Rapid Screening of Environmental Chemicals for Estrogen Receptor Binding Capacity

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Over the last few years, an increased awareness of endocrine disrupting chemicals (EDCs) and their potential to affect wildlife and humans has produced a demand for practical screening methods to identify endocrine activity in a wide range of environmental and industrial chemicals. While it is clear that *in vivo* methods will be required to identify adverse effects produced by these chemicals, *in vitro* assays can define particular mechanisms of action and have the potential to be employed as rapid and low-cost screens for use in large scale EDC screening programs. Traditional estrogen receptor (ER) binding assays are useful for characterizing a chemical's potential to be an estrogen-acting EDC, but they involve displacement of a radioactive ligand from crude receptor preparations at low temperatures. The usefulness of these assays for realistically determining the ER binding interactions of weakly estrogenic environmental and industrial compounds that have low aqueous solubility is unclear. In this report, we present a novel fluorescence polarization (FP) method that measures the capacity of a competitor chemical to displace a high affinity fluorescent ligand from purified, recombinant human ER-α at room temperature. The ER-α binding interactions generated for 15 natural and synthetic compounds were found to be similar to those determined with traditional receptor binding assays. We also discuss the potential to employ this FP technology to binding studies involving ER-β and other receptors. Thus, the assay introduced in this study is a nonradioactive receptor binding method that shows promise as a high throughput screening method for large-scale testing of environmental and industrial chemicals for ER binding interactions. *Key words*: competition binding, endocrine disruptor screening, estrogen, estrogen receptor, fluorescence polarization. *Environ Health Perspect* 106:551-557 (1998). [Online 6 August 1998]
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Reports in the last few years describing apparent increases in developmental, reproductive, or behavioral abnormalities as well as certain types of cancer in wildlife (*1–4*) or humans (*5–7*) have resulted in a worldwide intensification of research efforts to characterize endocrine disrupting chemicals (EDCs). Even though there are few, but significant, examples of a direct link from exposure to an EDC to adverse effects in humans (*8–10*), many of the mechanistic pathways mediating the deleterious effects resulting from EDC exposure of wildlife and laboratory animals have been elucidated (*1,11–15*). As a result, screening for endocrine activity has recently been mandated in the United States by the Food Quality Protection Act (Public Law 104-170) and the Safe Drinking Water Act amendments (Public Law 104-182) of 1996. Recent workshops have clarified the definition of EDCs (*16*), identified research needs (*16–18*), and reviewed potential EDC screening methods (*19–22*). While it is clear that a spectrum of *in vivo* screens may be required to characterize a compound as an EDC that can cause adverse effects in exposed organisms, *in vitro* assays are required to define the molecular mechanisms responsible for these effects. The enormous task of screening tens of thousands of natural and man-made chemicals for EDC activity suggests that *in vitro* assays are the most practical means to quickly identify compounds which may have the potential to cause adverse endocrine disruption effects in whole organisms.

Many of the adverse effects and biochemical mechanisms of action of EDCs identified over the last 20 years have been attributed to environmental estrogens (*11,23–30*). These substances mimic or block the activity of natural estrogens by specifically binding to the estrogen receptor (ER) nuclear protein, resulting in the transcriptional control of a variety of genes in target cells. ER-α is a 66 kDa transcription factor that regulates expression of genes involved in tissue growth and differentiation, functioning in diverse target tissues including reproductive, skeletal, cardiovascular, and mammary tumors (*31,32*). ER and other steroid hormone–receptors are activated by one or more endogenous ligands that bind with high affinity to the receptor's carboxy-terminal hormone binding domain. Ligand binding initiates a number of changes in the receptor including altered conformation, dimerization, and changes in interaction with other proteins (*33*). The hormone–receptor complex, with or without the involvement of other transcriptional accessory proteins, binds to DNA response elements through the receptor's DNA binding domain, thereby inducing or suppressing transcription of the target genes (*34–36*). Competitive inhibitors of ER (antiestrogens; e.g., tamoxifen), have been developed by the pharmaceutical industry to treat hormonally responsive breast cancer that blocks ER action in target tissues (*37,38*). The recent cloning of a second estrogen receptor, ER-β, led to the discovery that it and the classic estrogen receptor ER-α differ in their affinity for some estrogens and respond to ligand binding in different ways, depending on the DNA response element involved (*39*). For instance, 17β-estradiol (E_2) bound to ER-α enhanced transcription through the AP-1 response element regulated reporter; however, when E_2 interacted with ER-β, transcription of the same reporter was minimal. These studies highlight the complexity of ER interactions with hormone and DNA and shed light on the variety of subsequent downstream effects that might be observed in tissues sensitive to natural and synthetic estrogenic compounds (*40*). Thus, *in vitro* screening methods that evaluate the ability of a compound to bind ER and/or modulate ER-mediated gene regulation events are useful for identifying environmental estrogens (*21,41*).

