Binding Assays Using a Benzofurazan-Labeled Fluorescent Probe for Estrogen Receptor–Ligand Interactions

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Binding assays are widely used to study the estrogenic activity of compounds targeting the estrogen receptor (ER). The fluorescence properties of benzofurazan (BD), an environmentally sensitive fluorophore, have been used to characterize the ER-binding affinities of various environmental compounds. As an alternative method suitable for the screening of many endocrine disruptors, fluorescence polarization (FP)-based binding assays have been developed. The FP method is based on the principle that a fluorescent molecule, when excited with polarized light, will emit fluorescence with a degree of polarization inversely proportional to its rate of rotation. Small fluorescent molecules (e.g., probe) will rotate faster in solution than larger molecules, (e.g., ER-probe complex); hence, when the probe is bound to ER, its rotation is slowed, and the polarization value is increased. Therefore, the bound to free ligand ratio (bound/free) can be easily quantified according to the FP value without the need to evaluate separately bound and free ligand conditions. Although FP-based methods are well suited for high-throughput screening (HTS), they still require special instruments.

Commonly used fluorophores (such as fluorescein and rhodamine) are associated with several limitations, including the unstable fluorescence intensities and the alteration of excitation/emission spectra, depending on the molecular environment. Benzo[c]furan (2,1,3-benzoxadiazole [BD]) fluorophores are small molecules with large Stokes shifts. Additionally, BD derivatives have long excitation and emission wavelengths, ideal to prevent biomatrixes-derived interference. The fluorescence intensity of BD derivatives usually increases sharply as the solvent polarity decreases, although these compounds do not fluoresce in aqueous solution, their fluorescence intensity increases sharply in nonpolar solvents or hydrophobic environments, such as receptor binding sites. However, no studies have reported the use of BD derivatives in the context of ER binding affinity assays.

To overcome the existing methods/approaches’ disadvantages mentioned above, we attempted to design a fluorescent...
ligand that would be an effective and reliable reporter in ER binding assays. We synthesized a series of BD-labeled E2 derivatives and evaluated their binding affinities to ER via fluorescence intensity measurements. We further performed ER competitive binding assays with BD-labeled ligands and unlabeled estrogenic compounds.

**Results and Discussion**

**Preparation of BD-Labeled E2 Derivatives** First, we developed the fluorescent ligands for ER binding assays (Fig. 1). The benzofurazan-labeled estradiol derivatives (BD-E2, 2a–2c) were based on the structure of fluorescein-labeled estradiol (F-E2), 17 a 17α-substituted E2 derivative with a spacer containing an amino group. The fluorescent derivatization reagents were mixed with 17α-(4-aminobutynyl)estradiol, prepared as previously described (9; Chart 1). Following silica column chromatography and C18 solid-phase extraction, the purified BD-E2s were stored in MeOH after quantification via absorption spectra measurements.

The fluorescence properties of BD-E2s are described in Table 1. The Stokes shifts were approximately 120 (DBD-E2, ABD-E2) and 70 nm (NBD-E2). The effects of hydrophobicity on their fluorescence intensities was examined using various MeOH in water mixtures. As shown in Fig. 2, the fluorescence values (F<sub>obs</sub>) of BD-E2 compounds were increased with increasing MeOH proportions. From these results, we anticipated that the BD-E2 compounds’ fluorescence intensity increase occurred upon binding to ER owing to the more hydrophobic environment. Thus, the binding ability of BD-E2 to ER may probably be extrapolated based on the change of BD-E2 fluorescence intensity upon binding to the ER binding site.

