Identification of Environmental Chemicals with Estrogenic Activity Using a Combination of *In Vitro* Assays

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Environmental chemicals that function as estrogens have been suggested to be associated with an increase in disease and dysfunctions in animals and humans. To characterize chemicals that may act as estrogens in humans, we have compared three *in vitro* assays which measure aspects of human estrogen receptor (hER)-mediated estrogenicity. Chemicals were first tested for estrogen-associated transcriptional activity in the yeast estrogen screen (YES). This was created by expressing hER and two estrogen response elements linked to the *lacZ* gene in yeast. Second, chemicals that were tested in YES were then assayed for direct interaction with hER in a competition binding assay. Third, chemicals were tested in the estrogen-responsive MCF-7 human breast cancer cell line transiently transfected with a plasmid containing two estrogen response elements linked to the luciferase gene. Together, these assays have identified two metabolites of DDT, *o,p'*-DDD and *p,p'*-DDD, that have estrogenic activity. Interestingly, previous studies had reported that the DDD metabolites were nonestrogenic in whole animal models. Alachlor, the most frequently used herbicide in the United States, *cis*-nonachlor, and *trans*-nonachlor displayed weak estrogenic activity in the combined assays. The antifungal agent benomyl had no estrogenic activity. We propose that a combination of *in vitro* assays can be used in conjunction with whole animal models for a more complete characterization of chemicals with estrogenic activity. *Key words*: environmental estrogens, estrogen receptor, estrogens, MCF-7 cells, yeast estrogen screen. *Environ Health Perspect* 104:1084–1089 (1996)

The development and maintenance of reproductive tissues is, to a large extent, controlled by steroid hormones. The effects of hormones are mediated through binding intracellular receptors and the interaction of hormone-receptor complexes with DNA. Recently, it has become apparent that hormonal responses can be generated in cell culture and in animals by environmental chemicals functioning as hormones or antihormones (*1*). In 1968 Bitman et al. (*2*) demonstrated that the pesticide *o,p'*-DDT produced characteristic estrogen responses in the reproductive tracts of rats and birds. Subsequent studies have reported that DDT can induce feminization of male sea gull embryos (*3*). A more recent study correlated a decrease in the population of alligators in Lake Apopka, Florida with a DDT and dicofol spill in the lake (*4*). Investigations into the mechanism(s) responsible for the actions of DDT have shown that its effects appear to be primarily mediated by its interaction with the estrogen receptor (*5*). Thus, environmental chemicals such as DDT, which interact with the estrogen receptor and display estrogenic activity have been classified as environmental estrogens. Numerous other environmental estrogens have been identified, e.g., bisphenol A (*6*), a byproduct of autoclaving polycarbonate; the phthalates di-*n*-butylphthalate and bis(2-ethylhexyl)phthalate (*7*), also in plastic; and the detergents octylphenyl and nonylphenol (*8,9*). Several polychlorinated biphenyls (PCBs), industrialized chemicals associated with adhesives, fire retardants, and waxes, have also been shown to be estrogenic, inducing the development of ovaries in turtles that would have otherwise hatched as males (*10*).

The identification of a large number of environmental estrogens and their effects on various wildlife species has focused attention on the association of environmental estrogens with human health. The concentration of the DDT metabolite *p,p'*-DDE in the sera of women has been associated with an increased risk for breast cancer (*11,12*). A separate study reported that the sera concentration of *p,p'*-DDE was a risk factor for breast cancer in Caucasian and African American women but not in Asian women (*13*). Other studies, however, have reported no correlation between levels of environmental chemicals and incidence of breast cancer (*14*). In addition to the potential impact on women's health, environmental estrogens have been suggested to account for decreased semen quality and increased testicular cancer in men (*15*). Nonetheless, the findings correlating environmental estrogens with adverse human health are still the focus of scientific debate and investigation.

