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The Estrogen Receptor Relative Binding Affinities of 188 Natural and Xenochemicals: Structural Diversity of Ligands

Robert M. Blair,*[,1] Hong Fang,* William S. Branham,* Bruce S. Hass,* Stacey L. Dial,* Carrie L. Moland,* Weida Tong,† Leming Shi,† Roger Perkins,† and Daniel M. Sheehan*

**Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Jefferson, Arkansas 72079; and †R.O.W. Sciences, Jefferson, Arkansas 72079*

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We have utilized a validated (standardized) estrogen receptor (ER) competitive-binding assay to determine the ER affinity for a large, structurally diverse group of chemicals. Uteri from ovariectomized Sprague-Dawley rats were the ER source for the competitive-binding assay. Initially, test chemicals were screened at high concentrations to determine whether a chemical competed with [^{3}H]-estradiol for the ER. Test chemicals that exhibited affinity for the ER in the first tier were subsequently assayed using a wide range of concentrations to characterize the binding curve and to determine each chemical's IC_{50} and relative binding affinity (RBA) values. Overall, we assayed 188 chemicals, covering a 1×10^6-fold range of RBAs from several different chemical or use categories, including steroidal estrogens, synthetic estrogens, antiestrogens, other miscellaneous steroids, alkylphenols, diphenyl derivatives, organochlorines, pesticides, alkylhydroxybenzoate preservatives (parabens), phthalates, benzophenone compounds, and a number of other miscellaneous chemicals. Of the 188 chemicals tested, 100 bound to the ER while 88 were non-binders. Included in the 100 chemicals that bound to the ER were 4-benzyloxyphenol, 2,4-dihydroxybenzophenone, and 2,2′-methylenebis(4-chlorophenol), compounds that have not been shown previously to bind the ER. It was also evident that certain structural features, such as an overall ring structure, were important for ER binding. The current study provides the most structurally diverse ER RBA data set with the widest range of RBA values published to date.

Key Words: **estrogen receptor competitive-binding assay; relative binding affinity; estrogens; antiestrogens; alkylphenols; organochlorines; pesticides; parabens; phthalates.**

A variety of synthetic chemicals has been released into the environment, some in large quantities, during the last few decades. Rapidly increasing scientific evidence suggests that many of these chemicals, structures of which cross a wide range, can interfere with normal, hormonally regulated biological processes to adversely affect development and/or reproductive function in wildlife, experimental animals, and humans (see Colborn *et al*., 1993; Danzo, 1998; Daston *et al*., 1997; Kavlock *et al*., 1996; Sonnenschein and Soto, 1998; Toppari *et al*., 1996 for recent reviews). These environmental contaminants are able to alter the normal functioning of the endocrine and reproductive systems by mimicking or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor populations (Sonnenschein and Soto, 1998). Due to the ability of these types of chemicals to interfere with endocrine systems, they have been labeled as "endocrine disruptors." A major mechanism of endocrine disruption is binding of a xenochemical to the ER. Other mechanisms of endocrine disruption, besides receptor-mediated events, may include such mechanisms as inhibition or stimulation of hormone metabolism, actions involved in the regulation of various neural centers or the pituitary, or alterations in serum hormone-binding proteins. Endocrine disruptors are comprised of numerous types of chemicals, which can be categorized by usage (herbicides, fungicides, insecticides) or chemical structure (polychlorinated biphenyls (PCBs), dioxins, organochlorines and alkylphenols) (Colborn *et al*., 1993; Toppari *et al*., 1996), and by regulatory authority (U.S. Food and Drug Administration, U.S. Environmental Protection Agency), and their specific statutory authority.

In 1996, due to the increasing concern regarding the adverse health effects of endocrine disruptors, the U.S. Congress passed the Food Quality Protection Act (FQPA) and amended the Safe Drinking Water Act (SDWA). These laws required the EPA to develop and implement a screening strategy to assess the risk associated with estrogenic endocrine disruptors. Subsequently, the Administrator of the EPA determined that androgens and thyroid-active chemicals should also be included and that chemicals active in the screens should be rigorously tested in animal studies. In response to the passage of these laws, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), which was given the task of designing a screening and testing program to assess potential endocrine disrupting chemicals. It was recommended by EDSTAC that screening and testing should be accomplished

[1] To whom correspondence should be addressed at Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079. Fax: (870) 543-7682. E-mail: rblair@nctr.fda.gov.

in 3 steps, to include (1) initial priority setting, (2) Tier 1 screening, and (3) Tier 2 testing. One prioritization method currently being assessed involves high throughput assays of receptor-dependent responses. A second method currently being examined for the priority-setting process is a quantitative structure-activity relationship (QSAR) analysis (Tong *et al*., 1997; Waller *et al*., 1996). This type of analysis correlates a chemical's structural characteristics with biological activity and the models derived from these relationships can be used to predict the activity of untested environmental contaminants. Such models could augment, and possibly replace, high throughput screening for priority setting. According to EDSTAC recommendations, Tier 1 screening will consist of a number of *in vivo* and *in vitro* assays. Included in the list of suggested *in vitro* assays is the ER competitive-binding assay, which assesses a chemical's ability to bind to the ER. This assay can be used to rapidly ascertain whether an environmental contaminant is capable of acting through the same binding mechanism as endogenous estradiol. Relative binding-affinity data can be used for (1) priority setting using models developed from the RBA values and (2) Tier 1 screening using the methods/results described in this study. The final EDSTAC report can be found on the internet at http://www.epa.gov/opptintr/opptendo/finalrpt.htm.

It has been demonstrated that a number of the environmental contaminants are able to function in a manner similar to estradiol (Colborn *et al*., 1993; Daston *et al*., 1997; Roy *et al*., 1997; Sonnenschein and Soto, 1998; Toppari *et al*., 1996). These include such compounds as bisphenol A (Krishnan *et al*., 1993; Nagel *et al*., 1997; Perez *et al*., 1998), the alkylphenols (Nimrod and Benson, 1996; Soto *et al*., 1991; White *et al*., 1994), PCBs (Bergeron *et al*., 1994; Bitman *et al*., 1970; Korach *et al*., 1988), kepone (Gellert, 1978; Hammond *et al*., 1979;), and the parabens (Routledge *et al*., 1998). When the ER is bound by its endogenous hormone, subsequent activation of the ER results in conformational changes, protein interactions, and gene transcription (Beekman *et al*., 1993; Parker *et al*., 1993; Tsai and O'Malley, 1994). Therefore, xenoestrogen-induced alterations in normal endocrine function can result in adverse effects at the cellular level (Roy *et al*., 1997).

The majority of QSAR models developed to date are based on the biological activity of small groups of compounds with similar activity and structural features. However, the structural diversity of estrogenic chemicals is very broad. For the current study, chemicals were selected such that 1) a large data set was generated, 2) a wide diversity of structural features was represented and 3) a wide range of biological activity was measured. To our knowledge, the results presented here represent the largest and most diverse data set publicly available for chemicals binding to the ER. These data can be utilized to develop a highly robust 3D-QSAR model, as well as separate chemometric models (the development and implementation of which will be presented in a separate manuscript). In addition, these data are also useful for comparing ER-binding results from a large number of chemicals to small data sets using different assay conditions.

MATERIALS AND METHODS

Chemicals. Trizma base, Trizma hydrochloride, glycerol, EDTA, dithiothreitol, and sodium azide were obtained from Sigma (St. Louis, MO). The [2, 4, 6, 7, 16, 17–^{3}H]-E_2 (141 Ci/mmol) used in the competitive-binding assay was obtained from Dupont-New England Nuclear (Boston, MA). Hydroxylapatite was obtained from Bio-Rad Laboratories (Hercules, CA) and the UltimaGold scintillation cocktail was purchased from the Packard Instrument Company (Meriden, CT). The source and purity (when available) for each of the competing test compounds is provided in tabular form with the results from the competitive-binding assays.

Uterine cytosol (estrogen receptor) preparation. Adult (retired breeders; 244.87 ± 18.27 days of age), non-pregnant Sprague-Dawley rats (n = 188; NCTR:SDN, an outbred albino rat stock originating from CRL:CD [SD]BR rats, obtained in 1979 and maintained as a closed colony) were maintained in a controlled environment (23°C and 50% humidity) on a 12-h light/dark cycle (lights on at 0600 h). Animals received Purina rat chow and filtered tap water *ad libitum*. Females (a mean of 14 rats per cytosol batch) were ovariectomized a minimum of 10 days prior to receptor preparation. After sacrifice by CO_2 asphyxiation, uteri were excised, trimmed of excess fat and mesentery, weighed and placed in ice-cold TEDG buffer (10 mM Tris, 1.5 mM EDTA, 10 mM dithiothreitol, 10% glycerol, pH 7.4). The pooled uteri were placed in fresh, ice-cold TEDG buffer at a concentration of 1.0 g of tissue/10 ml buffer. After pre-cooling (4°C) the homogenization probe, uterine tissue was homogenized at 4°C with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) using 5-s bursts. The resulting homogenate was transferred to pre-cooled (4°C) ultracentrifuge tubes and centrifuged at 105,000 × g for 60 min at 4°C. After centrifugation, the ER-rich supernatant was decanted into 15-ml conical tubes (~3 ml/aliquot; ~12 aliquots/average cytosol batch), and was stored at –70°C until used in competition assays.

