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Estrogen Receptor Binding Assay Method for Endocrine Disruptors Using Fluorescence Polarization

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A rapid, simple and nonhazardous assay method for endcrine disruptors was developed using an estrogen receptor (ER) and fluorescence polarization (FP). Among the fluorescent compounds, the 17α-fluorescein-labeled estradiol derivative was selected as the most suitable ligand for the ER binding assay, since it showed the highest affinity to ER. In the Scatchard plot analysis, its convex curve exhibited a positive cooperative binding, indicating the induction of a conformational change of the ER with the binding of the ligand to form a dimer and to increase the affinity for the additional ligand. On the basis of the Hill plot analysis, its dissociation constant and Hill coefficient were 10.4 nM and 1.63, respectively. A competitive binding assay with an unlabeled 17*β*-estradiol (E2) yielded an IC_{50} value of 2.82 nM and a Hill coefficient of 1.67, thus providing a Ki value of 0.65 nM. In the same manner, the Hill coefficients for estrone, estriol, diethylstilbestrol, and tamoxifen were determined to be 0.99, 1.17, 1.59, and 2.44, respectively.

The estrogen receptor (ER) is a 67 kDa member of the superfamily of nuclear receptor proteins, such as glucocorticoid, mineralocorticoid, progesterone, androgen, retinoic acid, vitamin D, and thyroid receptors.[1−5] The receptor possesses transcriptional activity modulated by the binding of the agonist or antagonist ligand that affects cell growth and differentiation, and participates in the growth of estrogen-dependent breast cancer. In recent years, ER has also been considered to be a target protein by a number of synthetic chemicals called "endocrine disruptors", which have been suspected to cause a disruption in the endocrine system, produce undesirable effects on the reproduction system, and interfere with the process of fetal development in animals and humans.[6−8]

With regard to the screening of these endocrine disruptors, several ER binding assay methods using a radioactive ligand as a probe are usually used.[9−12] Most of them have employed tritium or iodine-125-labeled estradiol (E2; Figure 1) derivatives. Use of these derivatives requires exclusive facilities under strict management to avoid any radiation hazard. In addition, a filtration step is required to remove any unbound derivative from the probe bound to ER prior to the measurement of the radioactivity. To overcome these problems, Adamczyk et al. prepared some fluorescent ligands to develop an ER binding assay method with fluorometric detection.[13] However, their fluorescent ligands have not been used for the method, since fluorescence detection would not be suitable for the ER binding assay because of the fluorescence properties of the ligands (fluorescence intensities and excitation and emission spectra) dependent on the ligand molecular environment (for example, ligand bound or not bound to ER). Therefore, alternative methods suitable for the screening of many endocrine disruptors without the use of radioactive reagents are desired.

When a fluorescent molecule in solution is excited with plane-polarized light, it emits another plane-polarized light, the angle of which is expressed as fluorescence polarization (FP). The polarization value (P) is obtained from the calculation according to the following equation by a fluorescent ligand excited with a vertical polarized light,

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the measured vertical and horizontal fluorescence intensities, respectively. FP is related to the rotational correlation time of the molecule and, thus, is also related

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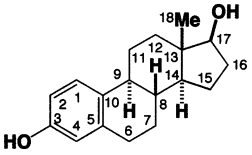


Figure 1. Chemical structure of 17β-estradiol.

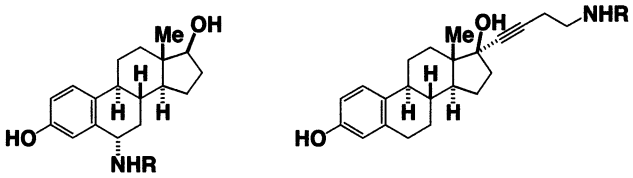
to $3\eta V/RT$, where η, T, V, and R are the viscosity, absolute temperature, molecular volume, and the gas constant, respectively. A large fluorescent molecule, such as an ER bound with a fluorescent ligand, slowly rotates during the excited state and produces a high polarization value. A small molecule, such as a fluorescent ligand, itself quickly rotates and produces a small polarization value. Therefore, the bound and free ligand ratio for an ER (bound/free) is easily quantified by the polarization value without physical separation of the free ligand from the bound ligand. That is, FP can be used for the high-throughput screening of many endocrine disruptors.