The well-documented effects of environmental estrogens in animals and their potential for adverse effects in humans have led to the development of assays for identifying chemicals with estrogenic activity. In 1993, McLachlan (*16*) proposed a screening approach to determine the functional characteristics of environmental chemicals. Soto et al. (*17*) have utilized the E-SCREEN assay, which measures the proliferation of estrogen responsive MCF-7 cells as a marker of the estrogenicity of chemicals (*17*). The drawback to the manner in which the E-SCREEN was used is that chemicals identified as estrogenic were not tested for interaction with the estrogen receptor by determining the proliferation of MCF-7 cells in the presence of the estrogen receptor antagonists tamoxifen or ICI 164,384. It has been suggested that the hormone activity of environmental chemicals can be measured by determining their interaction with hormone receptors and the production of functional responses in reporter gene assays (*16*). We have developed YES by expressing human estrogen receptor (hER) and two estrogen response elements (EREs) linked to the *lacZ* gene in yeast (*18*). Yeast do not contain steroid or nuclear receptors, but they do possess proteins homologous to mammalian cells necessary for activated transcription, allowing for the identification of chemicals that induce hER transcriptional activity. To further examine the activities of environmental chemicals in human cells, an estrogen-responsive reporter assay in MCF-7 cells transiently transfected with an ERE-luciferase plasmid was developed.

In this report we demonstrate that *o,p'*-DDD and *p,p'*-DDD, which have been previously identified as nonestrogenic in animal studies, display estrogenic activity. Furthermore, using this combination of assays, alachlor and *cis*-nonachlor and *trans*-nonachlor were identified as having weak estrogenic activity.

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Materials and Methods

17β-3,4,6,7[^{3}H](N) (99 Ci/mmol) estradiol was purchased from Amersham Corporation (Arlington Heights, IL) and *o,p′*-DDT [1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] was purchased from Sigma Chemical Co. (St. Louis, MO). *o,p′*-DDD [1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane] and *p,p′*-DDD [2,2-bis(4-chlorophenyl]-1,1-dichloroethane] were purchased from Aldrich Chemical Co. (Milwaukee, WI). Alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide], benomyl {[1-[(butylamino)carbonyl]-1*H*-benzimidazol-2-yl]carbamic acid methyl ester}, and *cis*- and *trans*-nonachlor were purchased from AccuStandard (New Haven, CT).

The yeast strain BJ2407 was transformed with the pSCW231-hER expression plasmid and the YRPE2 reporter plasmid that contains 2 EREs linked to the *lacZ* gene to create yeast strain hER-ERE as previously described (*18*). A single yeast colony was grown in SD-ura, trp medium overnight at 30°C. The next day, 50 µl of the overnight culture was diluted into 950 µl fresh medium and grown overnight in the presence of the test chemicals. All chemicals were prepared in DMSO and added to the medium so the concentration of DMSO did not exceed 2%. For the β-galactosidase assays, yeast cells were collected by centrifugation and resuspended in 700 µl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 35 mM β-mercaptoethanol). The cells were permeabilized by the addition of 6 µl $CHCl_3$ and 4 µl 0.1% SDS followed by vortexing for 25 sec. The reactions were equilibrated at 30°C for 10 min, then 160 µl *o*-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml Z-buffer) was added and the reactions returned to 30°C. The reactions were terminated by the addition of 400 µl 1M $NaCO_3$, the cell debris removed by centrifugation at 15,000 × *g* for 5 min, and the A_{420} of the samples measured. Miller units were determined using the following formula: [A_{420}/(A_{600} of 1/10 dilution of cells × volume of culture × length of incubation)] × 1000. The Miller units produced by the chemicals divided by the Miller units produced by vehicle was used to calculate fold-induction. The data are representative of two independent experiments of three replicates.