Estrogen receptor (ER) competitive-binding assays. [^{3}H]-E_2 (10 μl; 1 × 10^{-9} M final assay concentration) was incubated with 10 μl of increasing concentrations of radioinert competitor, 50 μl of uterine cytosol preparation and 230 μl of 50 mM Tris buffer (pH 7.4) in duplicate tubes. Reaction-mixture tubes were placed in a drum roller (Glas-Col, Terre Haute, IN) and incubated at 4°C for 20 h. Following the incubation period, 750 μl of a cold 60% hydroxylapatite (HAP) slurry (made in 50 mM Tris, pH 7.4) was added to each tube to separate the bound ligand from the free ligand. These tubes were incubated in an icewater bath for 20 min and vortexed for 10 s at 5-min intervals. Tubes were subsequently centrifuged (600 × g) at 4°C for 5 min. The supernatant was discarded and the resulting HAP pellet was resuspended in 2.0 ml of cold 50 mM Tris buffer and vortexed and centrifuged as above. After 3 washes, the supernatant was discarded and 2.0 ml of cold (4°C) 100% ethanol was added to each tube to extract the radiolabeled E_2 from the HAP. Tubes were incubated on ice for 15 min and vortexed at 5-min intervals. Following the ethanol incubation, the tubes were centrifuged (600 × g) at 4°C for 10 min. The resulting supernatant was decanted into vials containing 10 ml of scintillation cocktail. Radioactivity was measured on a Packard Tri-Carb 1600TR Liquid Scintillation Analyzer (Packard Instrument Company, Meriden, CT). In addition to the radioinert competitors, each assay included a zero tube (no competitor added; represented total binding of [^{3}H]-E_2; averaged approximately 15,000 dpm) and an E_2 standard curve (1 × 10^{-7}, 1 × 10^{-8}, 1 × 10^{-9}, 3.33 × 10^{-10}, 1 × 10^{-10}, and 3.33 × 10^{-11} M concentrations) for quality control purposes. The 1 × 10^{-7} M E_2 tube contained a 100-fold molar excess of radioinert E_2 compared to [^{3}H]-E_2 and thus represented non-specific binding (NSB; averaged approximately 800 dpm). Radioactivity counts (dpm) of the NSB tubes were subtracted from all tubes prior to calculation of percent [^{3}H]-E_2 bound. Data for each competitor and the E_2 standard curve were plotted as percent [^{3}H]-E_2 bound versus molar concentration, and the IC_{50} (50% inhibition of [^{3}H]-E_2 binding) for each competitor determined. The RBA

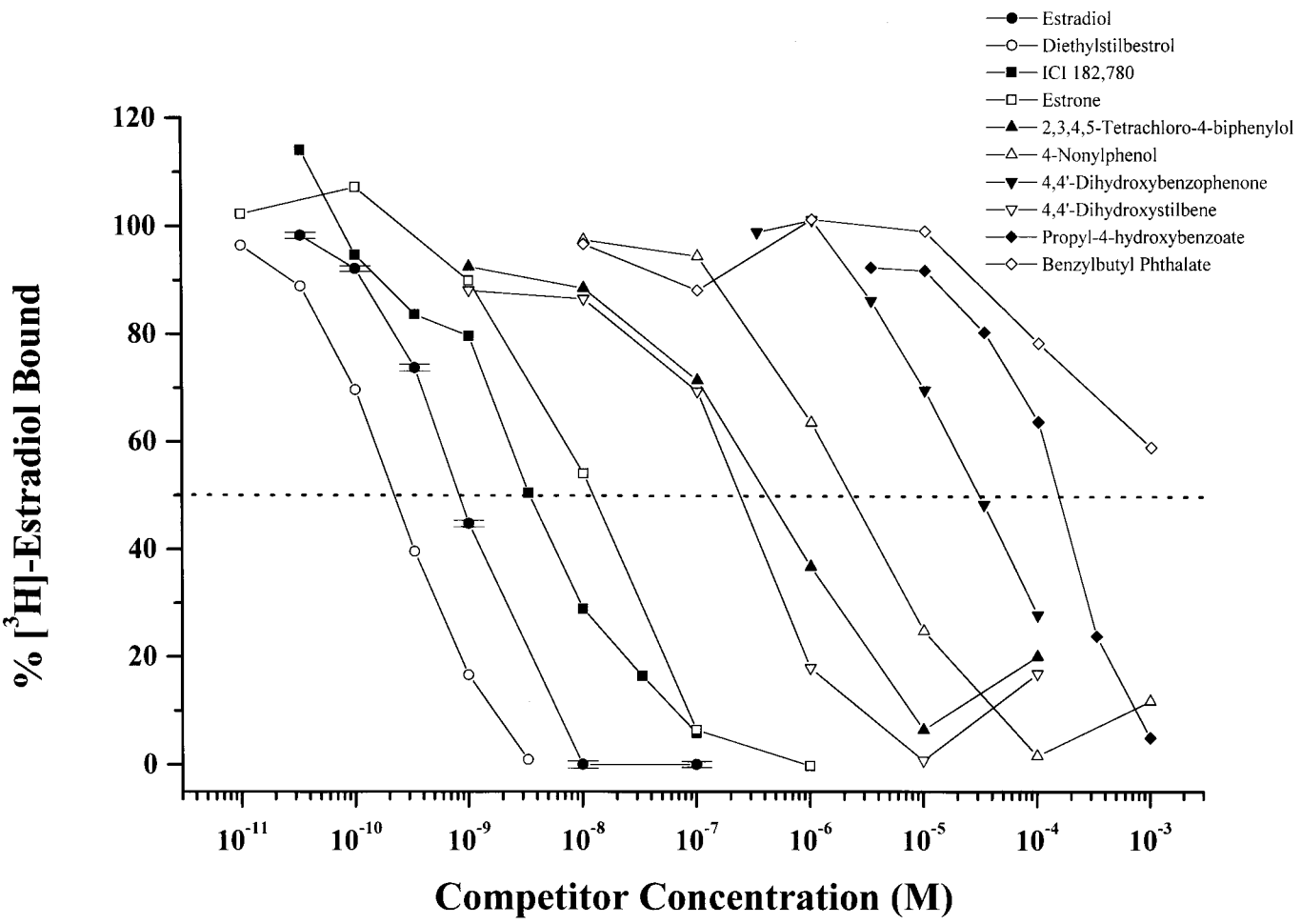

FIG. 1. Representative estrogen receptor binding curves. This figure demonstrates the variety of chemicals, based on both structure and affinity for the ER, assayed in the ER competitive-binding assay. It also clearly demonstrates the parallelism in the linear portion of the curve between different test competitor curves.

for each competitor was calculated by dividing the IC_{50} of E_2 by the IC_{50} of the competitor and was expressed as a percent ($E_2 = 100$). Details and validation of the ER competitive-binding assay will be published in a separate manuscript (unpublished).

Test chemicals were dissolved in 100% ethanol at the highest concentration possible. Stock solutions were then subsequently diluted in ethanol for analysis in the ER competitive-binding assay. Due to the large number of chemicals tested, the ER competitive-binding assays were set up in a tiered design. Unless known to bind to the ER, test chemicals were initially run at only 2 high concentrations spanning 3 log concentrations (Tier 1). If a test compound exhibited binding to the ER, then a second assay (Tier 2) was run using a wide range of concentrations, ranging generally from 1×10^{-4} to 1×10^{-9} M in 10-fold increments, though this varied depending upon the competitor. If necessary, a Tier 3 assay (consisting of one-half log molar concentrations which bracketed the IC_{50} observed in the Tier 2 assay) was run to more accurately determine a competitor's IC_{50}. In the Tier 1 assay, approximately 36 chemicals could be assayed in replicate per cytosol batch, while only 18 chemicals per cytosol batch could be assayed in replicate in either the Tier 2 or Tier 3 assay. In the final analysis, it required one rat per chemical assayed in replicate. Chemicals which failed to bind the ER were designated as "non-binders." All assays were replicated a minimum of 2 times and IC_{50} values of positive chemicals are the means of the replicates.

RESULTS

Mean IC_{50}s and RBAs for the 188 chemicals tested (100 binders and 88 non-binders) in the ER competitive-binding assay are presented in Tables 1–12. Of the 100 active test chemicals, 26 were strong binders (log RBA $>$ 0), 33 were moderate binders (log RBA between 0 and –2), and 41 were weak binders (log RBA $<$ –2). For purposes of clarity and convenience, test compounds were grouped in tables according to chemical or use classifications. Figure 1 shows representative ER-binding curves over the range of concentrations used for various chemicals in the ER competitive-binding assay.