Although numerous papers have been published using FP for the study of the interaction of biomolecules such as DNA−DNA, DNA−protein, protein−protein, and antigen−antibody,[14−17] to our knowledge, there is only one paper that reports a competitive ER binding assay method.[18] In this paper, the fluorescent ligand, a structurally modified diethylstilbestrol, exhibited a short excitation (360 nm) and emission (530 nm) wavelength and gave a weak fluorescence. FP is theoretically independent from the fluorescence properties of the molecule. However, the polarization value is actually calculated from the difference in the "$I_{||}$ minus background fluorescence" and "$I_{\perp}$ minus background fluorescence". Therefore, if an excessive fluorescence exists, a significant influence on the polarization value is possible. Since there are many fluorescent interfering compounds excitable at around 300−350 nm with an emission at 360−430 nm in the biomatrixes, the adopted fluorescent ligand would not be appropriate for the precise and selective detection of the endocrine disruptors in the biosamples. Thus, in this study, we attempted to use a fluorescent ligand with long wavelength excitation and emission and a strong fluorescence for the ER binding assay method.

Our initial effort was focused on finding an appropriate fluorophore for the labeling of E2. We then examined the possibility of introducing an amino group at the C6α or C17α position of E2 to attach the fluorophore through the group, and the resultant fluorescent ligands were evaluated in terms of their affinity for ER by the FP method. The competitive binding assay method for ER with the ligand and the unlabeled E2 was performed. Several other competitors were also examined to confirm the feasibility in the proposed assay method.

EXPERIMENTAL SECTION

Materials. Human recombinant estrogen receptor-α (hrER-α) was purchased from PanVera Corporation (Madison, WI). 17β-Estradiol and 6-ketoestradiol were from Sigma (St. Louis, MO). Estrone and fluorescein isothiocyanate (FITC) were from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical or guaranteed reagent grade and were used without further purification. Water was purified using a Milli-Q reagent system (Millipore, Bedford, MA).



1a: R = H
1b: R = $COCH_2NH_2$
1c: R = FITC
1d: R = $COCH_2NHFITC$

2a: R = H
2b: R = FITC

Figure 2. Chemical structures of 6α- or 17α-substituted estradiol derivatives (**1a**, **1b**, and **2a**) and their fluorescein-labeled derivatives (**1c**, **1d**, and **2b**).

Apparatus. The UV−vis absorption spectra were measured using a JASCO Ubest-50 spectrometer (Tokyo, Japan). The fluorescence spectra were measured using a Hitachi F-4010 fluorescence spectrometer (Tokyo, Japan). The proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a JEOL GSX-400 spectrometer (Tokyo, Japan) with tetramethylsilane as the internal standard. Mass spectra were measured on a Finnigan Mat TSQ 7000 (Finnigan Mat, San Jose, CA) with electrospray ionization (ESI). Fluorescence polarization assays were performed using a Beacon 2000 fluorescence polarization instrument (PanVera Corporation) with a 490-nm excitation filter and a 520-nm emission filter at the regulated temperature of 25 °C.

Synthesis. The 6α-substituted E2 derivatives (Figure 2; **1a**,**1b**) were stereoselectively synthesized from 6-ketoestradiol (6-ketoE2) as previously described.[19] Briefly, 6-ketoE2 protected with 2-(trimethylsilyl)ethoxymethyl (SEM) groups was converted to the corresponding oxime by reaction with hydroxylamine and then reduced with zinc to afford 6α-aminoestradiol (**1a** in Figure 2). We also prepared **1b** in Figure 2 with a spacer from **1a**. After **1a** was condensed with *tert*-butoxycarbonyl (Boc)-glycine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), the Boc group of the product was removed by treatment with HF to afford **1b**.

The 17α-substituted E2 derivative (Figure 2; **2a**) was also stereoselectively synthesized from estrone as the starting material (Figure 3). To a solution of estrone (**1**, 105 mg) in dry CH_2Cl_2 (1.6 mL) were added diisopropylethylamine (*i*-Pr_2NEt, 102 μL) and methoxymethyl chloride (MOMCl, 42 μL) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 12 h. The mixture was then extracted with diethyl ether. The organic extract was washed with water, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography on silica gel (10% ethyl acetate in *n*-hexane) to afford **2** (62.9 mg, 51%). ^{1}H NMR δ ($CDCl_3$): 7.21 (1H, d, J = 11 Hz), 6.86 (1H, dd, J = 2.5, 8.5 Hz), 6.80 (1H, d, J = 3.5 Hz), 5.15 (2H, s), 3.47 (3H, s), 2.91 (2H, m), 1.44−2.54 (13H, m), 0.91 (3H, s). ESI-MS: m/z 315 $(M + H)^+$. To a THF (1 mL) solution of 3-butyn-1-ol tetrahydropyranyl (THP) ether (92.5 mg) were added