Recombinant hER was produced in Sf9 insect cells by using the baculovirus expression system and prepared as ammonium sulfate precipitates. For competition binding assays, recombinant hER at a concentration of approximately 0.4 nM was incubated in the binding buffer [10 mM Tris, pH 7.4, 1 mM EDTA, 1mM EGTA (ethyleneglycol-bis(β-aminoethyl-ether)*N,N,N′,N′*-tetraacetic acid), 1 mM $NaVO_4$, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM PMSF (phenylmethyl sulfonyl flouride), and 0.2 mM leupeptin] for 1 hr at 25°C with 2.5 nM [^{3}H]17β-estradiol in the presence or absence of radioinert environmental chemicals or 17β-estradiol. Nonspecific binding of [^{3}H]17β-estradiol was assessed by adding a 300-fold molar excess of radioinert 17β-estradiol. For Scatchard analysis, recombinant hER was dissolved in the binding buffer with 0.5, 1, 2.5, 5, or 10 nM [^{3}H]17β-estradiol and a 300-fold molar excess of 17β-estradiol, 10 mM *p,p′*-DDD, or 50 µM alachlor. [^{3}H]17β-estradiol and test chemicals were dissolved in DMSO or ethanol and added to the reaction so the concentration of solvent did not exceed 2.5%. Free [^{3}H]17β-estradiol was removed by incubation with chardex (5% activated charcoal/0.5% dextran) for 10 min at 4°C and centrifugation for 3 min at 15,000 × *g*. Bound [^{3}H]17β-estradiol was measured by scintillation counting. The data shown are representative of two independent experiments with three replicates.

MCF-7 cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal bovine serum; Gibco-BRL, Gaithersburg, MD), BME amino acids, MEM nonessential amino acids, L-glutamine, sodium pyruvate, penicillin-streptomycin (BME and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin were diluted in the medium to a 1X concentration from either 100X or 50X stocks), and porcine insulin (10^{-8}M) (Sigma). Stocks were maintained in 75-cm^2 culture flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

MCF-7 stocks were transferred to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) for 48 hr prior to plating. Cells were plated at a density of 5 ×10^5 cells/35-mm plate and maintained at 37°C for an additional 24 hr in phenol red-free DMEM with 5% DCC-FBS. Cells were transfected in serum-free, phenol red-free DMEM using 12 µg of Lipofectamine (Gibco BRL) with 2 µg of vector pERE2luc, containing two copies of the vitellogenin ERE linked to the luciferase gene, and 1 µg of pCMVβ-galactosidase plasmid for 5 hr. After transfection, the medium was replaced with phenol red-free DMEM with 5% DCC-FBS and vehicle, 17β-estradiol, or environmental chemicals for 18 hr. All chemicals were prepared in DMSO or ethanol and added to the medium so the final concentration of solvent did not exceed 1%. Cells were harvested by incubation in lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) for 15 min at 25°C and cell debris was removed by centrifugation for 3 min at 15,000 × *g*. Protein concentrations were measured using the BioRad protein assay (BioRad Laboratories, Hercules, CA). β-galactosidase activity was determined by the addition of 40 µg protein to 500 µl Z-buffer and 100 µl ONPG and incubated at 37°C. Reactions were terminated by the addition of 500 µl 1 M $NaCO_3$, and the A_{420} for each sample was measured. The volume of sample measured in the luciferase assay was normalized for β-galactosidase activity and protein concentration. Luciferase activity was determined in a Monolight 2010 luminometer (Analytical Luminescence Laboratory) using manufacturer's reagents and instructions. Luciferase activity of samples treated with chemicals divided by the luciferase activity of those treated with vehicle was used to determine fold induction. The data shown are representative of at least two independent experiments with three replicates.

