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 17 β -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

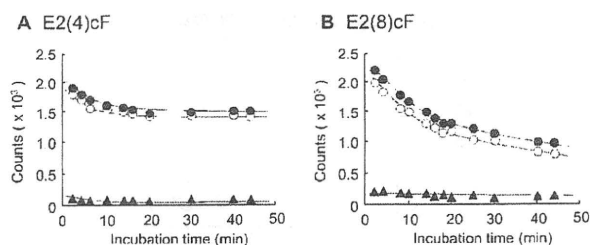


Fig. 3. Change in the fluorescence intensity of fluorescent ligands E2(4)cF and E2(8)cF in the presence and absence of unlabelled estrogen. E2(4)cF (A) and E2(8)cF (B) (100 nM) were incubated with ER (10 nM) at room temperature for 1 h. Total binding (filled circle) and non-specific binding (filled triangle) were obtained by the incubation with and without 17 β -estradiol (E2: 10 μ M), 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 3,000g for 10 min at 4 C. The fluorescence intensity was measured for 200 μ l 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 \pm 41	101 \pm 38	338 \pm 14
E2(4)cF	1450 \pm 77	178 \pm 86	1280 \pm 45
E2(6)cF	1020 \pm 16	309 \pm 88	713 \pm 74
E2(8)cF	1980 \pm 110	476 \pm 18	1500 \pm 97
E2(10)cF	522 \pm 57	374 \pm 32	148 \pm 36
E2(12)cF	657 \pm 120	155 \pm 20	502 \pm 99

The fluorescence intensity of E2(n)cF was measured in the presence or absence of the estrogen receptor ER α -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 3,000g 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 17 β -estradiol (10 μ M) 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

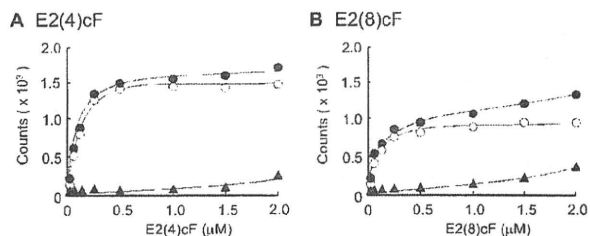


Fig. 4. Saturation binding of fluorescent ligands E2(4)cF and E2(8)cF to ER. Fluorescent ligands E2(4)cF (A) and E2(8)cF (B) were incubated with ER (10 nM) at room temperature for 1 h. Total binding (filled circle) and non-specific binding (filled triangle) were obtained by the incubation with and without 17 β -estradiol (E2: 10 μ M), 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 3,000g for 10 min at 4 C. The fluorescence intensity was measured for 200 μ l 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 ER α -LBD in the presence of unlabelled 17 β -estradiol (10 μ M) was investigated by saturation binding. Figure 4 shows the saturation binding analysis of the binding of these fluorescent ligands to ER α -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 ER α -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 ER α -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 $\frac{1}{5}$ -fold amount of receptor. The increase became greater

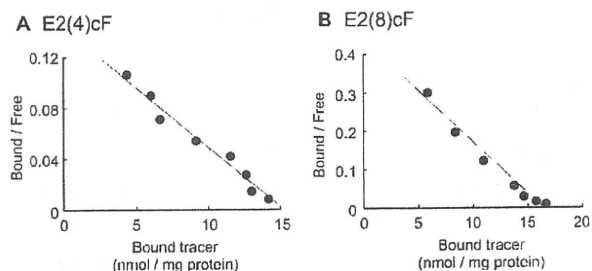


Fig. 5. Scatchard analysis of saturation binding of fluorescent ligands E2(4)cF and E2(8)cF to ER. The horizontal axis is the concentration (nmol/mg protein) of bound fluorescent ligands E2(4)cF (A) and E2(8)cF (B) to ER. The vertical axis is the ratio of the concentration of bound fluorescent ligand 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).