As expected, all of the selected steroidal estrogens (Table 1), synthetic estrogens (Table 2) and antiestrogens (Table 3) showed affinity for the ER. With the exception of one chemical in each of these 3 classes, all these chemicals exhibited moderate to strong binding affinity. This was especially evident for the synthetic estrogens, which exhibited strong affinity for the ER in 13 of the 16 chemicals tested. Of the synthetic estrogens,

TABLE 1
IC_{50}s and Relative Binding Affinities (RBA) for Steroidal Estrogens

Chemical name	Source	Purity[a] (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
17β-Estradiol	U.S. Biochemical	NA	$8.99 \times 10^{-10} \pm 0.27 \times 10^{-10}$	100.000	2.00
Estra-1,3,5(10)-trien-3-ol	NCI[b]	NA	$4.95 \times 10^{-9} \pm 0.85 \times 10^{-9}$	18.162	1.26
Estra-1,3,5(10)-trien-3-ol	Steraloids	NA	$8.85 \times 10^{-9} \pm 3.15 \times 10^{-9}$	10.158	1.01
Estriol	Sigma	99	$9.25 \times 10^{-9} \pm 1.75 \times 10^{-9}$	9.719	0.99
Estrone	Aldrich	99	$1.23 \times 10^{-8} \pm 0.32 \times 10^{-8}$	7.309	0.86
17α-Estradiol[c]	Sigma	99	$2.93 \times 10^{-8} \pm 0.80 \times 10^{-8}$	3.068	0.49
1,3,5(10)-Estratrien-3, 6α, 17β-triol	Steraloids	NA	$1.27 \times 10^{-7} \pm 0.43 \times 10^{-7}$	0.708	–0.15
3-Hydroxyestra-1,3,5(10)-trien-16-one[c]	NCI	NA	$1.75 \times 10^{-7} \pm 0.05 \times 10^{-7}$	0.514	–0.29
3-Deoxyestradiol	NCI	NA	$1.80 \times 10^{-7} \pm 0.20 \times 10^{-7}$	0.499	–0.30
16β-Hydroxy-16-methyl-3-methylether 17β estradiol	NCI	NA	$2.70 \times 10^{-6} \pm 0.20 \times 10^{-6}$	0.033	–1.48
3-Methylestriol	NCI	NA	$4.00 \times 10^{-6} \pm 0.00$	0.022	–1.65
3-Deoxyestrone[c]	NCI	NA	$1.43 \times 10^{-5} \pm 0.58 \times 10^{-5}$	0.006	–2.20

[a] Purity information as provided by the manufacturer; NA = purity not available.
[b] NCI = chemicals generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.
[c] Chemical exhibited a U-shaped binding curve.

diethylstilbestrol (DES), meso-hexestrol, and ethinyl estradiol (EE_2) exhibited greater affinity for the ER than E_2. The only other chemical analyzed that exhibited greater affinity than E_2 for the ER was the antiestrogen 4-hydroxytamoxifen.

A number of miscellaneous steroids were also analyzed in the ER competitive-binding assay (Table 4). The majority of these chemicals was inactive and did not bind to the ER. However, norethynodrel and 5α-androstane-3β,17β-diol exhibited moderate binding while 5α-androstane-3α,17β-diol showed weak binding. Unexpectedly, both of these latter chemicals exhibited U-shaped ER-binding curves in which the percent [^{3}H]-E_2 bound increased with increasing concentration of test compound after reaching a nadir below 50% (Fig. 2). Nonetheless, the initial portions of these curves were parallel to the E_2 standard curve. Of the 188 chemicals assayed, 22 (20 binders and 2 non-binders) demonstrated a U-shaped binding curve (identified individually in Tables 1–12). In 5 of these 22 chemicals, an increase in percent [^{3}H]-E_2 bound was evident for the 2–3 highest concentrations tested. In the remaining 17 chemicals, an increase in the percent [^{3}H]-E_2 binding was evident only at the highest concentration tested. The majority (15) of these 22 chemicals consisted of steroidal

TABLE 2
IC_{50}s and Relative Binding Affinities (RBA) for Synthetic Estrogens

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
Diethylstilbestrol (DES)	Research Plus	NA	$2.25 \times 10^{-10} \pm 0.05 \times 10^{-10}$	399.556	2.60
Meso-hexestrol	Aldrich	99	$3.00 \times 10^{-10} \pm 0.00$	299.667	2.48
Ethinyl estradiol	Research Plus	NA	$4.73 \times 10^{-10} \pm 0.60 \times 10^{-10}$	190.063	2.28
Dienestrol[a]	Sigma	99	$2.40 \times 10^{-9} \pm 0.50 \times 10^{-9}$	37.458	1.57
Diethylstilbestrol monomethyl ether	Steraloids	NA	$4.40 \times 10^{-9} \pm 0.50 \times 10^{-9}$	20.432	1.31
3,3′-Dihydroxyl hexestrol	NCI	NA	$5.85 \times 10^{-9} \pm 1.65 \times 10^{-9}$	15.368	1.19
Dimethylstilbestrol	NCI	NA	$6.20 \times 10^{-9} \pm 1.30 \times 10^{-9}$	14.500	1.16
Moxestrol[b]	R. H. Purdy	NA	$6.50 \times 10^{-9} \pm 1.40 \times 10^{-9}$	13.831	1.14
2,6-Dimethyl hexestrol[a]	NCI	NA	$7.00 \times 10^{-9} \pm 1.73 \times 10^{-9}$	12.843	1.11
Hexestrol, mono methyl ether	NCI	NA	$9.60 \times 10^{-9} \pm 1.40 \times 10^{-9}$	9.365	0.97
p-(α,β-Diethyl-*p*-methyl phenethyl)-meso phenol[a]	NCI	NA	$2.25 \times 10^{-8} \pm 0.75 \times 10^{-8}$	3.996	0.60
DL-hexestrol	NCI	NA	$2.50 \times 10^{-8} \pm 0.50 \times 10^{-8}$	3.596	0.56
Mestranol[a]	NA	NA	$3.97 \times 10^{-8} \pm 0.65 \times 10^{-8}$	2.264	0.35
α,α-Dimethyl-β-ethyl allenolic acid	NCI	NA	$9.50 \times 10^{-8} \pm 0.50 \times 10^{-8}$	0.946	–0.02
Diethylstilbestrol dimethyl ether[a]	Steraloids	NA	$1.60 \times 10^{-6} \pm 0.30 \times 10^{-6}$	0.056	–1.25
Doisynoestrol	NCI	NA	$4.90 \times 10^{-5} \pm 1.40 \times 10^{-5}$	0.002	–2.74

[a] Chemical exhibited a U-shaped binding curve.
[b] Moxestrol was a gift from Dr. R. H. Purdy, Southwest Foundation for Research and Education, San Antonio, TX 78284.

TABLE 3
IC_{50}s and Relative Binding Affinities (RBA) for Type I[a] and Type II[b] Antiestrogens

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
4-Hydroxytamoxifen	Zeneca	NA	$5.13 \times 10^{-10} \pm 1.12 \times 10^{-10}$	175.244	2.24
ICI 182,780[c]	Zeneca	NA	$2.40 \times 10^{-9} \pm 1.10 \times 10^{-9}$	37.458	1.57
Droloxifene (3-hydroxytamoxifen)	Zeneca	NA	$5.90 \times 10^{-9} \pm 3.05 \times 10^{-9}$	15.237	1.18
ICI 164,384[c]	Zeneca	NA	$6.20 \times 10^{-9} \pm 1.30 \times 10^{-9}$	14.500	1.16
Tamoxifen citrate	Zeneca	NA	$5.55 \times 10^{-8} \pm 0.05 \times 10^{-8}$	1.620	0.21
Toremifene citrate	Zeneca	NA	$6.50 \times 10^{-8} \pm 0.50 \times 10^{-8}$	1.383	0.14
Clomiphene citrate (mixture of *cis* and *trans* isomers)	Sigma	98	$1.25 \times 10^{-7} \pm 0.75 \times 10^{-7}$	0.719	–0.14
Nafoxidine	Sigma	NA	$1.25 \times 10^{-7} \pm 0.55 \times 10^{-7}$	0.719	–0.14
Triphenylethylene	Aldrich	99	$5.45 \times 10^{-5} \pm 0.55 \times 10^{-5}$	0.002	–2.78

[a] Type I antiestrogens include the partial agonists and partial antagonists.
[b] Type II antiestrogens are the pure antiestrogens. In this table they are represented by the ICI compounds.
[c] Chemical exhibited a U-shaped binding curve.

estrogens, synthetic estrogens, antiestrogens, or miscellaneous steroids.

Alkylphenolic compounds (Table 5) were a major group of chemicals assayed. All but 3 of the alkylphenols exhibited binding to the ER with their binding affinity ranging from moderate to weak. Of concern was the possibility that different sources or different lots of an individual chemical might bind to the ER with substantially different affinities. Therefore, we tested this possibility on 4-nonylphenol, a compound known to be a mixture of isomers. Nonylphenol was chosen due to its importance in National Toxicology Program studies (Chapin *et al*., 1999) and the fact that it is easily obtained from several different sources. We obtained 4-nonylphenol from Aldrich, Fluka, Schenectady International, and Lancaster. In addition, we tested 2 different lots of 4-nonylphenol from Aldrich and 2 different lots from Fluka. The IC_{50} values for 4-nonylphenol from Aldrich, Fluka, and Schenectady International ranged from 2.4×10^{-6} to 4.7×10^{-6}, thus demonstrating very little difference across sources. Also, there were no substantial differences in ER-binding affinities between different lots of 4-nonylphenol from the same source. The one source of 4-nonylphenol which exhibited a significantly lower affinity for the ER compared to the other sources was the Lancaster ($IC_{50} = 2.8 \times 10^{-5}$); however, this was 4-n-nonylphenol, an analytical standard, which is a pure, unbranched isomer unlike the other nonylphenol compounds which are a mixture of *para*-isomers.