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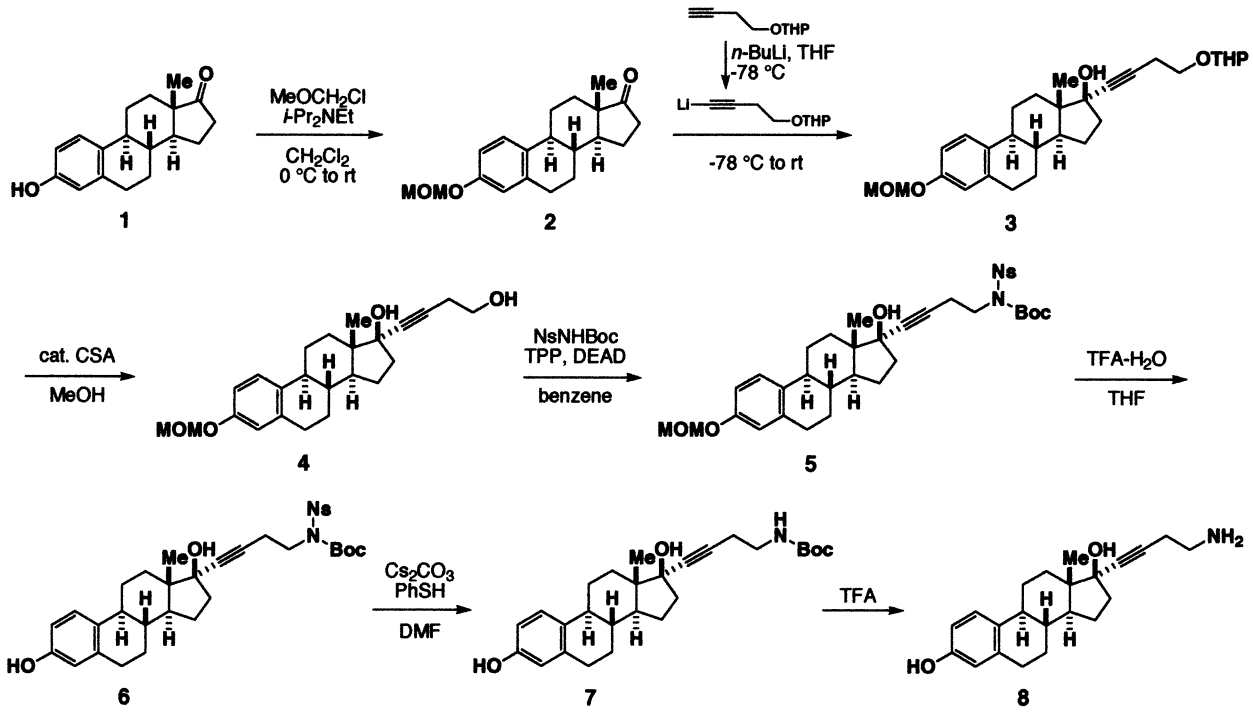

Figure 3. Synthesis of 17α-substituted estradiol derivative (for abbreviations, see Experimental Section).