For the 96-well plate transfections, MCF-7 cells were plated at a density of 1.5 × 10^3 cells/well in phenol red-free DMEM containing 5% DCC-FBS and allowed to attach overnight. The next day, cells were transfected in serum-free, phenol red-free DMEM using 75 µg of Lipofectamine with 12.5 µg pEREluc/96-well plate for 5 hr. After transfection, the medium was aspirated from the wells and replaced with phenol red-free DMEM containing 5% DCC-FBS and vehicle, 17β-estradiol, *o,p′*-DDT, or *p,p′*-DDD for 18 hr. For luciferase assays, the medium was removed and the cells were incubated in lysis buffer for 15 min at 25°C. Luciferase activity was determined in a Monolight 9600 luminometer (Analytical Luminescence Laboratory) using manufacturer's reagents and instructions. Luciferase activity of samples treated with chemicals divided by the luciferase activity of those treated with vehicle was used to determine fold induction. The data shown are representative of at least two independent experiments with six replicates.

Results

Identification of Environmental Chemicals with Estrogenic Activity in the Yeast Estrogen Screen

We have created the YES by expressing hER and two EREs linked to the *lacZ* gene in the yeast strain hER-ERE as previously described by Arnold et al. (*18*). Yeast strain hER-ERE was grown overnight in the pres-

ence or absence of 10 nM 17β-estradiol or increasing concentrations of various environmental chemicals. Ten nanomolar estradiol produced a 100-fold induction of β-galactosidase activity (Table 1). At 10 μM, *o,p′*-DDT increased β-galactosidase activity by 95-fold, to a similar extent as 10 nM 17β-estradiol. *p,p′*-DDD at 10 μM induced β-galactosidase activity to a level similar to that of *o,p′*-DDT. At 10 μM, alachlor, *cis*-nonachlor, and *trans*-nonachlor all induced β-galactosidase activity, but to a lesser extent than *o,p′*-DDT. Benomyl did not increase β-galactosidase activity in this assay.

Inhibition of [^{3}H]17β-estradiol Binding by Environmental Chemicals

The chemicals tested in YES were measured for their ability to inhibit the binding of [^{3}H]17β-estradiol to hER in competition binding assays. Recombinant hER was incubated with [^{3}H]17β-estradiol in the presence or absence of varying concentrations of radioinert chemicals. Unbound [^{3}H]17β-estradiol was removed by incubation with chardex, and bound [^{3}H]17β-estradiol was measured by scintillation counting. Not surprisingly, 17β-estradiol was the most effective chemical tested at inhibiting the binding of [^{3}H]17β-estradiol to hER with an IC_{50} (concentration of chemical necessary to inhibit the binding of [^{3}H]17β-estradiol by 50%) of 1 nM (Fig. 1). *o,p′*-DDT was the most effective environmental chemical at reducing [^{3}H]17β-estradiol binding with an IC_{50} of 1 μM or 1000-fold greater than the IC_{50} for estradiol. This result is consistent with previous studies which showed that *o,p′*-DDT has a 1000-fold lower binding affinity for ER in rat cytosol than estradiol (*19*). *p,p′*-DDD had an IC_{50} of 11 μM, which was approximately 10,000-fold greater than the IC_{50} for 17β-estradiol. The chemicals *o,p′*-DDD, alachlor, *cis*-nonachlor, and *trans*-nonachlor only weakly reduced the binding of [^{3}H]17β-estradiol, and none of the chemicals reduced binding by 50% at the concentrations tested. The inability of the chemicals to significantly reduce the binding of [^{3}H]17β-estradiol may have been due the insolubility of these chemicals at concentrations greater than 50 μM (unpublished observations).

A Scatchard analysis was performed with [^{3}H]17β-estradiol in the presence or absence of *p,p′*-DDD or alachlor to determine their mechanism of binding. Both *p,p′*-DDD and alachlor competitively inhibited [^{3}H]17β-estradiol binding to hER as demonstrated by the nonparallel slopes in the Scatchard plots (Fig. 2).