Several methods are used to follow ligand–ER binding interactions (*20,22,42,43*). All are competitive assays in which the test compound displaces a receptor-bound probe molecule, usually radioactive E_2. While there are compelling reasons why these assays are well suited for large-scale screening of ER competitors, they suffer from a number of

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important shortcomings, including the use of crude receptor preparations from cells or tissue, binding incubations at subphysiological temperatures, and the use of radioisotopes. Furthermore, these assays include unbound tracer separation/wash steps and are relatively time intensive. While such binding assays have been useful for characterizing estrogenic compounds with relatively high ER affinity and aqueous solubility, they are not necessarily suitable for screening compounds that may bind only weakly to the ER and have limited solubility. One approach to address these problems was the development of scintillation proximity assays in which the ER and the scintillant are bound to a solid phase, either a bead or a plate, so that only the receptor-bound radioactive E_2, not the excess free tracer, is close enough to excite the scintillant (*44*). This approach eliminates the need to separate free from bound ligand, but still relies upon radioisotopes and immobilization of the receptor, which could cause disadvantageous conformational changes. Furthermore, if antibodies are used to immobilize the ER, another possible interference source is introduced to the assay.

In this report, we describe a new estrogen/ER competition binding assay and its application to the rapid screening of environmental compounds for ER binding activity. This assay uses fluorescence polarization (FP) to monitor the displacement of a high affinity fluorescent ligand from purified recombinant human ER (*45*). Furthermore, this method can be run at physiological temperatures, requires less than half a day to complete, and involves no radioactivity. We present ER binding determinations made using this method for 15 natural and synthetic compounds, and compare each to ER binding affinity reported by others using traditional radioligand assays and ER from various sources. Test compounds include the endogenous steroids E_2, estrone, and dihydrotestosterone (DHT); the antiestrogen tamoxifen; the pesticides DDT, methoxychlor, kepone, and dieldrin; and the industrial chemicals bisphenol A, butyl benzyl phthalate, and certain alkylphenols (Fig. 1).

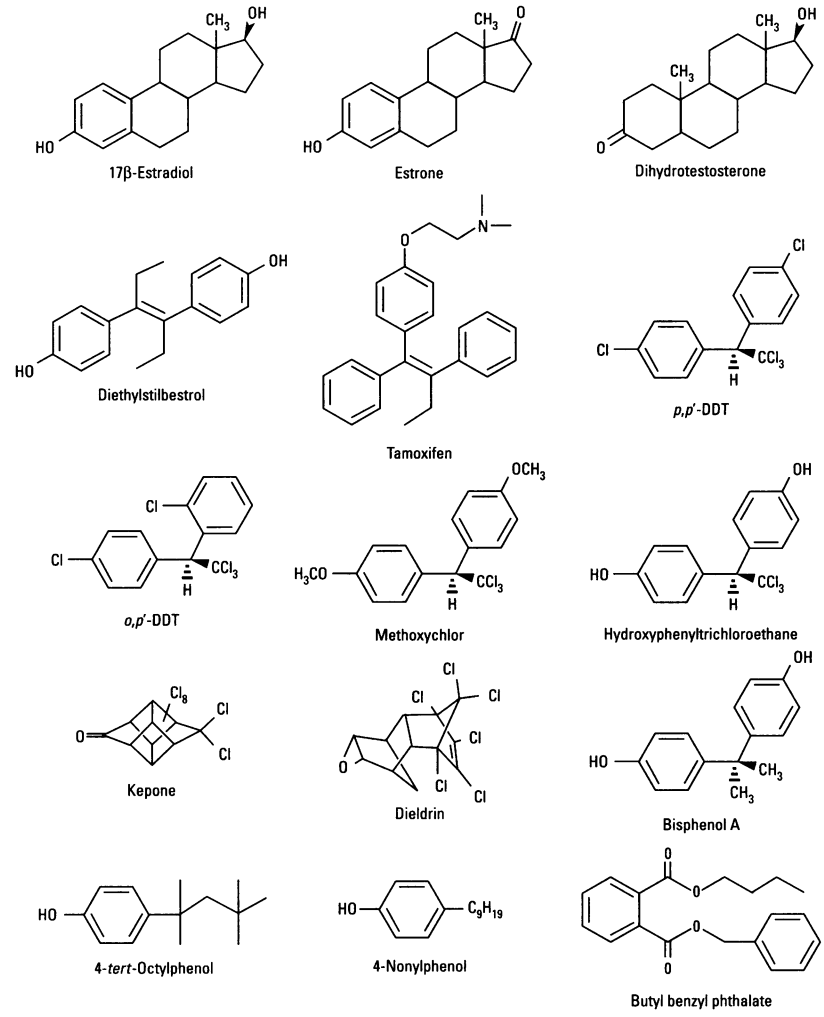

Figure 1. Structures and abbreviations of all natural and synthetic compounds used in the estrogen receptor binding study.