**Saturation Binding Assay** To assess the interactions of ER and BD-E2, the F<sub>obs</sub> values were determined for different human recombinant (hr)-ERα concentrations and used to calculate the K<sub>d</sub> values. Figure 3 shows the results of the saturation binding assays using BD-E2s. The F<sub>obs</sub> values for DBD-E2 (2a) showed a sigmoid curve, dependent on the concentration of hr-ERα. The F<sub>obs</sub> values for DBD-E2 and ABD-E2 (2a and 2c) were lower than those of NBD-E2. However, F<sub>obs</sub> values were almost fully saturated around the hr-ERα concentration of 160 nM. A Scatchard plot analysis of the F<sub>obs</sub> values was performed to obtain the K<sub>d</sub> values for the BD-E2 compounds-hr-ERα interaction. The Scatchard curves for the three BD-E2 derivatives showed convex forms (Fig. 4A), suggesting a homotropic allosteric effect. However, since the K<sub>d</sub> values could not be obtained from the convex Scatchard curves, the data were transformed into Hill plots, which normally give linear curves (Fig. 4B). The obtained K<sub>d</sub> values of BD-E2 compounds were 32.0 (2a), 47.0 (2b), and 23.4 nM (2c), respectively. Compared with F-E2 (K<sub>d</sub> value for ERα of 10.4 nM), each BD-E2 compound showed a moderate affinity for hr-ERα. The Hill coefficients obtained from the Hill plots’ slopes were 0.73 (2a), 1.39 (2b), and 1.34 (2c), respectively. These results suggest that the Hill coeffi-

**Fig. 1.** Chemical Structures of 17α-Estradiol (1) and the Benzofurazan-Labeled Fluorescent Ligands 2a–2c

**Table 1.** Absorption and Emission Data of Benzofurazan-Labeled Compounds in Three Solvents

| Solvent   | MeCN | EtOH | MeOH |
|-----------|------|------|------|
|           | λ<sub>ex</sub> (nm) | λ<sub>em</sub> (nm) | λ<sub>ex</sub> (nm) | λ<sub>em</sub> (nm) | λ<sub>ex</sub> (nm) | λ<sub>em</sub> (nm) |
| DBD-E2 (2a) | 427 | 542 | 430 | 544 | 429 | 552 |
| NBD-E2 (2b) | 462 | 533 | 467 | 529 | 466 | 537 |
| ABD-E2 (2c) | 427 | 548 | 428 | 555 | 428 | 555 |

Reagents and conditions: (a) MOMCl, DIEA, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to room temperature, 16 h, 79%. (b) 3-butyln-1-ol THP ether, n-BuLi, THF, −78 °C to room temperature, 16 h, 68%. (c) CSA, MeOH, room temperature, 30 min, 94%. (d) NsNHBOc, Ph<sub>3</sub>P, DEAD, benzene, room temperature, 16 h, (e) PhSH, Cs<sub>2</sub>CO<sub>3</sub>, DMF, room temperature, 1 h, 94% for two steps. (f) TFA, room temperature, 20 min.

Chart 1. Preparation of the 17α-Substituted E2 Derivative 9
cients of BD-E2 compounds are related to the electron-accepting ability of the benzofurazan skeleton substituent group (R), as was reported elsewhere\(^{22}\) (NO\(_2\) > SO\(_2\)NH\(_2\) > SO\(_2\)N(CH\(_3\))\(_2\)). A Hill coefficient greater than 1 indicates positive cooperativity: binding of the first ligand induces structural changes to the protein, increasing its affinity for additional ligands. Previous studies using calf uterine-\(^{23,24}\) and hr-ER\(^{17}\) have shown that ER formed a dimer after the conformational changes induced by ligand binding, with a Hill coefficient of approximately 1.6. The Hill coefficients of compounds 2b and 2c were comparable with that of tritium labeled E2 or F-E2.