It also appeared that length of a chemical's side chain influenced the ER-binding affinity of the alkylphenols. In general, the longer the side chain, the greater the binding affinity for the ER, such that nonylphenol > octylphenol > heptyloxyphenol > amylphenol > butylphenol > ethylphenol. However, there appears to be a limit to the number of side-chain carbons

TABLE 4
IC_{50}s and Relative Binding Affinities (RBA) for Other Miscellaneous Steroids

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
Norethynodrel[a]	Sigma	99	$4.00 \times 10^{-7} \pm 0.10 \times 10^{-7}$	0.225	–0.65
Norethynodrel[a]	Steraloids	NA	$4.40 \times 10^{-7} \pm 0.40 \times 10^{-7}$	0.204	–0.69
5α-Androstane-3β,17β-diol[a]	Sigma	NA	$7.50 \times 10^{-7} \pm 1.30 \times 10^{-7}$	0.120	–0.92
5α-Androstane-3α,17β-diol[a]	Sigma	99	$4.20 \times 10^{-5} \pm 0.16 \times 10^{-5}$	0.002	–2.67
5α-Dihydrotestosterone[a]	Sigma	99	$>1.00 \times 10^{-3}$	—	—
Aldosterone	Sigma	98	$>1.00 \times 10^{-4}$	—	—
Cholesterol	Sigma	99	$>1.00 \times 10^{-3}$	—	—
Corticosterone	Sigma	95	$>1.00 \times 10^{-4}$	—	—
Dexamethasone	Sigma	NA	$>1.00 \times 10^{-4}$	—	—
Epitestosterone	Sigma	99.9	$>6.00 \times 10^{-4}$	—	—
Etiocholan-17β-ol-3-one (*5β-dihydrotestosterone*)	Sigma	99	$>1.00 \times 10^{-4}$	—	—
Progesterone	Sigma	99	$>1.00 \times 10^{-3}$	—	—
Testosterone[a]	Sigma	98	$>1.00 \times 10^{-3}$	—	—

[a] Chemical exhibited a U-shaped binding curve.

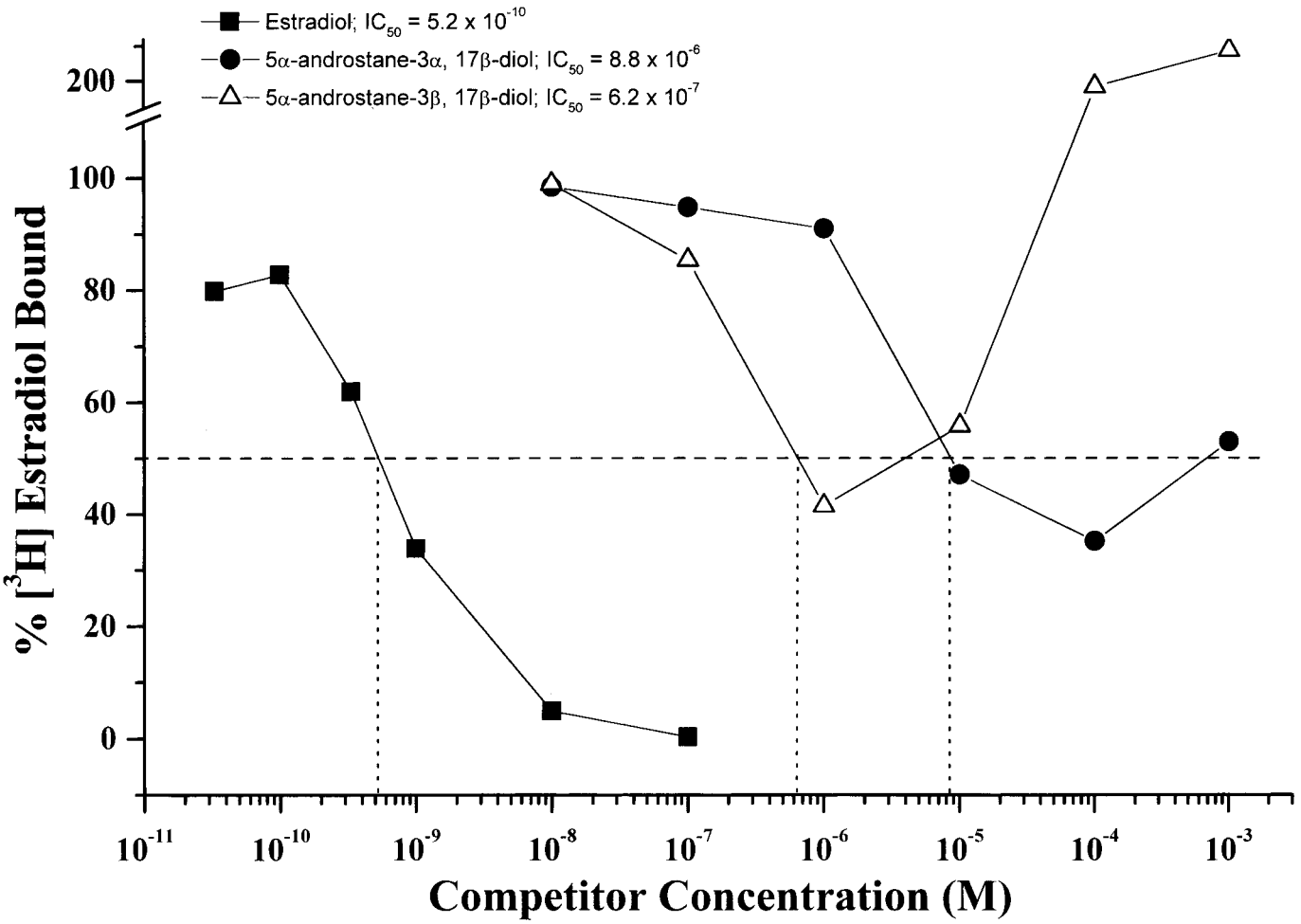

FIG. 2. U-shaped binding curves were evident for a few of the chemicals assayed in the ER competitive-binding assay. As described in the results, this may be due to an increased dissociation rate of the ER-E_2 complex in the presence of high concentrations of competitors.

that increases binding to the ER since 4-dodecylphenol (12 carbons) exhibited a lower RBA than 4-nonylphenol (9 carbons).

Table 6 provides the binding affinities of another major group of test chemicals, the diphenyl derivatives. Of the diphenyl methane derivatives (bisphenol As), only bisphenol B was moderately active at binding the ER, while the remaining chemicals in this group were weak binders or inactive at the concentrations tested. Three of the 5 diphenyl ethanes were moderate ER binders, while the biphenyl compounds were either weak binders or non-binders.

In this study, we tested 3 groups of organochlorines (Table 7): DDT isomers, methoxychlor and it's derivatives, and polychlorinated biphenyls (PCBs). Only dihydroxymethoxychlor olefin showed strong affinity for the ER. The remaining chemicals exhibited either moderate or weak RBAs or were inactive at the concentrations tested. Only one of the DDT isomers, o,p′-DDT, bound to the ER and did so with weak affinity. The majority of the methoxychlor derivatives (5 of 7) and PCBs (6 of 9) competed for the ER. A difference in ER binding between methoxychlor with different levels of purity was evident. The 95% methoxychlor exhibited weak affinity for the ER, while the 99% methoxychlor did not compete for the ER. It has been demonstrated previously that a phenolic contaminant in methoxychlor preparations is estrogenic and that this contaminant may be dihydroxymethoxychlor (HPTE), a metabolite of methoxychlor (Bulger *et al*., 1978a,b; Cummings, 1997). Since the pesticides (the DDT and methoxychlor isomers) within this group of chemicals exhibited affinity for the ER, it was important to determine whether other pesticides could also compete. Of the other pesticides tested (Table 8), only kepone bound with moderate affinity to the ER. None of the remaining pesticides analyzed in the ER competitive-binding assay exhibited any activity at the concentrations tested.

Table 9 shows the RBAs for several paraben compounds. All of the parabens examined in this study competed for the ER. Of the 7 chemicals analyzed, one bound the ER with moderate affinity while the rest exhibited weak binding. The parabens, like the alkylphenolic compounds, demonstrated a positive correlation between binding affinity and chain length.

TABLE 5
IC_{50}s and Relative Binding Affinities (RBA) for Alkylphenolic Compounds

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
4-Nonylphenol (Lot 14081-001)[a]	Schenectady	95.6	$2.40 \times 10^{-6} \pm 0.30 \times 10^{-6}$	0.037	–1.43
4-Nonylphenol (Lot 10004ES)[a]	Aldrich	Tech	$2.60 \times 10^{-6} \pm 0.30 \times 10^{-6}$	0.035	–1.46
4-Nonylphenol (Analysis #: 383810/1 51998)[a]	Fluka	85	$2.90 \times 10^{-6} \pm 0.80 \times 10^{-6}$	0.031	–1.51
4-Nonylphenol (Lot 14110BR)[a]	Aldrich	Tech	$3.05 \times 10^{-6} \pm 0.15 \times 10^{-6}$	0.029	–1.53
4-Nonylphenol (Analysis #: 347353/1 897)[a]	Fluka	85	$4.73 \times 10^{-6} \pm 0.93 \times 10^{-6}$	0.019	–1.72
4-Dodecylphenol (mixture of isomers)[a]	Aldrich	99.7	$4.85 \times 10^{-6} \pm 1.95 \times 10^{-6}$	0.019	–1.73
4-*tert*-Octylphenol	Aldrich	97	$6.00 \times 10^{-6} \pm 1.10 \times 10^{-6}$	0.015	–1.82
4-Octylphenol	Aldrich	99	$1.95 \times 10^{-5} \pm 0.15 \times 10^{-5}$	0.005	–2.34
4-*n*-Nonylphenol	Lancaster	98	$2.80 \times 10^{-5} \pm 0.10 \times 10^{-5}$	0.0032	–2.49
4-*tert*-Amylphenol	Aldrich	99	$1.65 \times 10^{-4} \pm 0.45 \times 10^{-4}$	0.0005	–3.26
4-*sec*-Butylphenol	Aldrich	96	$2.10 \times 10^{-4} \pm 0.30 \times 10^{-4}$	0.00043	–3.37
4-Chloro-3-methylphenol	Aldrich	99	$2.15 \times 10^{-4} \pm 0.15 \times 10^{-4}$	0.00042	–3.38
2-*sec*-Butylphenol	Aldrich	98	$3.15 \times 10^{-4} \pm 0.05 \times 10^{-4}$	0.00029	–3.54
4-*tert*-Butylphenol	Aldrich	99	$3.68 \times 10^{-4} \pm 0.83 \times 10^{-4}$	0.00024	–3.61
2-Chloro-4-methylphenol	Aldrich	97	$4.15 \times 10^{-4} \pm 1.75 \times 10^{-4}$	0.00022	–3.66
4-Chloro-2-methylphenol	Aldrich	97	$4.25 \times 10^{-4} \pm 1.05 \times 10^{-4}$	0.00021	–3.67
3-Ethylphenol	Aldrich	80	$6.60 \times 10^{-4} \pm 0.76 \times 10^{-4}$	0.00014	–3.87
4-Ethylphenol	Aldrich	99	$1.34 \times 10^{-3} \pm 0.04 \times 10^{-3}$	0.00007	–4.17
2-Ethylphenol	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
Eugenol	Supelco	99.2	$>1.00 \times 10^{-3}$	—	—
Isoeugenol (mixture of *cis* and *trans*)	Aldrich	98	$>1.00 \times 10^{-4}$	—	—

[a] Chemical exhibited a U-shaped binding curve.