Materials and Methods

Method theory. FP is used to study molecular interactions by monitoring changes in the apparent size of fluorescently labeled or inherently fluorescent molecules (*45–50*). The binding of a fluorescent molecule to another molecule can be quantified by the change in its speed of rotation, and FP is a measure of that speed or tumbling rate. When plane-polarized light is used to excite a solution of fluorescent molecules, those molecules parallel to the plane will become excited. If the molecules remain stationary during the period of excitation (4 nanoseconds for fluorescein) the emitted light will remain highly polarized. If the molecules tumble during the period of excitation, the emitted light will be random or depolarized. Polarization and the mathematically related parameter anisotropy are a measure of the tumbling rate of the fluorescent molecule and are directly related to its molecular volume (*45–50*). An increase in the volume of a fluorescent molecule (e.g., through its binding to a receptor or antibody) or a decrease in molecular volume (due to dissociation or enzymatic degradation) can be measured directly by FP. The observed value is a weighted average of the polarization values of the individual bound and free fluorescent molecules and is therefore a direct measure of the fraction bound. The polarization value is easily converted into the concentration of bound ligand, and the resultant bound versus free isotherm is analyzed like those generated by conventional techniques such as radioactivity (*45–50*).

The primary components of this assay are human recombinant ER and an intrinsically fluorescent nonsteroid estrogen [Fluormone ES1 (FES 1); PanVera Corporation, Madison, WI] that binds to ER with high affinity and was developed by Katzenellenbogen and colleagues (*51*). This ligand was chosen because of its high affinity, stable quantum yield in various solvents, and very low binding in the absence of ER. While methods to obtain ER from natural sources have been known for some time, full-length human recombinant ER (hrER) was first expressed by Brown and Sharp in 1990 (*52*). These studies were expanded by Obourn et al. (*53*), who showed that hrER made with baculovirus in insect cells compared favorably to receptor isolated from tissue preparations in terms of size, immunogenicity, hormone binding characteristics, phosphorylation state, DNA interactions in gel shift

assays, and thymine interference footprinting. hrER interacts normally with its DNA response element, further strengthening its use as a competent alternative to ER purified from animal tissues (*54,55*). The hrER produced in this laboratory using baculovirus has greater than 80% purity (data not shown). Using standard hydroxyapatite [^{3}H] estradiol binding assays, we have calculated a dissociation constant (K_d) between 0.1 and 0.5 nM hrER and observed cooperative binding at higher ER concentrations. These results are comparable to those obtained with ER purified from natural sources (unpublished observations).

Materials. Human recombinant estrogen receptor-α and β (hrER-α and hrER-β) and FES1 were obtained from PanVera Corporation. The 96-well microtitre plates were obtained from Costar (Acton, MA). All competing compounds were prepared at a standard concentration of 10 mM in ethanol: E_2, estrone, and methoxychlor (98% pure) were from Sigma, St. Louis, Missouri; diethylstilbesterol (DES; 99% pure), and dihydrotestosterone (DHT; 99% pure) were from Steraloids, Inc., Wilton, New Hampshire; *o,p'*-DDT (R/S isomer mix; 99% pure) was from Crescent Chemical Co., Hauppauge, New York; chlordecone (kepone; 87.5% pure) and dieldrin (98.8% pure) were from the EPA Chemical Repository stock, Research Triangle Park, North Carolina; bisphenol A (99% pure), butyl benzyl phthalate (BBP; 98% pure), *p,p'*-DDT (98%), and 4-*tert*-octylphenol (97%) were from Aldrich, Milwaukee, Wisconsin. 4-Nonylphenol (p-isomer mix; 85% pure) was obtained from Fluka Chemie AG, Buchs, Switzerland. Hydroxyphenyltrichloroethane (HPTE; 99% pure) was synthesized (*56*) by W.R. Kelce (Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, EPA, Research Triangle Park, NC).