Competitive Binding Assay Ideally, the optimal fluorescent ligand should retain the binding affinity for the receptor and detectable fluorescence intensity. NBD-E2 and ABD-E2 (2b and 2c) fluorescence intensities were determined in the context of a fixed concentration of hr-ER\(\alpha\) (16 nM). The differences in \(F_\text{obs}\) values for the ER-ABD-E2 complex (0% inhibition) and free ABD-E2 (100% inhibition) were higher than those for NBD-E2 (data not shown). Thus, we selected ABD-E2 as the fluorescent ligand to use in competitive binding assays in order to confirm whether decreases in the fluorescence intensity were based on changes in environmental polarity, namely, the displacement from the ER binding site by the unlabeled compound ligand.

The ER affinity of test compounds was determined using ABD-E2 (2c) and hr-ER\(\alpha\). The competition binding curves are shown in Fig. 5A. The IC\(_{50}\) of each compound was calculated from the fluorescence intensity values and converted into the inhibition constant (\(K_i\)) value using the Kenakin’s correlation\(^{25}\) with the \(K_d\) value obtained for compound 2c. The calculated \(K_i\) value of 0.10 nM was similar to that previously reported using a radioactive ligand.\(^{11}\) This result suggested that compound 2c was obviously displaced from the hr-ER\(\alpha\) binding site by the unlabeled compound ligand. After the data were converted using pseudo-Hill plot analysis, the Hill coefficient was obtained (Fig. 6A). The Hill coefficient of 1.61 obtained for the unlabeled E2 binding to hr-ER\(\alpha\) was similar to that previously reported,\(^{17,18}\) suggested that the positive cooperativity of compound 2c induces the same hr-ER\(\alpha\) conformational changes.

Next, we examined the affinities of 10 compounds, including physiological estrogens, pharmaceuticals, and industrial chemicals, to hr-ER\(\alpha\) using competitive binding assays. The
obtained inhibition curves of the tested compound are shown in Figs. 5A–5C. Most of the tested compounds showed sigmoidal curves, suggesting that they displaced ABD-E2 from the hr-ERα. The inhibition parameters of the tested compounds are summarized in Table 2. The relative binding affinities (RBAs) of these compounds, which were calculated on the basis of the IC_{50} value of E2 were easily compared with the values reported from other sources. For physiological estrogens, the order of competition was as follows: E2 > estriol (E3) > estrone (E1) > 17α-estradiol (17α-E2). The current IC_{50} values of E1 and E3 (2.53 and 2.43 nM, respectively) were lower than those reported in a previous study (9.9 and 5.0 nM, respectively) using F-E2.\(^{17,18}\) The order of
competition for pharmaceuticals and industrial chemicals was as follows: diethylstilbestrol (DES) > ethynylestradiol (EE2) > 4-hydroxytamoxifen (4-OH-TAM) > tamoxifen (TAM) > 4-nonylphenol (4-NP) > bisphenol A (BPA). Overall, the order of affinity of these compounds (obtained by competition with ABD-E2) was well correlated with the results obtained by the conventional method using FP (Fig. 7A).

In a previous report, the relationship between estrogenic compounds’ chemical structures and their estrogenic activities were examined, and the molecules were classified into three categories (agonists, partial agonists, and antagonists) based on their Hill coefficients. Because we found that all tested compound competed with compound 2c, we assessed the Hill coefficients obtained from pseudo-Hill plots (Figs. 6A–6C). DES and EE2 are strong estrogenic compounds, as indicated by their Hill coefficients of 1.80 and 1.34, respectively. The magnitude of Hill coefficient values opposed that obtained using F-E2 (1.59 and 1.73, respectively). However, we consider that these results were attributed to differences in ligand structures. In contrast, TAM and 4-OH-TAM are anti-estrogens that are used for the treatment of estrogen-dependent breast cancer; the Hill coefficient of TAM was found to be 3.42. This value increased by 40% compared with that obtained in the context of F-E2 competition, but met the criteria for the antagonist category according to the Hill coefficient proposed by Ohno et al. Therefore, and overall we considered that our results of competitive binding assays using compound 2c, reproduced those published previously. The correlations of Hill coefficients obtained with compounds 2c and F-E2 are shown in Fig. 7B. Although we observed a clear correlation between the values obtained by fluorescent measurements and those obtained by FP, further studies of the binding mechanisms of BD-E2 to ER are needed to evaluate the transcriptional activities, namely, agonism/antagonism of ER.