n-butyllithium (*n*-BuLi, 0.36 mL at 1.6 M in *n*-hexane) and then a THF (1 mL) solution of **2** at −78 °C. The reaction mixture was slowly warmed to room temperature. After stirring for 15 h, the reaction was quenched by the addition of water. The reaction mixture was then extracted with ethyl acetate, and the organic extracts were washed with water, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography on silica gel (20% ethyl acetate in *n*-hexane) to afford **3** (84.1 mg, 89%). ^{1}H NMR δ ($CDCl_3$): 7.22 (1H, d, J = 9.5 Hz), 6.85 (1H, dd, J = 2.5, 8.5 Hz), 6.78 (1H, d, J = 3.5 Hz), 5.15 (2H, s), 4.66 (1H, s), 3.48−3.57 (5H, m), 2.86 (2H, m), 1.26−2.58 (24H, m), 0.86 (3H, s). ESI-MS: m/z 469 $(M + H)^+$. The THP group of the E2 derivative of **3** was removed by treatment of **3** with catalytic 10-camphorsulfonic acid (CSA, 4.2 mg) in methanol (2 mL) for 10 min at room temperature. The concentration of the reaction mixture left a crude product, which was purified by column chromatography on silica gel (33% ethyl acetate in *n*-hexane) to afford **4** (50.1 mg, 73%). ESI-MS: m/z 385 $(M + H)^+$. A protected nitrogen function was then introduced by the Mitsunobu reaction to afford **5**. Thus, to a mixture of **4**, *N*-(*tert*-butoxycarbonyl)-2-nitrobenzenesulfonamide[20,21] (NsNHBoc, 700 mg) and triphenylphosphine (TPP, 417 mg) in dry benzene (5 mL) was added diethyl azodicarboxylate (DEAD, 680 μL at 2.2 M in CH_2Cl_2), and the resulting mixture was stirred for 16 h at room temperature. The reaction was then quenched by the addition of water, and the mixture was extracted with ethyl acetate. The organic extract was washed with water, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography on silica gel (33% ethyl acetate in *n*-hexane) to afford **5** (120 mg, 69%). ^{1}H NMR δ ($CDCl_3$): 8.37 (1H, br), 8.35 (1H, br), 7.88 (1H, d, J = 2.5 Hz), 7.77 (1H, d, J = 3.5 Hz), 7.21 (1H, d, J = 8.5 Hz), 6.84 (1H, dd, J = 2.5, 8.5 Hz), 6.77 (1H, d, J = 3.5 Hz), 5.15 (2H, s), 3.48 (3H, s), 2.84 (2H, m), 2.73 (2H, t, J = 8.5 Hz), 1.24−2.45 (25H, m), 0.87 (3H, s). ESI-MS: m/z 669 $(M + H)^+$. Next, the three protective groups on the nitrogen and phenol were sequentially removed. First, the MOM group was removed by treatment with a mixture of trifluoroacetic acid (TFA)/water/THF (1:1:4, 3 mL) for 20 min at room temperature, and the crude product was purified by column chromatography on silica gel (17% ethyl acetate in *n*-hexane) to afford **6** (49.8 mg, 45%). ^{1}H NMR δ ($CDCl_3$): 8.33 (1H, br), 8.32 (1H, br), 7.77 (1H, d, J = 2.5 Hz), 7.75 (1H, d, J = 3.5 Hz), 7.17 (1H, d, J = 11 Hz), 6.64 (1H, dd, J = 2.5, 8.5 Hz), 6.62 (1H, d, J = 3.5 Hz), 4.51 (1H, s), 2.81 (2H, m), 2.73 (2H, t, J = 8.5 Hz), 1.24−2.56 (25H, m), 0.87 (3H, s). ESI-MS: m/z 625 $(M + H)^+$. Second, the Ns group was removed by stirring **6** with thiophenol (PhSH, 10 μL) and cesium carbonate (Cs_2CO_3, 78.2 mg) in DMF (1.2 mL) for 1 h at room temperature,[20,21] and the resultant product was purified by thin-layer preparative chromatography on silica gel (50% ethyl acetate in *n*-hexane) to afford **7** (24.5 mg, 70%). ^{1}H NMR δ ($CDCl_3$): 7.16 (1H, d, J = 9 Hz), 6.64 (1H, dd, J = 2.5, 8.5 Hz), 6.57 (1H, d, J = 3.5 Hz), 6.41 (1H, br), 4.51 (1H, s), 3.30 (3H, d, J = 7.3 Hz), 2.82 (2H, m), 1.21−2.46 (24H, m), 0.87 (3H, s). ESI-MS: m/z 440 $(M + H)^+$. Finally, the Boc group was removed with TFA (0.5 mL) after 10 min reaction at room temperature. The reaction mixture was then evaporated to afford **8** (24.2 mg) as the trifluoroacetate salt. ^{1}H NMR δ (CD_3OD): 7.07 (1H, d, J = 8.3 Hz), 6.54 (1H, dd, J = 2.7, 8.5 Hz), 6.47 (1H, J = 2.7 Hz), 3.30 (2H, m), 2.97 (2H, m), 2.79−2.86 (2H, m), 1.34−2.53 (13H, m), 0.86 (3H, s). ESI-MS: m/z 340 $(M + H)^+$.