FES1-hrER direct binding experiment. Recombinant hrER-α was serially diluted from 200 nM to 0.391 nM in screening buffer (40 mM Tris-HCl, pH 7.5; 50 mM KCl; 5% glycerol; 10% dimethylformamide; 0.02% sodium azide; 50 µg/ml bovine gamma globulin) to a final volume of 100 µl in borosilicate test tubes. FES1 was added to each tube (final concentration 2 nM), followed by mixing. After a 1-hr incubation (equilibrium was reached after 60 min; data not shown) at room temperature, the fluorescence anisotropy of each tube was measured on a Beacon 2000 Fluorescence Polarization Instrument (PanVera Corporation) with 360 nm excitation filter and 530 nm emission filter. Anisotropy at each ER concentration was then converted to fraction bound ligand using the following equation:

$$F_b = (A-A_f)/(A_b-A_f),$$

where F_b = fraction of ligand bound, A = observed anisotropy, and A_b and A_f = anisotropy values of the bound and free ligand, respectively (*45–50*). Bound ER (ER_b) was assumed to be equal to bound ligand (L_b), and therefore calculated by multiplying F_b by the total ligand concentration (L_t). Free ER (ER_f) was calculated by subtracting ER_b from ER_t. The equilibrium K_d was calculated from the ER-bound versus ER-free isotherm using a nonlinear least-square curve fitting program (Prism, Graphpad Inc., San Diego, CA). We did not observe nonspecific binding of the FES1 to glass or plastic tubes or to ER. In the case of glass, free FES1 has a very low polarization value, consistent with its unhindered mobility. Binding of FES1 to the test tube (leading to immobilization and removal from the light path) would have dramatically raised the polarization value and decreased the fluorescence intensity. In the case of plastic, we could not detect a nonspecific binding component in the binding isotherms. We analyzed the curves by nonlinear least-squares regression using models with and without a nonspecific binding term and found no difference between the analysis. Indeed, it is a hallmark of FP assays that nonspecific binding is largely eliminated because the assay does not depend on a solid support surface.

Competitive binding studies. Fifteen compounds were tested for their ability to displace the fluorescent ligand ES1 from an ER–ES1 complex. The large ER–ES1 complex tumbles slowly and therefore has a high anisotropy value. As increasing concentrations of a competing ligand displace the ES1 from the complex, the free ES1 molecules tumble more rapidly and have a low anisotropy value. The measured anisotropy is a weighted average of the bound and free ES1 molecules. As more ES1 molecules are displaced from the complex, the measured anisotropy approaches the free anisotropy value.

Specifically, a 200-µM working stock of each competing test compound was prepared from the original 10-mM ethanol stock and was serially diluted in triplicate in screening buffer to the desired concentrations. hrER-α and FES1 were added to a final concentration of 2 nM and 3 nM, respectively. Negative (ER + FES1, equivalent to 0% inhibition) and positive (free FES1, equivalent to 100% inhibition) controls in the absence of competitor were measured in triplicate. After 60 min at room temperature, the anisotropy values in each tube were measured using the Beacon 2000 system. The anisotropy values were converted to percent inhibition using the following formula: $I_{\%} = (A_0-A)/(A_0-A_{100}) \times 100$, where $I_{\%}$, A_0, A_{100}, and A are the percent inhibition, A at 0% inhibition, A at 100% inhibition, and observed A value, respectively. Polarization values were converted to percent inhibition to make the data more intuitive to the reader and to normalize the day-to-day differences in the starting 0% inhibition polarization values. The percent inhibition versus competitor concentration curves were analyzed by nonlinear least-squares curve fitting and yielded an IC_{50} value (the concentration of competitor needed to displace half of the bound ligand). To compare binding affinities of the test compounds to those reported in the literature, IC_{50} values were converted to relative binding affinities (RBA) using E_2 as a standard. The E_2 RBA was set equal to 100 [RBA = (IC_{50}/IC_{50} of E_2) × 100].

RBAs are a convenient way to compare test compounds because they tend to minimize variability in operators, instrumentation, preparations, and assay conditions. While not necessary, IC_{50} values can be converted into K_i (inhibition constants). Several corrections have been presented in the literature (*57–59*). For example, in this study we used Kenakin's correction to convert the IC_{50} for E_2 (13 nM) into a K_i of 0.6 nM (*57–59*), similar to the K_d of the E_2–ER complex using a direct binding study. The correction is

$$K_i = (0.5\ B_T)(IC_{50})(K_d)/[(L_T \times R_T) + 0.5\ B_T(R_T-L_T+0.5\ B_T-K_d)],$$

where B_T, IC_{50}, L_T, and R_T are bound ligand, concentration of competitor at 50% inhibition, total ligand, and total receptor, respectively.