### Conclusion

In this study, we exploited the structural and binding properties of known fluorescein-labeled E2 compounds and developed novel fluorescent ligands (BD-E2) destined to ER binding assays. Although BD-E2 compounds yield little fluorescence in aqueous solution, they exhibit spectroscopic properties with high fluorescence intensities and large Stokes shifts in a hydrophobic environment. By evaluation of the bound ligands based on changes in the fluorescence intensity, we established a simple, rapid, reliable ER binding assay. The binding properties obtained through this fluorescence-based ER binding assay indicated good correlations with the results obtained by FP analysis and conventional methods using radioactive ligands. On the other hands, it is anticipated that the enhancement of the benzofurazan skeleton electron-accepting ability will contribute to the increase of BD-E2 compounds’ binding affinity to ER. We expect that this approach will be a valuable tool not only for HTS of estrogenic compounds but also for studies of drug discovery or cell imaging.

| Compound                        | IC50 (nM) | RBA (%) | Log RBA | Ki (nM) | Hill coefficient |
|---------------------------------|-----------|---------|---------|---------|-----------------|
| 17β-Estradiol (E2)              | 2.24      | 100     | 2.00    | 0.11    | 1.61            |
| Estrone (E1)                    | 2.53      | 88.71   | 1.95    | 0.13    | 0.93            |
| Estriol (E3)                    | 2.43      | 92.33   | 1.97    | 0.12    | 1.23            |
| 17α-Estradiol (17α-E2)          | 2.70      | 82.99   | 1.92    | 0.13    | 1.46            |
| Ethynylestradiol (EE2)          | 2.23      | 100.54  | 2.00    | 0.11    | 1.34            |
| Diethylstilbestrol (DES)        | 1.64      | 136.64  | 2.14    | 0.08    | 1.80            |
| Bisphenol A (BPA)               | 377.56    | 0.59    | −0.23   | 18.71   | 0.95            |
| 4-α-Nonylphenol (4-NP)          | 347.96    | 0.64    | −0.19   | 17.24   | 1.02            |
| Tamoxifen (TAM)                 | 51.04     | 4.39    | 0.64    | 2.53    | 3.42            |
| 4-Hydroxytamoxifen (4-OH-TAM)   | 16.70     | 13.43   | 1.13    | 0.83    | 3.60            |

Fig. 7. Correlations between the Binding Affinity (A) or Hill Coefficient (B) Obtained for hr-ERα and Compound 2c Interactions, and the FP Method Using F-E2

The log RBA and Hill coefficient obtained with compound 2c and those obtained with F-E2 are plotted on the X and Y axes, respectively.
Experimental