The chemicals with the longer side chains (2-ethylhexyl, heptyl, and benzyl 4-hydroxybenzoates) showed greater affinity for the ER compared to the parabens with shorter side chains (butyl, propyl, ethyl, and methyl 4-hydroxybenzoates).

Under the conditions of our ER competitive-binding assay, none of the 8 phthalate compounds exhibited an IC_{50} (Table 10). However, 2 of these chemicals, benzylbutyl phthalate and BIS(2-ethylhexyl) phthalate, did compete for the ER (Fig. 3a).

TABLE 6
IC_{50}s and Relative Binding Affinities (RBA) for Diphenyl Derivatives

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
Diphenyl methane derivatives (bisphenol A's)					
2,2-Bis-(4-hydroxyphenyl)-butane (*bisphenol B*)	Aldrich	NA	$1.05 \times 10^{-6} \pm 0.46 \times 10^{-6}$	0.086	–1.07
Bisphenol A	Aldrich	99	$1.17 \times 10^{-5} \pm 0.64 \times 10^{-5}$	0.008	–2.11
2,2′-Methylenebis (4-chlorophenol)	Aldrich	90	$2.55 \times 10^{-5} \pm 0.15 \times 10^{-5}$	0.004	–2.45
BIS (4-hydroxyphenyl)-methane	Aldrich	98	$9.50 \times 10^{-5} \pm 0.50 \times 10^{-5}$	0.0009	–3.02
4,4′-Sulfonyldiphenol	Sigma	99	$1.05 \times 10^{-4} \pm 0.35 \times 10^{-4}$	0.0009	–3.07
Diphenolic acid	Aldrich	95	$1.20 \times 10^{-4} \pm 0.30 \times 10^{-4}$	0.0007	–3.13
4,4′-Methylenebis (2,6-di-*tert*-butylphenol)	Aldrich	98	$>1.00 \times 10^{-4}$	—	—
BIS (2-hydroxyphenyl)-methane	Aldrich	98	$>1.00 \times 10^{-5}$	—	—
Diphenyl ethane derivatives					
4,4′-Dihydroxystilbene[a]	NCI	NA	$3.20 \times 10^{-7} \pm 0.90 \times 10^{-7}$	0.281	–0.55
2,2′,4,4′-Tetrahydroxybenzil	NCI	NA	$4.30 \times 10^{-7} \pm 0.00$	0.209	–0.68
4,4′-Ethylene diphenol	NCI	NA	$2.45 \times 10^{-6} \pm 0.35 \times 10^{-6}$	0.037	–1.44
4-Phenethylphenol	NCI	NA	$4.40 \times 10^{-5} \pm 0.60 \times 10^{-5}$	0.002	–2.69
4-Stilbenol	NCI	NA	$>1.00 \times 10^{-4}$	—	—
Biphenyl derivatives					
4-Phenylphenol	Aldrich	90	$9.80 \times 10^{-5} \pm 5.20 \times 10^{-5}$	0.001	–3.04
3-Phenylphenol	Aldrich	90	$2.45 \times 10^{-4} \pm 0.45 \times 10^{-4}$	0.0004	–3.44
2-Phenylphenol	Aldrich	99	$>1.00 \times 10^{-4}$	—	—

[a] Chemical exhibited a U-shaped binding curve.

TABLE 7
IC_{50}s and Relative Binding Affinities (RBA) for Organochlorines

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
DDTs					
o,p′-DDT	Supelco	98.5	$6.43 \times 10^{-5} \pm 0.89 \times 10^{-5}$	0.001	–2.85
o,p′-DDD	Supelco	99.2	$>3.00 \times 10^{-4}$	—	—
p,p′-DDD	Supelco	98.5	$>1.00 \times 10^{-4}$	—	—
o,p′-DDE	Supelco	99.8	$>5.00 \times 10^{-4}$	—	—
p,p′-DDE	Supelco	99.4	$>1.00 \times 10^{-4}$	—	—
p,p′-DDT	Supelco	99.2	$>1.00 \times 10^{-3}$	—	—
Methoxychlor derivatives					
Dihydroxymethoxychlor olefin	NIEHS[a]	98	$3.40 \times 10^{-8} \pm 4.00 \times 10^{-8}$	2.644	0.42
Dihydroxymethoxychlor (*HPTE*)	NIEHS	98	$3.55 \times 10^{-7} \pm 0.15 \times 10^{-7}$	0.253	–0.60
Monohydroxymethoxychlor olefin	NIEHS	98	$3.90 \times 10^{-7} \pm 0.80 \times 10^{-7}$	0.231	–0.64
Monohydroxymethoxychlor	NIEHS	98	$6.90 \times 10^{-7} \pm 0.10 \times 10^{-7}$	0.130	–0.89
Methoxychlor	Sigma	95	$1.44 \times 10^{-4} \pm 0.66 \times 10^{-4}$	0.001	–3.20
Methoxychlor	Supelco	99	$>1.00 \times 10^{-4}$	—	—
Methoxychlor olefin	Supelco	95	$>1.00 \times 10^{-4}$	—	—
PCBs					
2′,3′,4′,5′-Tetrachloro-4-biphenylol[b]	Ultra Scientific	95	$3.95 \times 10^{-7} \pm 0.15 \times 10^{-7}$	0.228	–0.64
2′,5′-Dichloro-4-biphenylol	Ultra Scientific	95	$2.50 \times 10^{-6} \pm 0.30 \times 10^{-6}$	0.036	–1.44
4-Chloro-4′-biphenylol	Ultra Scientific	95	$1.35 \times 10^{-5} \pm 0.15 \times 10^{-5}$	0.007	–2.18
2-Chloro-4-biphenylol	Ultra Scientific	95	$5.25 \times 10^{-5} \pm 2.55 \times 10^{-5}$	0.002	–2.77
3,3′,5,5′-Tetrachloro-4,4′-biphenyldiol	Ultra Scientific	95	$1.60 \times 10^{-4} \pm 0.10 \times 10^{-4}$	0.001	–3.25
2,4′-Dichlorobiphenyl	Ultra Scientific	99	$3.65 \times 10^{-4} \pm 1.15 \times 10^{-4}$	0.0002	–3.61
2,2′,4,4′-Tetrachlorobiphenyl	Ultra Scientific	98.4	$>1.00 \times 10^{-4}$	—	—
3,3′,4,4′-Tetrachlorobiphenyl	Ultra Scientific	99	$>3.00 \times 10^{-4}$	—	—
4,4′-Dichlorobiphenyl	Ultra Scientific	98.6	$>3.00 \times 10^{-4}$	—	—

[a] NIEHS = chemicals provided by Dr. Tom Burka, National Institute of Environmental Health Sciences.
[b] Chemical exhibited a U-shaped binding curve.

At a concentration of 1×10^{-3} M, benzylbutyl phthalate and BIS(2-ethylhexyl phthalate) exhibited 66% and 78% [^{3}H]-E_2 bound, respectively. Since the phthalates are apparently such weak binders, it is possible that they had not reached equilibrium during the 20-h, 0°C incubation period of the ER competitive-binding assay. Therefore, an assay was conducted that examined benzylbutyl phthalate binding at 4°C at twice-daily intervals for 3 days (Fig. 3b). The results of this assay demonstrated that extending the incubation time had no effect on binding of benzylbutyl phthalate to the ER. As a final test, we conducted an assay in which the 20-h, 4°C incubation period was replaced by a 30-min, 30°C incubation followed by a 30-min, 4°C incubation (Fig. 3c). Under these assay conditions, ER binding of benzylbutyl phthalate ($IC_{50} = 7.2 \times 10^{-5}$ M), diethyl phthalate ($IC_{50} = 5.0 \times 10^{-3}$ M), and dimethyl phthalate ($IC_{50} = 9.9 \times 10^{-3}$ M) was achieved, with benzylbutyl and dibutyl phthalate exhibiting U-shaped binding curves.

The remaining test chemicals consisted of benzophenone compounds (Table 11) and several miscellaneous classes of chemicals (Table 12). Only 2 of the 5 benzophenone compounds competed for the ER. Of the remaining 39 chemicals assayed (Table 12), 3 showed moderate affinity and 4 exhibited weak affinity for the ER, while the other 32 were inactive at the concentrations tested.

There were also a number of test chemicals that competed for the ER, but did so with such a weak affinity that an IC_{50} was not attainable (Table 13). While the chemicals that exhibited this slight affinity for the ER were structurally diverse, including 4 organochlorines and 2 phthalates, they were also environmentally important.