Results and Discussion

While not necessary, a low K_d for the ER/FES1 interaction, similar to that for E_2/ER would reduce the amount of ER needed and make development of this assay easier. Direct binding studies of FES1 to hrER-α using the FP method are presented in Figure 2. In this experiment, a constant amount of FES1 was titrated with increasing concentrations of hrER-α. At each receptor concentration, the FP value was measured and used to calculate the amount of bound (FES1–hrER-α) and free hrER-α. The FES1–hrER-α interaction had a K_d of 0.3 nM and exhibited positive cooperativity. These results compare favorably to the affinity obtained for radioactive E_2 and native ER, and their observed cooperativity (*60*).

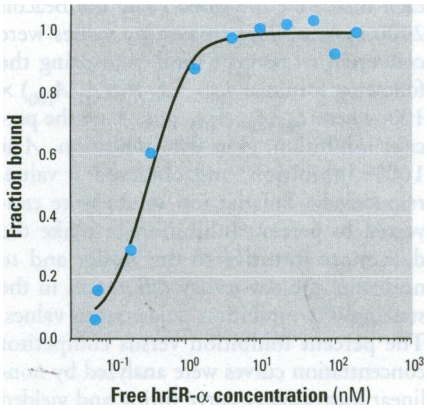

Figure 2. The binding isotherm of human recombinant estrogen receptor α (hrER-α) and fluorescent estrogen (FES1). Increasing concentrations of hrER were incubated with 2 nM FES1 for 60 min at room temperature (22°C) followed by measure of fluorescence polarization. Polarization data was converted to fraction bound and plotted against free hrER-α (see Materials and Methods).

The competition binding curves generated for 15 compounds that compete to varying degrees with FES1 binding to hrER-α are shown in Figure 3. On each graph, the y-axis is reported as the percent inhibition of ES1 binding. The resulting classic competitive binding curves can be analyzed by a variety of methods in order to assess the potency of the competitor molecule. The IC_{50} values for each test compound were calculated by nonlinear least-squares regression and are shown in Table 1. To more easily compare the hrER-α binding capacity of test compounds to each other and to values reported by others, the RBA for each compound in relation to the natural ligand E_2 was also calculated and is reported in Table 1 (E_2 RBA = 100). All compounds examined in this study demonstrated some ability to displace the fluorescent ligand FES1 from the hrER-α. However, it should be recognized that the androgenic steroid DHT; the