Table 1. Fold-induction of β-galactosidase by various environmental chemicals in the YES

Chemicals	Concentration (μM)	Fold-induction
DMSO		0
Estradiol	0.0001	50
	0.0010	80
	0.0100	100
o,p′-DDT	0.100	40
	1.0	85
	10.0	95
o,p′-DDD	0.100	0
	1.0	10
	10.0	35
p,p′-DDD	0.100	25
	1.0	65
	10.0	85
Alachlor	0.100	0
	1.0	10
	10.0	40
Benomyl	0.100	0
	1.0	0
	10.0	0
cis-Nonachlor	0.100	0
	1.0	0
	10.0	20
trans-Nonachlor	0.100	0
	1.0	0
	10.0	12

Fold induction represents the β-galactosidase activity induced by the chemical compared to the β-galactosidase activity in the presence of DMSO. Each value represents the mean ± SE of two independent experiments with three replicates. The SE was less than 10% for the experiments performed.

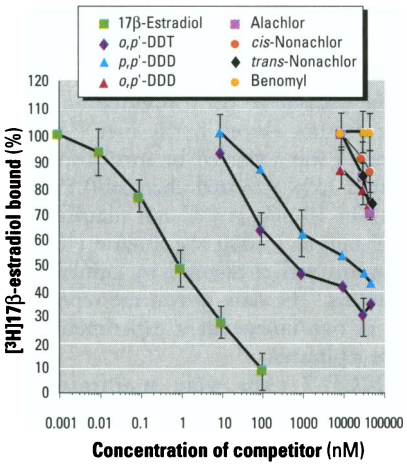

Figure 1. Inhibition of specific [^{3}H]17β-estradiol-binding to human estrogen receptor (hER) by environmental chemicals. Recombinant hER was incubated with 2.5 nM [^{3}H]17β-estradiol in the presence or absence of increasing concentrations of radioinert 17β-estradiol, *o,p′*-DDT, *o,p′*-DDD, *p,p′*-DDD, benomyl, alachlor, *cis*-nonachlor, or *trans*-nonachlor. Data are representative of at least three independent experiments, and each data point is presented as mean ± SE.

Activation of an ERE–Luciferase Reporter in MCF-7 cells by Environmental Chemicals

To examine the ability of the environmental chemicals to facilitate hER-mediated transcriptional activation in mammalian cells, MCF-7 human breast cancer cells were transiently transfected with a plasmid containing two EREs linked to the luciferase gene. The cells were incubated in the presence or absence of increasing concentrations of environmental chemicals for 18 hr, and then cell extracts were assayed for luciferase activity. Consistent with the results of the competition binding assays, 17β-estradiol at 100 pM was the most effective chemical at inducing luciferase activity, with a 46-fold induction above control (Fig. 3). *p,p′*-DDD at 100 nM induced luciferase activity to a level similar to 100 pM estradiol. *o,p′*-DDT induced luciferase activity to the same extent as *p,p′*-DDD, but at a concentration of 1 μM (Fig. 3). This is inconsistent with the competition binding studies which showed that *o,p′*-DDT was more effective than *p,p′*-DDD at inhibiting [^{3}H]17β-estradiol bind-

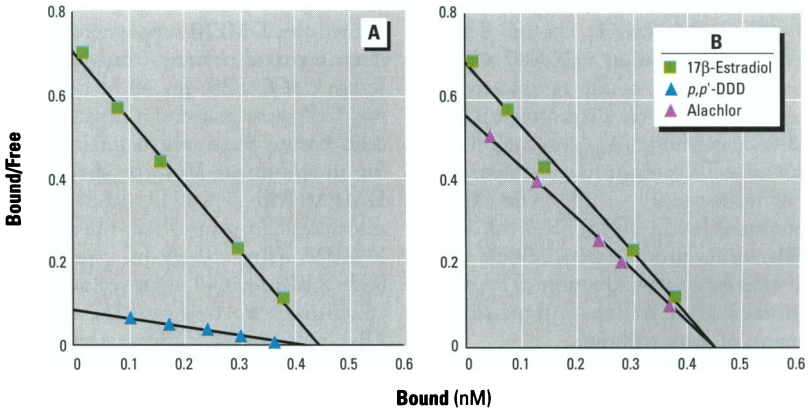

Figure 2. Scatchard analysis of human estrogen receptor (hER) with [^{3}H]17β-estradiol in the presence or absence of *p,p′*-DDD and alachlor. Recombinant hER was incubated with increasing concentrations of [^{3}H]17β-estradiol and 10 μM *p,p′*-DDD (A) or 50 μM alachlor (B). The data were analyzed by the method of Scatchard (*28*) and are representative of at least two independent experiments.