Each E2 derivative was reacted with FITC to produce the fluorescein-labeled E2 derivatives (F-E2: Figure 2; **1c**, **1d**, and **2b**) in DMF containing 5% pyridine for 10−15 h at room temperature. Following purification by reversed-phase HPLC, the

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purified F-E2 derivatives were stored in ethanol after being quantified by the measurement of their absorption and fluorescence spectra for use in the ER binding assays.

Binding Assays. hrER-α was usually stored at −80 °C and had not been subjected to vortex mixing during handling.

Saturation binding assays using FP were performed to examine the affinities of the F-E2 with hrER-α. A 10-μL portion of the 5 μM F-E2 stock solution in ethanol was placed in a glass vial, and the solvent was removed using a dry nitrogen gas stream. The dried F-E2 was then redissolved with 1 mL of 10 mM Tris−HCl buffer (pH 7.4) containing 50 mM KCl, 10% glycerol, 0.1 mM dithiothreitol, 0.02% sodium azide, 1 μg/mL bovine gamma globulin, and diluted with the same buffer. Each concentration of hrER-α diluted with the buffer was added to 0.2 nM F-E2 (final concentration) in borosilicate test tubes to a final volume of 100 μL. After being allowed to stand for 1 h at room temperature, each sample was subjected to the FP method. The measured polarization values (P_{obs}) were transformed into the bound receptor concentrations (R_b) using the following equation,

$$R_b = ((P_{obs} - P_{min}) \times L_t)/(P_{max} - P_{min})$$

where, P_{max}, P_{min}, and L_t are the polarization values when the fluorescent ligand was completely bound and not bound to hrER-α and the total ligand concentration, respectively. The free receptor concentration (R_f) was calculated by subtracting R_b from the total receptor concentration. The bound ligand concentration (B) was equal to R_b, and the free ligand concentration (F) was calculated by subtracting B from L_t. On the basis of the above calculations, the Scatchard and the Hill plots were analyzed.

A competitive binding assay was carried out to investigate the ability of the unlabeled E2 (17β-estradiol) to displace the fluorescent ligand from the hrER-α−F-E2 complex. Unlabeled E2 as a competitor of the 10 mM stock solution in ethanol was also prepared as described above. hrER-α and F-E2 were then mixed and preincubated at 4 °C for 30 min to form the hrER-α−F-E2 complex, of which the final concentrations were 5 and 0.2 nM, respectively, followed by the addition of each concentration of unlabeled E2. After being allowed to stand for 1 h at room temperature, each sample was subjected to the FP method in triplicate. The pseudo-Hill plot was also analyzed as previously described.

RESULTS AND DISCUSSION

Selection of a Fluorophore under the Proposed Conditions. Twenty-two fluorophores (benzofurazans, cyanines, fluoresceins, lucifer yellow, and rhodamines) affording an excitation wavelength longer than 400 nm were tested in phosphate-buffered solution (pH 7.4). Those with good solubilities and strong fluorescences in aqueous solution were selected. Among them, fluorescein (λ_{ex} = 490 nm, λ_{em} = 520 nm) and tetramethylrhodamine (λ_{ex} = 540 nm, λ_{em} = 570 nm) were the best. Because tetramethylrhodamine was found to adsorb onto the vessels at concentrations below 20 nM, fluorescein was selected for further evaluation.

Synthesis of E2 Derivatives with Fluorescein (F-E2). Although the synthesis of the 17α-substituted E2 derivative with a spacer (C_6) having amino group had been reported,[13] we synthesized a new one with a shorter spacer (C_4), through which fluorescein was labeled. The shorter spacer provided an advantage in suppressing the "propeller effect",[22] an effect caused by the independent rotation of the fluorophore moiety attached to the ligand through the spacer. 6α-Substituted E2 derivatives were also synthesized as previously described[19] to compare the effect of the substituent group at the E2 position on the binding to hrER-α. Finally, the three fluorescein-labeled E2 derivatives were obtained as shown in Figure 2 (**1c**, **1d**, and **2b**).