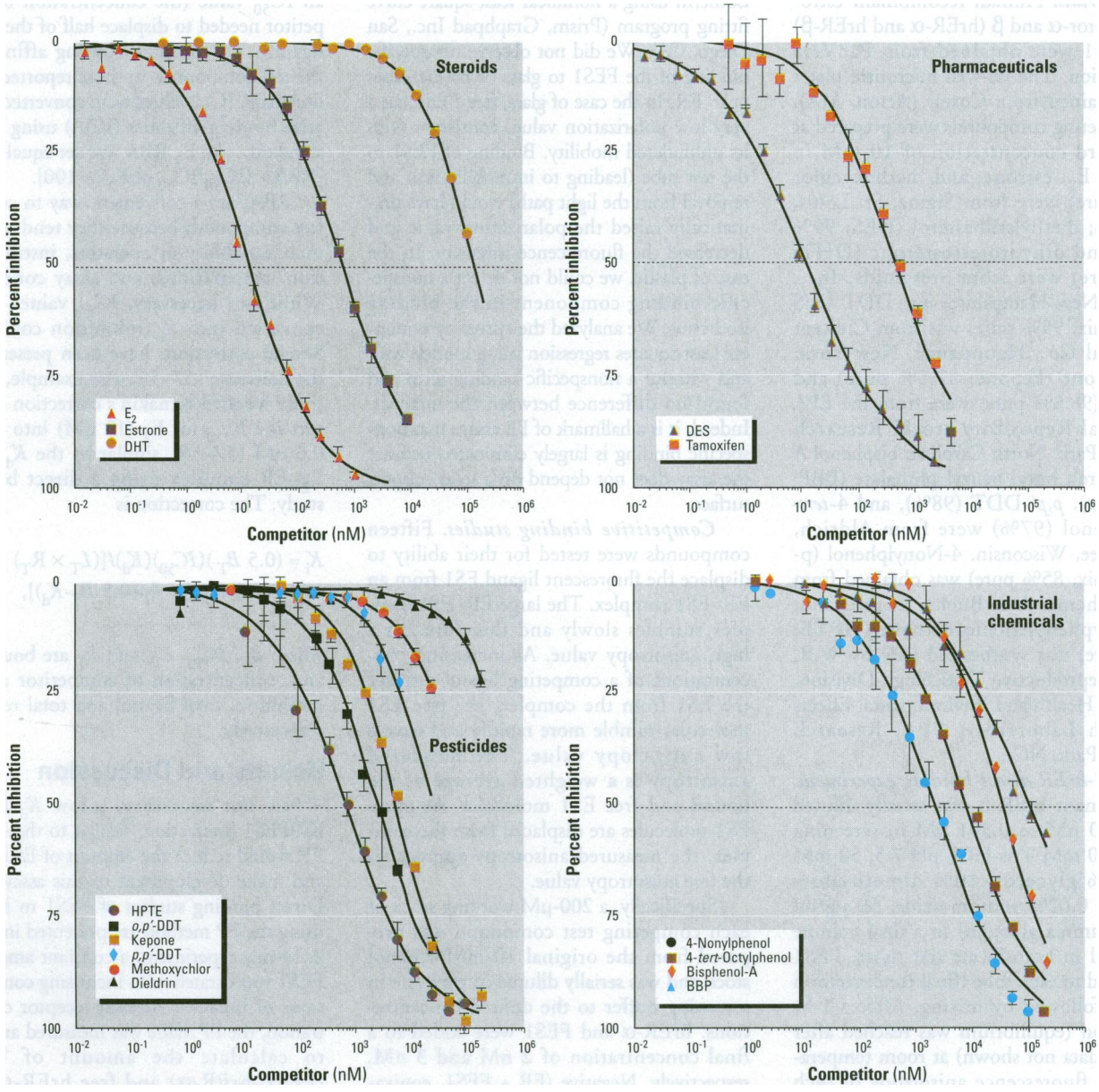

Figure 3. Competition binding curves of various compounds against a human recombinant estrogen receptor α/fluorescent ligand complex (hrER-α–FES-1). Abbreviations: E_2, 17β-estradiol; DHT, dihydrotestosterone; DES, diethylstilbestrol; HPTE, hydroxyphenyltrichloroethane; BBP, butyl benzyl phthalate. Increasing concentrations of competitor were incubated with 2 nM hrER-α and 3 nM FES1 for 60 min at room temperature (22°C) followed by measurement of fluorescence polarization. Polarization data was converted to percent inhibition and plotted against competitor concentration (see Materials and Methods). Data points and error bars represent the mean percent inhibition value ± 1 standard deviation ($n = 3$).

pesticides dieldrin, *p,p'*-DDT, and methoxychlor; and the plasticizer BBP, which induce little to no estrogen activity in other *in vitro* or *in vivo* estrogen screens (*43,61–67*), all failed to displace more than 50% of FES1 from hrER-α at treatment concentrations exceeding 100 μM (Fig. 3 and Table 1).

The E_2 metabolite estrone displayed an RBA of 2.1 in the FP assay, which is weaker than a determination of 60 made by Kuiper et al. using *in vitro*-translated rat ER at 4°C in a gel filtration (*64*), and the RBA obtained from traditional 4°C binding assays involving mouse uterine ER (*63*) or human ER from MCF-7 cells (*68*). The pharmaceutical estrogen DES was found to have an hrER-α RBA slightly greater than E_2, which is similar to the determination made by Shelby et al. with mouse ER (*43*), but lower than the values obtained by others with mouse, rat, or human ER (*63,64,69*). The RBA value determined with the FP binding assay for the pharmaceutical antiestrogen tamoxifen is similar to those reported by others (*43,64*).

Using the FP binding assay, the *o,p'*-DDT isomer of the pesticide DDT was found to have 4–10 times higher binding affinity for hrER-α than the *p,p'*-DDT isomer component. While this relationship between isomer and ER affinity is the same as found by others using MCF-7 or mouse ER (*61,63*), both isomers display higher affinity for the ER in the FP binding assay (Table 1). In the case of the estrogenic pesticide methoxychlor (*12,25*), this parent compound was found to have a much lower hrER-α RBA using the FP assay than its estrogenic metabolite HPTE. Furthermore, these ER binding values for methoxychlor and HPTE correspond closely to previous determinations (*43,63,64*). The estrogenic pesticide kepone (*10,11,24*) had an hrER-α RBA value in the FP assay that is similar to the ER binding interactions reported from other laboratories (*43,63*). At the same time, the pesticide dieldrin was found to have only the extremely weak interaction with hrER-α that has been reported by others (*66,70*).

The industrial chemical bisphenol A, which has been shown to have estrogenic effects *in vitro* and *in vivo* (*26,67,71,72*), maintained a weak but significant hrER-α interaction in the FP binding assay. This RBA value for bisphenol A is similar to that reported by Kuiper et al. (*64*), but it is significantly lower than observed by Waller et al. (*63*). The ER binding capacity of the estrogenic alkylphenols 4-*tert*-octylphenol and 4-nonylphenol (*30*) determined with the FP method are similar to those obtained from the mouse uterine ER assay by Waller et al. (*63*), but higher than determinations made with ER derived from trout or MCF-7 cells (*27,61*). The plasticizer BBP, which has been shown to exhibit weak estrogenic properties in some assay systems (*62*), was shown to have only minimal binding interactions with the hrER-α that were barely detectable with the FP assay (Table 1).