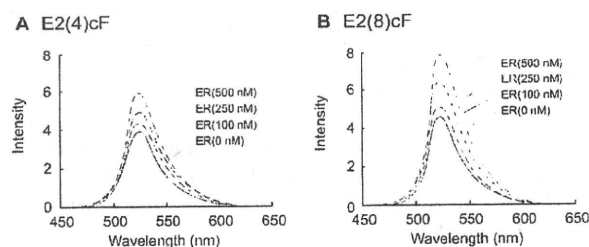


Fig. 6. Fluorescence spectra of E2(4)cF and E2(8)cF in the presence or absence of ER. For E2(4)cF (A) or E2(8)cF (B) (500 nM each), ER of 100, 250 and 500 nM concentrations were added at room temperature. Emission spectra were observed by excitation at 498 nm. Excitation at 485 nm afforded almost the 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 $\frac{1}{2}$ -fold, 67% with equivalent amounts of receptor (data not shown). The peak maximum remained unchanged ($\lambda_{\text{ex}} = 498$ nm). When excited at 498 nm, the fluorescence intensity at the emission wavelength also increased to 16% of the initial value with a $\frac{1}{5}$ -fold amount of receptor. The increment was enhanced by increasing the amount of receptor; i.e. 37% with $\frac{1}{2}$ -fold, 62% with equivalent amounts of receptor. No shift in the peak maximum was observed ($\lambda_{\text{em}} = 520$ nm). When excited at 485 nm, which is the wavelength set on a plate reader, the fluorescence intensity at the emission wavelength ($\lambda_{\text{em}} = 520$ nm) increased to 16% of the initial value with a $\frac{1}{5}$ -fold amount of receptor. The increment was enhanced by increasing the amount of receptor; i.e. 26% with $\frac{1}{5}$ -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

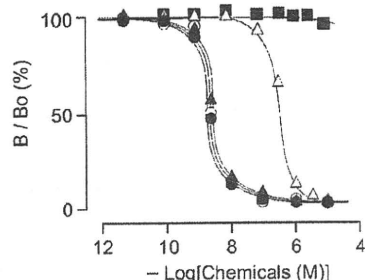


Fig. 7. Concentration-dependent curves in a competition binding assay using fluorescent ligand E2(4)cF as a tracer for ER. To a solution of ER (10 nM) was added fluorescent tracer E2(4)cF (5 nM) and compounds of 17 β -estradiol (filled circle), estriol (open circle), estrone (filled triangle), 4-nonylphenol (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 3,000g for 10 min at 4 C. The fluorescence intensity was measured for 200 μ l 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]17 β -estradiol. The concentrations of estrogen receptor ER α -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), 17 β -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]17 β -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 17 β -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 17 β -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
<i>n</i> -Nonylphenol	2510 ± 566	2240 ± 730	ND
Bisphenol A	1050 ± 243	1090 ± 225	1160 ± 110
4-Octylphenol	2290 ± 166	2120 ± 427	ND
<i>t</i> -Octylphenol	4040 ± 524	4760 ± 647	ND
4-Cyclohexylphenol	700 ± 165	642 ± 234	ND
Diethylphthalate	NB	NB	NB
Di- <i>n</i> -butylphthalate	NB	NB	NB
Tributyltin chloride	NB	NB	NB
Triphenyltin Chloride	NB	NB	NB

ND not determined, implies that the IC₅₀ (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 (IC₅₀ = 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 [³H]17β-estradiol.

A receptor-binding assay for measuring the fluorescence intensity has been reported for G protein-coupled receptors such as serotonin 5HT₃ 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 [³H]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 (CH₂)₄ and octamethylene (CH₂)₈ exhibit much larger changes in fluorescence intensity. As such, cF, attached to 17 α -ethinylcarboxyestradiol via polymethylene (CH₂)₄ in E2(4)cF and (CH₂)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 (CH₂)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 micro-well 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 17 β -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 ERR γ as the target receptor of bisphenol A (9, 11).

Bisphenol A was found to bind strongly to ERR γ , one of 48 human NRs, with high constitutive basal activity (9). Bisphenol A's binding to ERR γ was further demonstrated by X-ray crystallographic analysis of the complex between bisphenol A and ERR γ (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

of the various compounds, and thus it is critical to develop the efficient assay systems.

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