Materials Commercial materials and solvents were used without further purification. 17β-Estradiol, estrone, estradiol, 4-nitro-7-fluoro-2,1,3-benzoxadiazole (NBD-F), 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DDBD-F), DES, and 2,2-bis(4-hydroxyphenyl)propane (BPA) were all purchased from Tokyo Chemical Industry (Tokyo, Japan). 4-Fluoro-7-sulfamoylbenzofurazan (ABD-F) and 4-(tert-butoxycarbonyl)-2-nitrobenzenesulfonamide27,28 (NsNHBOc, 700.2 mg) and triphenylphosphine (Ph,P, 417.1 mg) in dry benzene (5 mL) was added to diethyl azodicarboxylate (DEAD, 685 μL at 40% in toluene), and the reaction mixture was then stirred at room temperature for 16 h. The reaction was quenched by the addition of water, and the mixture was diluted with EtOAc. The organic layer was washed with water, dried over Na2SO4, filtered, and evaporated. The crude sample was removed by flash chromatography (hexane:EtOAc, 2:1) to originate 7 (561.5 mg) as a colorless solid. Next, benzene thiol (PhSH, 105 μL) and cesium carbonate (Cs2CO3, 826.2 mg) were added to the solution of 7 in N,N-dimethylformamide (DMF, 10 mL), and the mixture was stirred at room temperature for 1 h. The reaction was quenched by the addition of water, and the mixture was extracted with EtOAc. The organic layer was then washed with 5% HCl, sat. NaHCO3 solution, and water; dried over Na2SO4; filtered; and evaporated. The residue was purified by flash chromatography (hexane:EtOAc, 3:1) to obtain 8 (119.3 mg, 94%) as a pale-yellow oil. 1H-NMR (CDCl3): δ (ppm) 0.87 (3H, s), 1.42 (9H, s), 2.45 (2H, t, J = 6.6 Hz), 3.29 (2H, t, J = 5.9 Hz), 4.79 (1H, brs), 6.51 (1H, d, J = 2.9 Hz), 6.63 (1H, dd, J = 8.4, 2.9 Hz), 7.16 (1H, d, J = 8.4 Hz).

Preparation of BD-E2 Derivatives The fluorescent ligands (BD-E2 derivatives) were synthesized by reaction with 7-fluoro-2,1,3-benzoxadiazole (BD-F) reagent using the two procedures described below.

Procedure A: 9 (0.04 mmol) was dissolved in pyridine (0.5 mL). After the addition of BD-F (5.7 mg) in MeCN (1.1 mL), the reaction mixture was warmed at 60 °C for 4 h. The mixture was cooled, added to 5% HCl, and extracted with EtOAc. The organic layer was washed with sat. NaHCO3 solution, and water, dried over Na2SO4, filtered, and evaporated. After purification by preparative TLC (hexane:EtOAc, 1:1), a yellow oil was obtained, which was further purified by solid-phase extraction using Agilent Bond Elut C18 (200 mg, 3 mL) with MeCN–water to give BD-E2 compounds. 1H-NMR (CDCl3): δ (ppm) 0.86 (3H, s), 2.40 (2H, t, J = 6.6 Hz), 3.10–3.20 (2H, m), 4.80–4.90 (1H, brs), 6.47 (1H, d, J = 2.4 Hz), 6.54 (1H, dd, J = 8.3, 2.7 Hz), 7.08 (1H, d, J = 8.3 Hz).

Preparation of 17α-Substituted E2 Derivative The overview is represented in Chart 1. The solution of estrone (3, 998.0 mg, 3.69 mmol) in dry CH2Cl2 (10 mL) was added to methoxyethyl chloride (MOMCl, 806 μL) and N,N-disopropyl ethylamine (DIEA, 973 μL) at 0 °C. Then, the reaction mixture was stirred and warmed to room temperature. After 16 h, the mixture was diluted with diethyl ether. The organic layer was washed with water, dried over Na2SO4, and filtered, and evaporated. The residue was purified by flash chromatography (hexane:EtOAc, 6:1) to originate 4 (916.8 mg, 79%) as a colorless solid. 1H-NMR (CDCl3): δ (ppm) 0.91 (3H, s), 3.48 (3H, s), 5.15 (2H, s), 6.79 (1H, d, J = 2.7 Hz), 6.85 (1H, dd, J = 8.5, 2.7 Hz), 7.21 (1H, d, J = 8.3 Hz).