ing to hER. In this assay, *o,p′*-DDT and *p,p′*-DDD appear to be full agonists of hER-mediated transactivation because they increased luciferase activity to the same extent as estradiol, albeit at higher concentrations.

None of the other environmental chemicals, at the concentrations tested, induced luciferase activity to the same extent as estradiol (Fig. 3). At 1 µM alachlor, luciferase activity was increased 23-fold or approximately 50% of the maximal activity induced by estradiol. 10 µM alachlor did not further increase luciferase activity, indicating that alachlor is only a partial agonist of hER. *o,p′*-DDD increased luciferase activity 15-fold above control at a concentration of 1 µM. Higher concentrations of *o,p′*-DDD were not tested for induction of luciferase activity due to their toxic effects on MCF-7 cells. *cis*-Nonachlor and *trans*-nonachlor displayed minimal estrogenic activity in this assay. At the maximum concentration tested, *trans*-nonachlor (4.5 µM) induced luciferase activity 14-fold above control levels and *cis*-nonachlor (20 µM) induced luciferase activity seven-fold above control levels. Benomyl was not able to induce luciferase activity even at 20 µM (Fig. 3).

To demonstrate that the chemicals interacted with the hER in MCF-7 cells, MCF-7 cells were incubated in the presence of environmental chemicals alone or environmental chemicals and a 100-fold molar excess of 4-OH-tamoxifen, an ER antagonist. The luciferase activity induced by all of the chemicals tested was eliminated in the presence of 4-OH-tamoxifen (Fig. 4), demonstrating that the chemicals interact with the hER in MCF-7 cells.

Next, we examined the feasibility of testing environmental chemicals for estrogenic activity using MCF-7 cells in a 96-well plate. MCF-7 cells in 96-well plates were transfected with the ERE–luciferase reporter plasmid, treated with various concentrations of 17β-estradiol, *o,p′*-DDT, *p,p′*-DDD, or vehicle for 18 hr, and then assayed for luciferase activity. Luciferase activity was induced by environmental chemicals in MCF-7 cells plated in 96-well plates (Fig. 5). The induction of luciferase activity by the chemicals was reduced compared to the induction seen in the 35-mm wells, as shown in Figure 3. The maximum induction by the chemicals was 46-fold in 35-mm wells, whereas the maximum induction in the 96-well plates was fourfold.

Discussion

We have used a combination of three assays to study the estrogenic activity of several environmental chemicals. This