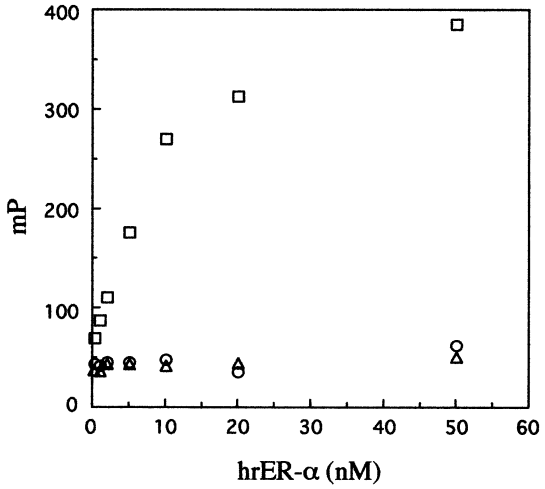

Figure 4. The binding isotherms of 6α- or 17α−F-E2 (0.2 nM) with the increasing concentration of hrER-α by the FP method: 6α−F-E2, **1c** (○); 6α−F-E2, **1d** (△); and 17α−F-E2, **2b** (□), as shown in Figure 2.

Binding Assay. Saturation Binding Assays for the Fluorescent Ligands to hrER-α. To delineate the interactions of the three F-E2 (Figure 2; **1c**, **1d**, and **2b**) with hrER-α, the FP values were determined by increasing the concentration of hrER-α with a fixed concentration of F-E2. The changes in the FP values caused by the interaction of each F-E2 with hrER-α were calculated. As shown in Figure 4, the FP values for 6α−F-E2 (Figure 2; **1c** and **1d**) in the concentration range of hrER-α from 0.1 to 50 nM remained constant at a low value. The results indicated that 6α−F-E2 (**1c** and **1d**) had low affinities to hrER-α and were inappropriate ligands for the ER binding assay. For 17α−F-E2 (Figure 2; **2b**), a significant increase in its FP value was observed according to the increase in the hrER-α concentration. The Scatchard plot analysis of the FP values was performed to obtain the dissociation constant (K_d) of 17α−F-E2 to hrER-α. As shown in Figure 5A, the shape of the Scatchard curve showed a typical convex curve, which suggested a positive cooperative binding; the binding of the first ligand to an oligomeric protein afforded an increase in the affinity of the protein to the additional ligands, namely, a homotropic allosteric effect. 17α−F-E2 might act as an allosteric effector to increase the affinity of the additional ligand, as observed for the tritium-labeled E2.[2,23,24]

Since the K_d value was not obtainable from the convex curve in the Scatchard curve, the data were transformed into a Hill plot

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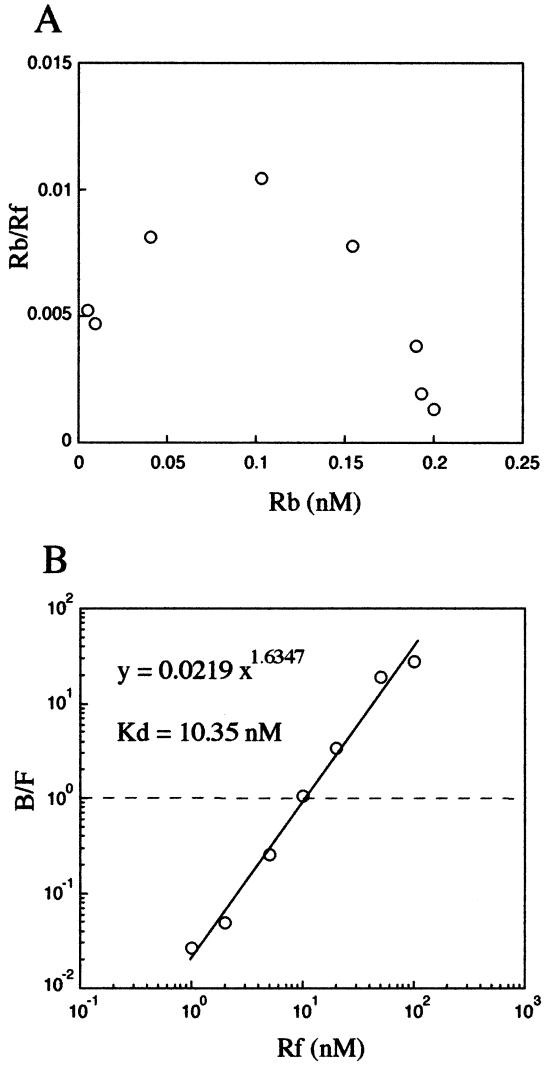

Figure 5. Scatchard (A) and Hill plot (B) analyses on the interaction of 17α−F-E2 (0.2 nM) with hrER-α using the proposed saturation binding study. Abbreviations: R_b and R_f, concentrations of bound and free receptor; *B* and *F*, concentrations of bound and free 17α−F-E2, respectively.