When considering the ability of the FP binding assay used in this study to replicate the ER binding determinations made by others, a few important points should be highlighted. First, there is not necessarily a correspondence between the ER RBAs reported by other laboratories, employing various binding assay techniques or receptor sources, for this diverse set of compounds reported in Table 1. The possible range of receptor purity obtained in these dissimilar preparations, as well as the fact that ER from distinct species may differ significantly in the ligand binding domain (*73*), may account for such differences. Even when the high purity and stability of the recombinant human ER used in the FP assay is taken into account, the extrapolation of receptor binding results from human to other species is unclear. It is possible that using a purified ER preparation in this assay unduly simplifies the ligand–ER interaction, but FP assays are ideally suited for assessing the effects of added binding components. Second, when the FP binding assay's ability to evaluate receptor occupancy at true equilibrium conditions and at a relevant temperature is considered, it is not unexpected that RBA values obtained by FP might differ from those obtained using traditional 4°C binding assays. It is possible that the relatively high ER RBA values obtained from the FP assay for some chemicals (e.g., the DDT isomers and BBP) are a reflection of this method's more realistic experimental conditions. At the same time, the observation that some estrogens displayed a weaker than expected affinity for ER in the FP assay (estrone, DES, and bisphenol A) may also be due to temperature or equilibrium condition effects. Finally, when considering industrial or environmental compounds that are difficult to obtain as pure samples (e.g., 4-*tert*-nonylphenol), differences in ER affinity between laboratories may be related to sample source. Taken together, the ER RBA values obtained in this study are within the range of determinations reported by other laboratories. Most importantly, the ER binding interactions determined with the FP binding assay in this study are similar to the evaluations of estrogen activity made for these same compounds using other *in vitro* methods (*43,61,67,68,70*).

Because screens involving both ER subtypes (α and β) will be required to completely evaluate the endocrine disruption potential of environmental estrogens, we applied the FP technology to binding studies involving ER-β as well as ER-α. The K_i for this FES1–ER-β interaction was estimated at 0.15 nM (data not shown), which compares favorably with the K_i determined by others using a direct binding study between [^{3}H] estradiol and hrER-β (*64*). The effect of increasing amounts of E_2 on the integrity of an FES1–hrER-β complex is shown in Figure 4. Thus, the FP receptor binding method presented in this study has considerable potential for analyses involving ER-β. In addition, while our current studies describe an FP competition assay for human ER subtypes, this same strategy

Table 1. Relative binding affinities (RBAs) of tested compounds

Compound	FP IC_{50}	FP RBA	RBA values from literature[a]	References
E_2	13 nM	100		
Estrone	626 nM	2.1	60h, 60m	(*63,64*)
DHT	126–146 μM[b]	0.01-0.009	0.05h, 0.026m	(*63,64*)
DES	11 nM	118	470h, 470r, 130m, 370m	(*43,63,64,69*)
Tamoxifen	423 nM	3.1	7.0h, 6.0m	(*43,64*)
p,p'-DDT	14–50 μM[b]	0.09–0.03	0.00026m	(*63*)
o,p'-DDT	2.7 μM	0.4	0.01r, 0.00031f, 0.17m, 0.090m	(*43,61,63,69*)
Methoxychlor	81–193 μM[b]	0.02–0.007	0.01h, 0.01m,[c] 0.0038m	(*43,63,64*)
HPTE	750 nM	1.7	1.3m, 5.2m	(*43,63*)
Kepone	5.7 μM	0.2	0.01m,[c] 0.019m	(*43,63*)
Dieldrin	470–4,500 μM[b]	0.003–0.0003	0.002h,[c] 0.0005r[c]	(*66,74*)
Bisphenol-A	32 μM	0.04	0.05h, 0.018m	(*63,64*)
4-*tert*-Octylphenol	7.5 μM	0.2	0.1t, 0.2m	(*27,63*)
4-Nonylphenol	3.9 μM	0.3	0.021f, 0.05t, 0.01m,[c] 0.313m	(*27,43,61,63*)
BBP	73–120 μM[b]	0.02–0.01	0.3t, 0.0034m	(*63,75*)

Abbreviations: FP, fluorescence polarization; IC_{50}, concentration that inhibits 50%; E_2, 17β-estradiol; DHT, dihydrotestosterone; DES, diethylstilbestrol; HPTE, hydroxyphenyltrichloroethane; BBP, butyl benzyl phthalate. The IC_{50} of each compound was compared to the IC_{50} for E_2.