3-Butyn-1-ol tetrahydropyran (THP) ether29 (972.0 mg, 6.31 mmol) in dry tetrahydrofuran (THF; 4 mL) was added to n-BuLi (3.8 mL at 1.6 M in n-hexane) at −78 °C under nitrogen gas stream. After stirring for 1 h, the solution of 4 in dry THF (15 mL) was added to the reaction mixture and stirred for 16 h in order to slowly warm the solution to room temperature. The reaction was quenched by the addition of water, and the mixture was extracted with diethyl ether. The organic layer was washed with water, dried over Na2SO4, and evaporated. The crude product was purified by flash chromatography (hexane:EtOAc, 6:1) to obtain 5 (928.3 mg, 68%) as a pale-yellow oil. 1H-NMR (CDCl3): δ (ppm) 0.86 (3H, s), 2.56 (2H, t, J = 6.9 Hz), 3.48 (3H, s), 3.54 (2H, m), 3.85 (2H, m), 4.66 (1H, brs), 5.15 (2H, s), 6.77 (1H, d, J = 2.7 Hz), 6.83 (1H, dd, J = 8.5, 2.7 Hz), 7.21 (1H, d, J = 8.5 Hz).
17α-[4-(N,N-Dimethylaminosulfonyl)-7-nitrobenzo-2-oxa-1,3-diazole-4-yl]amino-1-butynyl]estra-1,3,5(10)-trien-3-ol (DDB-E2, 2a) Yellow solid (0.4% by procedure A). 

\[ \text{1H-NMR (CDCl}_3\]: \delta (ppm) 0.96 (3H, s), 2.76 (2H, t, \( J = 6.51 \text{Hz} \)), 3.64 (2H, brs), 6.19 (1H, d, \( J = 8.1 \text{Hz} \)), 6.56 (1H, d, \( J = 2.7 \text{Hz} \)), 6.63 (1H, dd, \( J = 8.4, 2.8 \text{Hz} \)), 7.15 (1H, d, \( J = 8.51 \text{Hz} \)), 7.91 (1H, d, \( J = 7.8 \text{Hz} \)). FAB-MS m/z: 565 ([M + H]⁺), 587 ([M + Na]⁺).

HR-MS m/z: 565.2261 (Calcd for \( \text{C}_{30}\text{H}_{36}\text{N}_{4}\text{O}_{5}\text{S} \): 565.2485).

17α-[4-(7-Nitrobenzo-2-oxa-1,3-diazole-4-yl]amino-1-butynyl]estra-1,3,5(10)-trien-3-ol (NBD-E2, 2b) Yellow solid (1% by procedure B). 

\[ \text{1H-NMR (CDCl}_3\]: \delta (ppm) 0.77 (3H, s), 2.77 (2H, t, \( J = 6.4 \text{Hz} \)), 3.72 (2H, brss), 6.43 (1H, d, \( J = 2.6 \text{Hz} \)), 6.45 (1H, d, \( J = 8.8 \text{Hz} \)), 6.50 (1H, dd, \( J = 8.4, 2.6 \text{Hz} \)), 6.88 (1H, d, \( J = 8.8 \text{Hz} \)), 8.48 (1H, d, \( J = 8.4 \text{Hz} \)). FAB-MS m/z: 503 ([M + H]⁺), 525 ([M + Na]⁺).

HR-MS m/z: 503.2301 (Calcd for \( \text{C}_{28}\text{H}_{30}\text{N}_{4}\text{O}_{5} \): 503.2294).

Spectroscopic Measurement The purified BD-E2 derivatives were quantified by measurement of their absorption spectra, compared with model compounds as follows: DDB-NMe₂ in MeOH (\( \varepsilon = 1.06 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \) at 442 nm), NBD-NHMe in MeCN (\( \varepsilon = 2.30 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \) at 458 nm), and ABD-NHMe in MeCN (\( \varepsilon = 1.49 \times 10^4 \text{ M}^{-1} \text{cm}^{-1} \) at 426 nm).

Fluorescence spectra of the derivatives were measured using various solvents (0.1–0.5 \( \mu \text{M} \)) at room temperature. The emission spectra were obtained by excitation at the maximum absorption wavelength of the fluorophores.