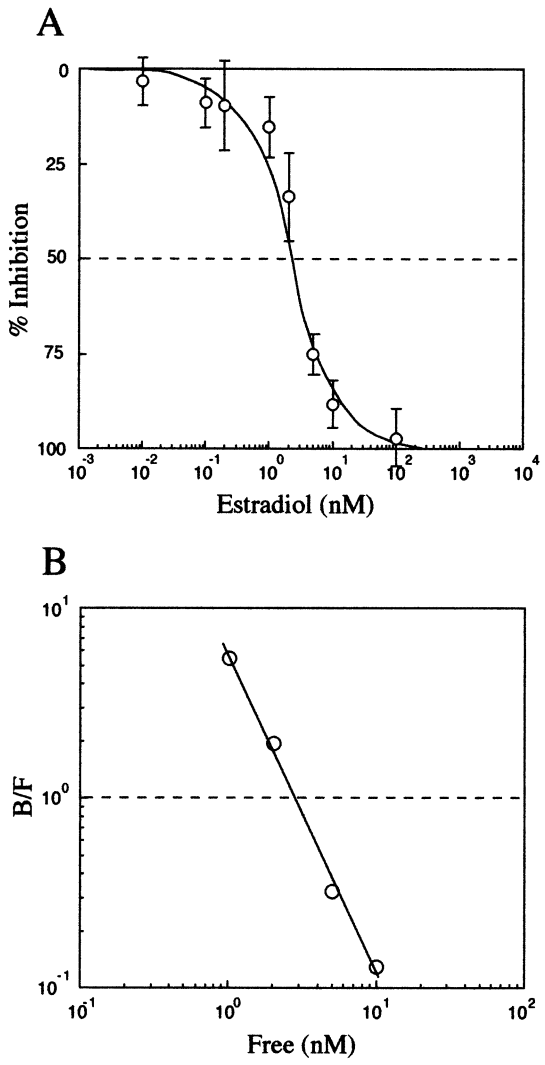

Figure 6. Inhibition curve (A) and pseudo-Hill plot analysis (B) of unlabeled E2 by the proposed competitive binding assay. Abbreviations: *B* and *F*, concentrations of bound and free 17α−F-E2, respectively.; Free, concentration of free competitor. The percentage of inhibition was calculated on the basis of the FP value for hrER-α−17α−F-E2 complex without a competitor as 0% inhibition (positive control) and for 17α−F-E2 (0.2 nM) alone as 100% inhibition (negative control). These results are the mean ± SD in triplicate.

analysis, which normally gives a linear curve. As shown in Figure 5B, the obtained K_d value was 10.4 nM, suggesting that it had the appropriate affinity to perform a competitive binding assay. The diethylstilbestrol (DES) derivative, with a K_d value of 0.3 nM,[18] was used as the fluorescent ligand. The high affinity of the DES derivative to hrER-α seemed to result in the use of high concentrations of the competitors to displace the derivative. Thus 17α−E2 was proved to be a more appropriate ligand than the DES derivative.

The Hill coefficient (*n*) obtained by the slope in Hill plot was an index of the degree of positive ($n > 1$) or negative cooperativity ($n < 1$). The Hill coefficient obtained was 1.63 (Figure 5B), which was consistent with the values reported in the previous papers using calf uterine ER[23,24] and hrER-α[2] obtained with the tritium-labeled E2. The present data also supported the results from a previous report[25] that hrER-α was an allosteric protein and formed a dimer with the conformational change induced by the binding with the ligand. To support the dimer formation of hrER-α, we took the bovine serum albumin conjugated with FITC ($M_w \approx$ 69 000) and Anti-FITC antibody ($M_w \approx$ 150 000) as the model proteins corresponding to the hrER-α monomer and then dimer and measured the fluorescence polarization. The values were ~200 and 400 mP, respectively (data not shown), suggesting that hrER-α formed a dimer by binding with the ligand.