[a]The letters after the RBA value indicate the source of ER in the assay: h, human; m, mouse; r, rat; t, trout; f, MCF-7 cells.

[b]These values represent the 95% confidence limit range (leading to a range of associated RBA values); in these cases, IC_{50}s were difficult to determine usually because of solubility limitations.

[c]RBA values prefaced were calculated from IC_{50} values that we estimated from the cited literature.

is being used to design assays for other receptors in the steroid hormone receptor superfamily such as the androgen, glucocorticoid, and thyroid hormone receptors. In each case, the limiting factor is the availability of a fluorescent hormone analog with characteristics suitable for use in FP. Ongoing research in our laboratory is aimed at developing novel fluorescent ligands for other receptors.

The FP receptor binding assays in general offer several advantages over other receptor binding technologies. First, the FP method uses no radioactivity. Second, FP measurements are done in solution, allowing molecules to be studied at true equilibrium and at relevant temperatures. When considering the task of screening environmental contaminants and other compounds that may have relatively weak binding interactions with the ER, the ability to study equilibrium binding at temperatures relevant to potential exposure scenarios is advantageous. Third, this method gives a direct measure of a tracer's bound/free ratio; no separation of bound and free tracer is required. The filtering, precipitation, and/or centrifugation steps common to other binding assays is eliminated. Thus, because manipulation or alteration of the sample is not required in this assay, artifactual loss of signal through handling does not occur. For example, during the wash steps of filter binding assays, significant release and loss of the precipitated signal may occur. Fourth, because the same tube is used for reaction and FP measurement, each reaction mixture's approach to equilibrium can be monitored by repeated measurements (e.g., every 6 sec), allowing direct determination of the binding reaction kinetics. Fifth, FP measurements do not have deleterious effects on sample or receptor. Samples can be analyzed, treated, and then reanalyzed. Different treatments of the same sample might involve the addition of detergent, dissociating agent, or excess unlabeled ligand or even a temperature shift. Mixtures of estrogenic compounds can also be easily analyzed in a stepwise or dynamic fashion with this assay. Lastly, the overall simplicity of this FP receptor binding assay results in a method that can easily be adapted to a high throughput (microtiter plate) format.

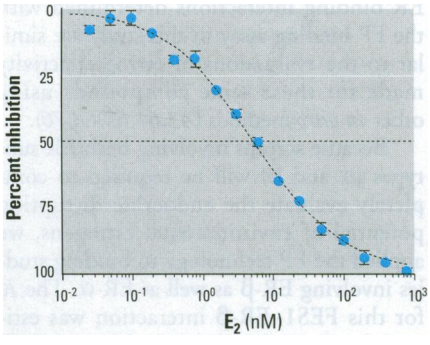

Figure 4. Competition binding curve of 17β-estradiol (E_2) against an estrogen receptor β (hrER-β)–FES1 complex. Increasing concentrations of unlabeled E_2 were incubated with 250 pM FES1 and 1 nM hrER-β overnight at 22°C followed by measure of fluorescence polarization. Polarization data was converted to percent inhibition and plotted against E_2 concentration. An IC_{50} (concentration that inhibits 50%) of 5.5 nM was determined by nonlinear least squares (see Materials and Methods). Data points and error bars represent the mean fraction bound value ± 1 standard deviation ($n = 3$).

A limitation of FP competition assays is that a relatively pure preparation of receptor is required in order to bind a significant percentage of fluorescent ligand. In the present study, the effects of secondary factors are not assessed. On the other hand, the effect of accessory proteins, added to the assay matrix, can be evaluated with a systematic approach. A caveat to using fluorescence detection methods in screening a large panel of organic compounds is the potential for fluorescence from the test compounds. FP measurements are less sensitive to this type of interference because fluorescence in the test compound can be subtracted out before the FP measurement is made.

In conclusion, the novel FP technology presented here represents the first receptor binding assay that is practical for use as a large-scale screening tool to characterize the ER binding interactions of pharmaceutical, environmental, and industrial compounds. Furthermore, considering the highly uniform source of receptor used and the simplicity of this assay, the potential that this method could be standardized between laboratories is considerable. Thus as part of a comprehensive battery of *in vitro* and *in vivo* tests for endocrine activity, the FP binding assay presented in this report can provide mechanistically pure information to define the mode of action of an EDC and otherwise assist in EDC hazard identification. Furthermore, the highly uniform receptor binding data derived from FP binding studies should also have application in the development of quantitative structure–activity models to prioritize compounds for further testing of endocrine activity.

References and Notes

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