Estrogen Receptor Binding Assay To perform the binding assays of BD-E2 compounds and hr-ERα, full-length ERα was purchased from ThermoFisher Scientific (MA, U.S.A.). hr-ERα was stored at \(-80^\circ \text{C} \) and was not subjected to vortex mixing during handling. The buffer solution for the fluorescence measurements was 10 mM Tris–HCl buffer (pH 7.4) containing 50 mM KCl, 0.1% glycerol, 0.1 mM dithiothreitol, 0.02% sodium azide, and 1 \( \mu \text{g}/\text{ml} \) bovine \( \gamma \)-globulin. All BD-E2s and competing compounds were prepared as standard solutions in MeOH, and the solvent was removed using a dry nitrogen gas stream. The fluorescence intensities were observed with excitation at 428 (2a, 2c) or 465 nm (2b) and emission at 546 (2a, 2c) or 533 nm (2b).

Saturation Binding Assay Direct binding studies were performed to examine the affinity of BD-E2 with hr-ERα. The final concentration of BD-E2 was fixed to 1 nM, and the final concentration of hr-ERα varied from 0.8 to 160 nM (0.8, 1.6, 4, 6, 8, 16, 40, 60, 80, 120, and 160 nM). Each sample volume was 100 \( \mu \text{L} \). Equal volumes of BD-E2 and hr-ERα at different concentrations were added to 96-well black plates, to a total 100 \( \mu \text{L} \) in triplicate. After being allowed to stand at room temperature for 1 h, the fluorescence intensity of each sample was measured. The average of the measured fluorescence values \( (F_{\text{obs}}) \) was transformed into the bound ERα \( (R_b) \) using the following equation:

\[ R_b = \left( \frac{F_{\text{obs}} - F_{\text{max}}}{F_{\text{obs}} - F_{\text{min}}} \right) \times L_f \]

where \( F_{\text{max}} \), \( F_{\text{min}} \), and \( L_f \) are the fluorescence values for BD-E2 completely bound to hr-ERα, BD-E2 without hr-ERα (negative control), and the total ligand concentration, respectively. Free hr-ERα \( (R_f) \) 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 \( L_f \). On the basis of the above calculations, Scatchard and Hill plots were designed and analyzed.

Competitive Binding Assays Competitive binding studies were performed to evaluate the ability of the tested compounds to displace BD-E2 compounds from ER. As the concentration of the competitor increased, fluorescence intensities were observed. The purified BD-E2 compounds were fixed to 16 and 10 nM, respectively. Each sample volume was 100 \( \mu \text{L} \). Initially, hr-ERα and BD-E2 were mixed in glass test tubes to form the ER/BD-E2 complex. Competitor solutions of different concentrations were prepared in 96-well black plates, and ER/BD-E2 solution was then added. After being allowed to stand at room temperature for 1 h, the fluorescence intensity of each sample was measured. The fluorescence values were converted to percent inhibition using the following equation:

\[ \% \text{Inhibition} = \left( \frac{F_0 - F_{100}}{F_{100} - F_{100}} \right) \times 100 \]

where \( F_0 \) and \( F_{100} \) are the fluorescence values for the ER/BD-E2 complex without competitor as 0% inhibition (positive control) and for BD-E2 alone as 100% inhibition (negative control), respectively. The percent inhibition versus competitor concentration curves were analyzed by nonlinear least-squares curve fitting and yielded IC₅₀ values. The RBA for each competitor was calculated by dividing the IC₅₀ value of E2 by the IC₅₀ of the competitor and was expressed as a percent. Pseudo-Hill plots were also analyzed as previously described. The concentrations of bound competitor (\( B_b \)) and free competitor (\( F_c \)) were converted to \( B/F \) ratios as follows:

\[ B_t = \% \text{Inhibition} / 100 \times B_f \]

\[ F_c = C_t - B_t \]

\[ B/F = (B_t - B_f) / B_c \]

where \( B_t \) and \( C_t \) are the bound ligand and total competitor concentrations, respectively.

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