DISCUSSION

To our knowledge, this is the first paper to report the ER-binding affinities of such a large number (188) of structurally diverse chemicals assayed under identical conditions. Other published results (Andersen *et al*., 1999; Bolger *et al*., 1998; Kuiper *et al*., 1997, 1998; Perez *et al*., 1998; Shelby *et al*., 1996; Waller *et al*., 1996) report the ER RBAs for smaller and less structurally diverse data sets.

In the current study, the rats utilized as the source of the ER were retired breeders. Although this is not a common practice, ER levels in ovariectomized, retired breeders are comparable to both immature, intact animals (Clark *et al*., 1978; van Doorn *et al*., 1982) and adult, ovariectomized rats (Medlock *et al*., 1991). Similar binding results were evident between the current study and a previous study using immature rats (Perez *et al*., 1999), further indicating that the age of the rats used as an ER source is not critical. While ER levels are similar between

TABLE 8
IC_{50}s and Relative Binding Affinities (RBA) for Other Pesticides

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
Kepone	Supelco	Neat	$7.00 \times 10^{-6} \pm 1.00 \times 10^{-6}$	0.013	–1.89
2,4,5-T	Supelco	98	$>1.00 \times 10^{-3}$	—	—
2,4-Dichlorophenoxyacetic acid (*2,4-D*)	Supelco	99	$>1.00 \times 10^{-4}$	—	—
α-Chlordane (mix of isomers)	Supelco	Neat	$>1.00 \times 10^{-3}$	—	—
Alachlor	Supelco	98.8	$>1.00 \times 10^{-4}$	—	—
Aldrin	Supelco	98	$>6.00 \times 10^{-4}$	—	—
Atrazine	Supelco	98	$>1.00 \times 10^{-4}$	—	—
Carbaryl	Supelco	99	$>1.00 \times 10^{-4}$	—	—
Carbofuran	Aldrich	98	$>1.00 \times 10^{-4}$	—	—
Dieldrin (pure)	Supelco	98	$>1.00 \times 10^{-4}$	—	—
Dieldrin (technical grade)	Aldrich	90	$>1.00 \times 10^{-4}$	—	—
Endosulfan	Supelco	99	$>1.00 \times 10^{-3}$	—	—
Heptachlor	Supelco	99.5	$>1.00 \times 10^{-4}$	—	—
Hexachlorobenzene	Supelco	Neat	$>1.00 \times 10^{-3}$	—	—
Lindane	Supelco	99	$>1.00 \times 10^{-4}$	—	—
Metolachlor	Supelco	98.7	$>1.00 \times 10^{-4}$	—	—
Mirex	Supelco	99	$>1.00 \times 10^{-4}$	—	—
Prometon	Supelco	Neat	$>1.00 \times 10^{-3}$	—	—
Simazine	Supelco	99	$>3.33 \times 10^{-5}$	—	—
Vinclozolin	Supelco	98.2	$>1.00 \times 10^{-4}$	—	—

these rats of different ages, the use of retired breeders has three distinct advantages. First, retired breeders have greater uterine weights, which results in more total ER available for use in binding studies. Second, due to the larger uterine weights, fewer animals are required. Lastly, the use of retired breeders is essentially an animal-sparing process, since the ability to use these animals, which would be disposed of under normal situations, allows us to conduct these studies without having to purchase and subsequently sacrifice new animals.

The reason for the U-shaped binding curves observed in the present study remains unclear. However, it has been shown that high concentrations of steroids and antiestrogens can markedly accelerate the dissociation rate of the ER-[^{3}H]-E_2 complex (Borgna and Ladrech, 1982). Chemicals in the present study that exhibited this response did so at concentrations greater than 1×10^{-5} M, with the majority of them being at concentrations greater than 1×10^{-4} M. Therefore, the U-shaped binding curves presented here are most likely due to the high doses assayed and the kinetics of the binding assay, and as such they are probably not associated with the U-shaped dose-response curves observed at low doses *in vivo* for some chemicals (vom Saal *et al*., 1997).

Of the variety of chemical classes analyzed in the current study, two were of particular interest. These were the phthalates and the parabens. The phthalic acid esters (phthalates) are widely used as plasticizing agents; however, since they are not covalently bound within the plastic, the phthalates can be released into the environment (Autian, 1973; Giam *et al*., 1978; Thomas and Thomas, 1984). Since phthalates are capable of altering reproductive function in rats (Ema *et al*., 1998; Wine *et al*., 1997) and since estrogens are a primary regulator of female reproduction, it is possible that phthalates are working through the ER. Previous studies have suggested that di-*n*-butyl phthalate, benzylbutyl phthalate and BIS(2-ethylhexyl)

TABLE 9
IC_{50}s and Relative Binding Affinities (RBA) for Alkyl Hydroxy Benzoate Preservatives (Parabens)

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
2-Ethylhexyl 4-hydroxybenzoate[a]	Pfaltz & Bauer, Inc.	99	$4.95 \times 10^{-6} \pm 0.05 \times 10^{-6}$	0.018	–1.74
Heptyl 4-hydroxybenzoate[a]	Pfaltz & Bauer, Inc.	97	$1.10 \times 10^{-5} \pm 0.10 \times 10^{-5}$	0.008	–2.09
Benzyl 4-hydroxybenzoate	Aldrich	99	$3.15 \times 10^{-5} \pm 0.35 \times 10^{-5}$	0.003	–2.54
Butyl 4-hydroxybenzoate	Sigma	99	$1.05 \times 10^{-4} \pm 0.35 \times 10^{-4}$	0.0009	–3.07
Propyl 4-hydroxybenzoate	Aldrich	99	$1.50 \times 10^{-4} \pm 0.10 \times 10^{-4}$	0.0006	–3.22
Ethyl 4-hydroxybenzoate	Aldrich	99	$1.50 \times 10^{-4} \pm 0.10 \times 10^{-4}$	0.0006	–3.22
Methyl 4-hydroxybenzoate	Aldrich	99	$2.45 \times 10^{-4} \pm 0.65 \times 10^{-4}$	0.0004	–3.44

[a] Chemical exhibited a U-shaped binding curve.

TABLE 10
IC_{50}s and Relative Binding Affinities (RBA) for Phthalates

Chemical name	Source	Purity (%)	Mean IC_{50} (M)	RBA (%)	Log RBA
Benzylbutyl phthalate	Aldrich	98	$>1.00 \times 10^{-3}$	—	—
BIS (2-ethylhexyl) phthalate	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
Dibutyl phthalate	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
Diethyl phthalate	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
Di-isobutyl phthalate	Fluka	98	$>1.00 \times 10^{-3}$	—	—
Di-isononyl phthalate	Fluka	Tech	$>1.00 \times 10^{-3}$	—	—
Dimethyl phthalate	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
n-Dioctyl phthalate	Fluka	98	$>1.00 \times 10^{-3}$	—	—

phthalate are weak binders or weakly estrogenic in a variety of systems (Bolger *et al*., 1998; Coldham *et al*., 1997; Jobling *et al*., 1995; Nakai *et al*., 1999; Soto *et al*., 1995; Waller *et al*., 1996; Zacharewski *et al*., 1998). However, we were unable to determine IC_{50} values for any of the phthalate compounds analyzed in our ER competitive-binding assay. Nonetheless, two of the phthalates, benzylbutyl phthalate and BIS(2-ethylhexyl) phthalate, competed with E_2 for the ER. Utilizing rainbow trout ER, Jobling *et al*. (1995) was able to establish an IC_{50} for benzylbutyl phthalate as well as binding for BIS(2-ethylhexyl) and di-*n*-butyl phthalate. However, the binding curves were not parallel with that of E_2, suggesting that the phthalates may be working through or influenced by an alternative mechanism. Other studies have also reported IC_{50} values for benzylbutyl phthalate and di-*n*-butyl phthalate, using a human recombinant ER (hrER) (Bolger *et al*., 1998; Nakai *et al*., 1999) and rat uterine cytosol (Zacharewski *et al*., 1998). Zacharewski and colleagues (1998) also demonstrated that dihexyl phthalate competed with [^{3}H]-E_2 for the ER, though not with strong enough affinity to attain an IC_{50} value. However, these studies utilized one-day ER competitive-binding assays, which included either a 30-min, 30°C (Zacharewski *et al*., 1998) or a 60-min, room temperature (Bolger *et al*., 1998; Nakai *et al*., 1999) incubation period. Replacing the standard 20-h, 4°C incubation with a 30-minute, 30°C incubation followed by a 30-min, 4°C incubation increased the ability of the phthalates to bind the ER. Waller and co-workers (1996) reported pK_is for both benzylbutyl phthalate and di-*n*-butyl phthalate using an 18-h, 4°C incubation in their ER competitive-binding assay. However, these authors measured binding to a mouse uterine cytosol ER preparation and differences in binding affinities may be related to variation in species sensitivity. When comparing the data presented here to data collected from competitive-binding assays that utilized a short-term incubation period at room temperature or above (Arcaro *et al*., 1998; Bolger *et al*., 1998; Klotz *et al*., 1996), it was evident that differences in RBA values were relatively small. In general, RBA values for low affinity xenobiotics were slightly higher in the short term, high temperature assays compared to the assay utilized in the current study, while high affinity chemicals exhibited similar binding affinities. This suggests that differences in assay conditions can lead to the observed differences in binding affinities and that high-temperature assays might be somewhat more sensitive to certain chemicals. However, high-temperature assays run the risk of ER degradation during the assay.