Competitive Binding Assay for Unlabeled E2. We subsequently performed the competitive binding assay using 17α−F-E2 to determine the K_d value of the unlabeled E2 (17β-estradiol). An increase in the concentration of the unlabeled E2 as a competitor resulted in a decrease of the FP value (Figure 6). To determine the concentration of 50% inhibition (IC_{50}), the inhibition curve was analyzed. As shown in Figure 6A, the IC_{50} value of the unlabeled E2 was 2.82 ± 0.22 nM (mean ± SD). The relative standard deviations (RSD) at each point on the inhibition curve were 2.4−7.3% ($n = 3$). Next, the IC_{50} value of the unlabeled

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Table 1. Binding Parameters of Tested Compounds[a]

compd	IC_{50} (nM)	RBA (%)	Hill coeff
17β-estradiol (E2)	2.82 ± 0.22	100	1.67
diethylstilbestrol (DES)	2.35 ± 0.18	120	1.59
estrone	9.85 ± 0.84	28	0.99
estriol	4.95 ± 0.28	56	1.17
tamoxifen	165 ± 11	1.7	2.44

[a] Abbreviations: IC_{50}, concentration of 50% inhibition; RBA, relative binding affinities calculated on IC_{50} value of 17β-estradiol as 100%. IC_{50} are expressed in the mean ± SD.

E2 was converted into the K_i value by the Kenakin's correction[26] using the K_d value (10.4 nM) obtained for 17α−F-E2. The calculated K_i value of 0.65 nM was similar to the K_d value obtained by the tritium-labeled E2,[27] indicating that 17α−F-E2 was obviously displaced by the unlabeled E2 at the binding site of hrER-α and was the appropriate ligand to afford the K_i value. After the data were transformed into a pseudo-Hill plot analysis,[28] the Hill coefficient was obtained (Figure 6B). The value of 1.67 obtained from the slope of the linear curve was similar to that of 17α−F-E2 (1.63) in the saturation binding assay. The proposed competitive binding assay method was rapid (3 assays/3−4 h) and simple, as compared to previous reports[21,22] using a radioactive ligand that required a process of bound/free separation and took 10−20 h before the radioactivity measurement.

To explore the feasibility of the proposed competitive binding assay method, we employed another competitor estrone, a metabolite of E2 with a weaker estrogenic activity. Calculated from the inhibition curve and pseudo-Hill plot for the estrone, the IC_{50} value and Hill coefficient were 9.85 ± 0.43 nM and 0.99, respectively (Table 1). The Hill coefficient (close to 1), in agreement with that previously reported,[23] exhibited noncooperative binding. Since the present Hill coefficients were determined in a homogeneous medium similar to physiological conditions, the difference between E2 and estrone (1.67 and 0.99) supported the postulation that the binding of the two ligands induced different conformational changes in hrER-α.

As a fluorescent ligand developed in the present experiment, 17α−F-E2 had the appropriate affinity (K_d = 10.4 nM) and the strong fluorescence as described above. A low concentration range of 0.2 nM 17α−F-E2 was available in the present competitive binding assay as compared with the DES derivative (3 nM) as a fluorescent ligand.[18] Furthermore, the low concentrations of E2 or estrone were able to displace 17α−F-E2 from the 17α−F-E2−ER complex. As a result, the present IC_{50} values for E2 and estrone (2.82 ± 0.22 and 9.8 5± 0.84 nM, respectively) were lower than those in a previous report (13 and 626 nM, respectively)[18] using the DES derivative. Although the report that the A ring of estrogens (Ph-OH) mainly contributes to the binding of ER should be favorable for our data showing similar affinities of E2 and estrone for ER, the reason for the discrepancy is not yet clear.

We similarly examined estriol, DES, and tamoxifen using the proposed competitive binding assay method and analyzed their inhibition curves by the pseudo-Hill plot. These results are summarized in Table 1. Since most endocrine disruptors were hydrophobic, an organic solvent was usually required to dissolve and suppress the nonspecific binding to the experimental supports or hrER-α. The solvent, however, could potentially cause damage to hrER-α. For the present binding assays, in contrast, the organic solvent was not required, because nonspecific binding was not observed in their Scatchard and Hill plot analyses. The IC_{50} values for estriol, DES, and tamoxifen were 4.95 ± 0.28, 2.35 ± 0.18, and 165 ± 11 nM, respectively. These values were again lower than the previous report using the DES derivative as a fluorescent ligand (estriol, data not found; DES, 11; and tamoxifen, 423 nM),[18] indicating that the proposed competitive binding assay method can sensitively detect endocrine disruptors.

In conclusion, the proposed competitive binding assay method is a rapid, simple, and nonhazardous homogeneous method. A Hill plot analysis can yield insights into the binding manner between ER and chemicals used in the binding assays.

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