It has been demonstrated in numerous studies that parabens compounds produce a variety of toxic and physiological effects. Chang and Voelkel (1986) reported that both methyl and propyl parabens are present in a commercial preparation of naloxone (Narcan®) at high concentrations (1.8 mg/ml of methyl [1.2×10^{-2} M] and 0.2 mg/ml of propyl [1.1×10^{-3} M] parabens). They also demonstrated that the vasodilatory effects of Narcan are due entirely to the parabens. Various paraben compounds have also been shown to cause severe damage to rat hepatocytes (Nakagawa *et al*., 1998; Sugihara, *et al*., 1997) and reduced ciliary beat frequency in rat trachea (Jian and Po, 1993a,b). Nakagawa *et al*. (1998) indicated that hepatocyte toxicity was correlated with alkyl side-chain length such that butyl and iso-butyl parabens were more toxic than propyl and

TABLE 11
IC_{50}s and Relative Binding Affinities (RBA) for Benzophenone Compounds

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
4,4′-Dihydroxybenzophenone	Aldrich	99	$2.60 \times 10^{-5} \pm 0.40 \times 10^{-5}$	0.003	–2.46
2,4-Dihydroxybenzophenone	Aldrich	99	$3.65 \times 10^{-5} \pm 0.45 \times 10^{-5}$	0.002	–2.61
2,2′-Dihydroxy-4-methoxybenzophenone	Aldrich	98	$>1.00 \times 10^{-4}$	—	—
2,2′-Dihydroxybenzophenone	Aldrich	98	$>1.00 \times 10^{-4}$	—	—
2-Hydroxy-4-methoxybenzophenone	Aldrich	98	$>1.00 \times 10^{-4}$	—	—

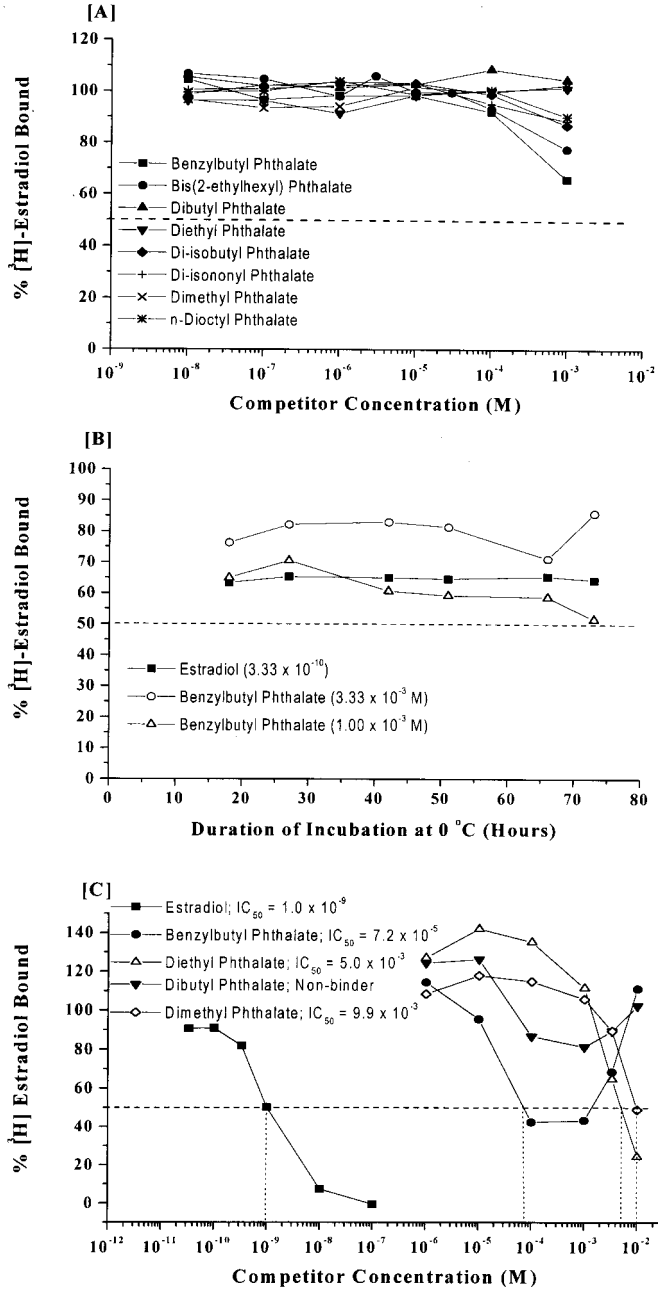

FIG. 3. Estrogen receptor binding affinity curves for the phthalate compounds. **A** depicts the binding curves for the entire phthalate group using our standard ER competitive-binding assay. None of the phthalates competed strongly for the ER; however, benzylbutyl and BIS(2-ethylhexyl) phthalate showed slight competition for the ER. **B** shows curves from a single equilibrium test with benzylbutyl phthalate. Two concentrations of benzylbutyl phthalate were assayed in our ER competitive-binding assay with extended incubation periods. Extending the incubation period did not increase affinity for the ER. **C** demonstrates phthalate binding in an alternative assay procedure in which the 20-h, 4°C incubation was replaced by a 30-min, 30°C incubation period followed by a 30-min, 4°C incubation period. Replacing the normal incubation conditions with a short-term, high-temperature incubation resulted in 3 of the 4 phthalates tested binding to the ER with very weak affinity.

iso-propyl parabens, which in turn were more toxic than ethyl and methyl parabens. This correlates well with the binding affinity data in the current study, which demonstrated increasing RBA values with increasing alkyl-side chain length. Despite these obvious toxic effects, parabens compounds are present in a wide variety of products including cosmetics (Rastogi *et al*., 1995), pharmaceutical products (Chang and Voelkel, 1986; Pompy *et al*., 1991; van Faassen *et al*., 1990), cigarettes (Castano, *et al*., 1988), and honey bee royal jelly (Ishiwata *et al*., 1995). In fact, in 1981, the Food and Drug Administration reported that four parabens compounds (methyl, ethyl, propyl, and butyl parabens) were used as preservatives in more than 13,200 formulations, including most cosmetic products, due to their antimicrobial properties (Elder, 1984). Although the antimicrobial activity of the parabens compounds is positively correlated with alkyl-chain length, their water solubility is negatively correlated. Therefore, the short-chain parabens compounds are generally used in formulations (Dal Pozzo and Pastori, 1996). Nonetheless, we and others (Routledge *et al*., 1998) have demonstrated that even the smaller parabens compounds are weak ER competitors. Cytotoxicity (Nakagawa *et al*., 1998) and antimicrobial activity (Dal Pozzo and Pastori, 1996) are not the only characteristics associated with alkyl side-chain length. Percutaneous absorption (Lee and Kim, 1994; Twist and Zatz, 1986) and transactivation of the ER (Routledge *et al*., 1998) also increase with alkyl side-chain length. Thus, due to their inherent estrogenicity and their wide range of applications, it is apparent that the parabens compounds pose a potential hazard as endocrine disruptors.

The determination of ER-binding affinities in a group of chemicals with such a wide variety of structural diversity is also beneficial because it increases one's knowledge of chemicals which are inadvertently estrogenic. For example, to our knowledge, 4-benzyloxyphenol, 2,4-dihydroxybenzophenone, and 2,2′-methylenebis(4-chlorophenol) have not previously been demonstrated to bind the ER. The 4-benzyloxyphenol (commonly called monobenzone) is a therapeutic agent used as a depigmenting compound. It has been widely used in the treatment of vitiligo, a skin disease which effects 1–3% of the population and is characterized by depigmentation of areas of the skin (Kenney, 1988). This chemical has also been examined as an alternative branding method in beef cattle (Schwartzkopf *et al*., 1994). Benzoresorcinol (2,4-dihydroxybenzophenone) is commonly used as a plastic additive (Spyropoulos 1998). It is also a metabolite of benzophenone-3, an absorber of ultraviolet light used extensively as a sunscreen and color fastener (Okereke *et al*., 1993, 1994). Dermal treatment of Sprague-Dawley rats with benzophenone-3 resulted in detection of it's metabolites, including 2,4-dihydroxybenzophenone, in plasma five min after administration (Okereke *et al*., 1994). This metabolite was detected in most tissues within six h after administration, with the highest concentration observed in the liver (Okereke *et al*., 1993). 2,2′-Methylenebis(4-chlorophenol), commonly called dichlorophen, is used as an

TABLE 12
IC_{50}s and Relative Binding Affinities (RBA) for Miscellaneous Compounds

Chemical name	Source	Purity (%)	Mean IC_{50} (M) ± SEM	RBA (%)	Log RBA
Acids					
Castor oil	Sigma	NA	$>1.00 \times 10^{-4}$	—	—
Cinnamic acid	Supelco	99.9	$>1.00 \times 10^{-3}$	—	—
Folic acid	Sigma	NA	$>1.00 \times 10^{-4}$	—	—
Suberic acid	Sigma	99	$>1.00 \times 10^{-4}$	—	—
Alcohols					
1,8-Octanediol	Aldrich	98	$>1.00 \times 10^{-4}$	—	—
Benzyl alcohol	Supelco	99.7	$>1.00 \times 10^{-2}$	—	—
Hexyl alcohol	Supelco	98.9	$>1.00 \times 10^{-2}$	—	—
Nerolidol	Supelco	97.7	$>1.00 \times 10^{-3}$	—	—
Aldehydes					
2-Furaldehyde (*Furfural*)	Supelco	99.4	$>1.00 \times 10^{-3}$	—	—
Heptanal	Supelco	92.9	$>1.00 \times 10^{-2}$	—	—
Vanillin	Aldrich	99	$>1.00 \times 10^{-4}$	—	—
Amines					
4,4′-Diaminostilbene dihydrochloride	Aldrich	95	$>1.00 \times 10^{-4}$	—	—
4,4′-Methylenebis (N,N-dimethylaniline)	Aldrich	98	$>1.00 \times 10^{-3}$	—	—
4,4′-Methylenedianiline	Aldrich	97	$>2.33 \times 10^{-4}$	—	—
4-Aminophenyl ether (*4,4′-Oxydianiline*)	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
Butyl-4-aminobenzoate	Aldrich	99	$>1.00 \times 10^{-4}$	—	—
Ethers/esters					
4-Heptyloxyphenol6[a]	Aldrich	97	$6.75 \times 10^{-5} \pm 0.75 \times 10^{-5}$	0.0013	–2.88
4-Benzyloxyphenol	Aldrich	99	$2.50 \times 10^{-4} \pm 0.50 \times 10^{-4}$	0.00036	–3.44
Cineole	Supelco	90	$>1.00 \times 10^{-2}$	—	—
Dibenzo-18-crown-6	Aldrich	98	$>1.00 \times 10^{-5}$	—	—
Ethyl cinnamate	Supelco	99.1	$>1.00 \times 10^{-3}$	—	—
Hydrocarbons					
1,6-Dimethylnaphthalen	Aldrich	99	$>1.00 \times 10^{-4}$	—	—
Benzo[a]fluorene	Aldrich	98	$>3.33 \times 10^{-5}$	—	—
sec-Butylbenzene	Aldrich	99	$>1.00 \times 10^{-3}$	—	—
Chrysene	Aldrich	98	$>1.00 \times 10^{-5}$	—	—
n-Butylbenzene	Aldrich	99	$>2.00 \times 10^{-4}$	—	—
trans, trans-1,4-Diphenyl-1,3-butadiene	Aldrich	98	$>1.00 \times 10^{-4}$	—	—
Others					
Aurin	Sigma	Practical	$2.80 \times 10^{-6} \pm 1.80 \times 10^{-6}$	0.032	–1.49
Nordihydroguariaretic acid	Aldrich	97	$2.90 \times 10^{-6} \pm 1.60 \times 10^{-6}$	0.031	–1.51
Phenolphthalein	Aldrich	NA	$6.73 \times 10^{-6} \pm 1.79 \times 10^{-6}$	0.013	–1.87
Phenol red	Aldrich	95	$1.60 \times 10^{-4} \pm 0.60 \times 10^{-4}$	0.001	–3.25
Phenolphthalin	Sigma	99	$4.25 \times 10^{-4} \pm 0.75 \times 10^{-4}$	0.0002	–3.67
2-Chlorophenol	Aldrich	99	$>2.00 \times 10^{-4}$	—	—
Amaranth	Aldrich	80	$>1.00 \times 10^{-4}$	—	—
Caffeine	Sigma	Ultra	$>1.00 \times 10^{-4}$	—	—
Dopamine	Sigma	99	$>1.00 \times 10^{-4}$	—	—
Melatonin	Aldrich	97	$>1.00 \times 10^{-4}$	—	—
Thalidomide	BiolMol	99	$>1.00 \times 10^{-3}$	—	—
Triphenyl phosphate	Aldrich	99	$>1.00 \times 10^{-4}$	—	—

[a] Chemical exhibited a U-shaped binding curve.

agricultural fungicide, a germicide in soaps and shampoos, and therapeutically as an anthelmintic.

Even absent the computational models, certain structural features important for ER binding can be discerned. A ring structure is of primary importance to ER-binding affinity. Although chemicals with a ring structure may or may not bind the ER, chemicals lacking a ring structure apparently will not bind the ER. In the present study, we were specifically attempting to identify potentially estrogenic chemicals so the vast majority of chemicals tested possessed a ring structure. Of the 6 chemicals that did not possess ring structures, none exhibited affinity for the ER. With the exception of 4 chemicals (kepone, norethynodrel, 5α-androstane-3α, 17β-diol, and 5α-androstane-3β,17β-diol), all test chemicals that actively bound the ER contained benzene rings. In addition to a ring structure, all the actively competing test chemicals, with the exception of

TABLE 13
Test Compounds that Competed for the ER but Did Not Attain a Measurable IC_{50}

Chemical name	Chemical class	Mean % [^{3}H]-estradiol bound ± SEM	Concentration (M)
Dihydrotestosterone	Miscellaneous steroid	60.23 ± 0.74	3.33×10^{-5}
3,3′,4,4′-Tetrachlorobiphenyl	Organochlorine	61.89	3.33×10^{-4}
Benzylbutyl phthalate	Phthalate	66.13 ± 5.06	1.00×10^{-3}
o,p′-DDE	Organochlorine	71.65 ± 1.63	5.33×10^{-4}
BIS(2-ethylhexyl) phthalate	Phthalate	77.74 ± 6.75	1.00×10^{-3}
o,p′-DDD	Organochlorine	78.42 ± 1.62	3.33×10^{-4}
Methoxychlor olefin	Organochlorine	80.46 ± 3.01	1.00×10^{-4}

triphenylethylene, o,p′-DDT, and 2,4-dichlorobiphenyl, contained an oxygen atom on the ring. It is well known that a phenolic ring is important for estrogenicity (Anstead *et al*., 1997) and it has been suggested that the hydroxyl group on the phenol ring acts as both a hydrogen bond donor and acceptor in the ER-binding site (Duax and Weeks, 1980). Substitution of the hydroxyl hydrogen atom by an alkyl group such as a methyl group significantly decreases the chemical's affinity for the ER. For example, diethylstilbestrol dimethyl ether (RBA = 0.056) exhibited a greatly lowered affinity for the ER when compared to the non-methylated diethylstilbestrol (RBA = 399.56). In addition, methoxychlor, which contains 2 methyl groups, showed a much lower ER-binding affinity (RBA = 0.001) than dihydroxymethoxychlor (RBA = 0.253), which contains no methyl groups. However, not all substitutions result in significant suppression of ER-binding affinity. Some of the antiestrogens (tamoxifen, clomiphene, nafoxidine, and toremifene) remain strong binders despite the lack of a phenolic ring. In general, the results indicate that chemicals with 2 ring structures separated by 2 carbon atoms (steroidal and synthetic estrogens and diphenyl ethanes) have higher RBAs compared to chemicals with a single ring structure (alkylphenols, phthalates and parabens) or 2 rings separated by one carbon atom (bisphenol A derivatives and benzophenone compounds). Interestingly, ER binding was even observed for very small chemicals such as ethylphenol and 2-chloro-4-methylphenol, further confirming the importance of the phenolic ring for binding. Crystallographic data of the ER complex with a number of chemicals, including E_2, DES, raloxifene and 4-hydroxytamoxifen, demonstrated that the critical spacing of hydrophobic and hydrogen bond interactions play a major role in determining binding affinity (Brzozowski *et al*., 1997; Shiau *et al*., 1998). Although hydrophobic interactions and hydrogen bonding are important for high-affinity ligands, they are clearly less important for low-affinity ligands (unpublished data).

Since the data from the ER competitive-binding assays are being utilized for the development of a computational QSAR model (Shi *et al*., unpublished), a large number of test chemicals, which provided a wide range of structural diversity with approximately similar numbers of binders in each decade of RBA values, was intentionally selected. These included chemicals that were both ER binders and non-binders, and were environmentally and commercially relevant. Specifically, the data for chemicals that do bind are being used to develop a robust comparative molecular field analysis (CoMFA) QSAR model, which can be used to predict the ER-binding affinity for specific chemicals of interest, including some 8,000 FDA-regulated chemicals. This allows one to identify new avenues for future research. Due to the large number of chemicals tested and their structural diversity, this data set will also be useful in the development of other computational predictive models. In addition to the CoMFA model, other QSAR models (classical, hologram, and pharacophore-based models), as well as a number of classification models (e.g., K-Nearest Neighbor and Self Independent Modeling of Chemical Analogy), which qualitatively predict whether a chemical will bind or not bind, are currently being explored. These models, individually or in a complementary sequence, may allow more complicated biological systems to be modeled. The strengths and weaknesses of various QSAR models/programs (Hansch, CoMFA, HQSAR, etc.) have been previously presented (Tong *et al*., 1997, 1998). The published ER crystal structure (Brzozowski *et al*., 1997; Shiau *et al*., 1998) is also being utilized, in combination with the current data set, to examine a "uniform docking" approach, which will enable one to identify the binding conformation of a diverse group of chemicals. Using an array of molecular modeling approaches, common structural features that are related to biological activity can be identified. Once identified, these structural features can be used to rapidly identify untested, potentially estrogenic chemicals.

In summary, the current study has analyzed a large number of structurally diverse chemicals for their ability to bind to the rat uterine ER. Compared to other published data sets, this is the largest and most diverse group of chemicals tested to date using a single assay. By examining such a large group of chemicals, we were able to identify several chemicals not previously known to bind the ER. Relative binding affinities of these diverse chemicals were often highly correlated with specific structural features. In particular, an overall ring structure was important to a chemical's ability to bind the ER; the presence of a phenolic ring was indicative of a chemical's affinity for the ER; and alterations in hydroxylation and alkyl side-chain length also affected a chemical's RBA. These data

are useful in a number of areas. For example, the RBA data are being utilized as a training set for the construction of a robust CoMFA QSAR model as well as other QSAR and chemometric models; these models can be useful as priority setting tools in the EDSTAC process. The large RBA data set presented here can also be used for comparisons with other ER-binding results generated under varying assay conditions.

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