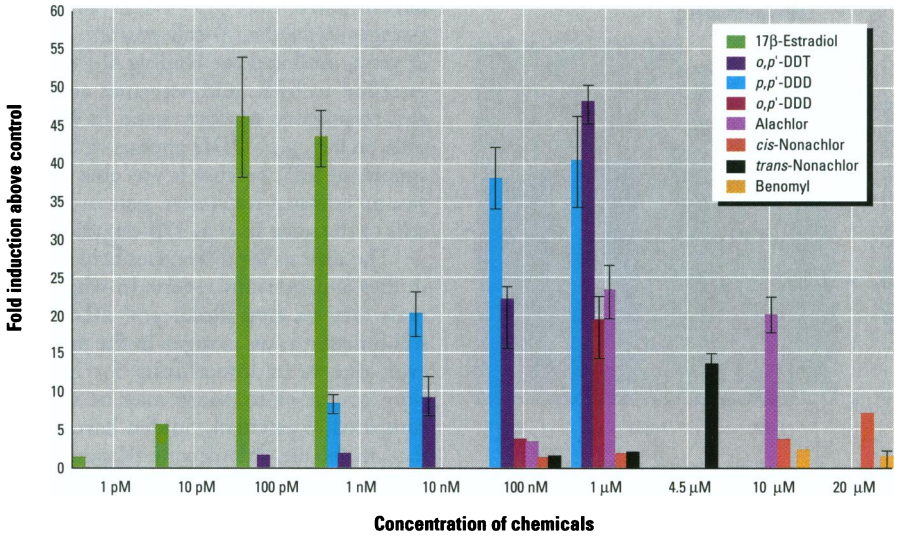

Figure 3. Induction of luciferase activity in MCF-7 cells by environmental chemicals. MCF-7 cells were transiently transfected with pEREluc and pCMV-β and then treated with increasing concentrations of 17β-estradiol, environmental chemicals, or vehicle. Cell extracts were measured for luciferase activity and data were expressed as fold-induction compared to luciferase activity of vehicle. Data are presented as mean ± SE and are representative of two independent experiments of three replicates.

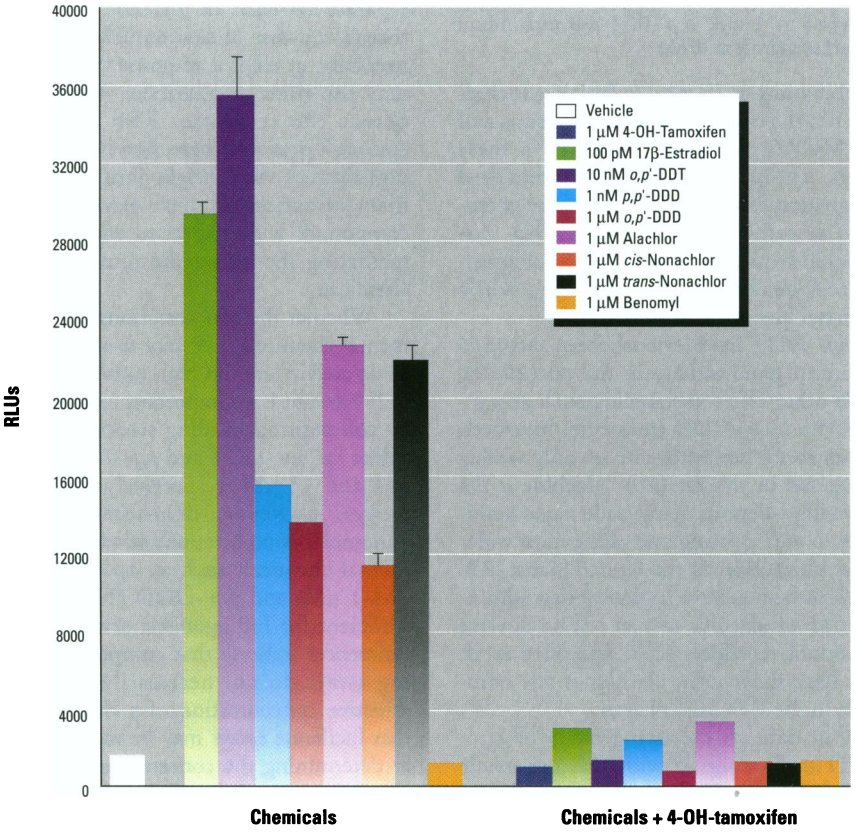

Figure 4. Inhibition of environmental chemical-induced luciferase activity by 4-OH-tamoxifen. MCF-7 cells, which were transiently transfected with pEREluc and pCMV-β, were treated with vehicle, 17β-estradiol, or environmental chemicals (the minimum concentration to elicit maximum luciferase activity) in the presence or absence of 100-fold molar excess 4-OH-tamoxifen. Luciferase activity of cell extracts is expressed as relative light units (RLUs). Data are presented as mean ± SE and are representative of two independent experiments of three replicates.

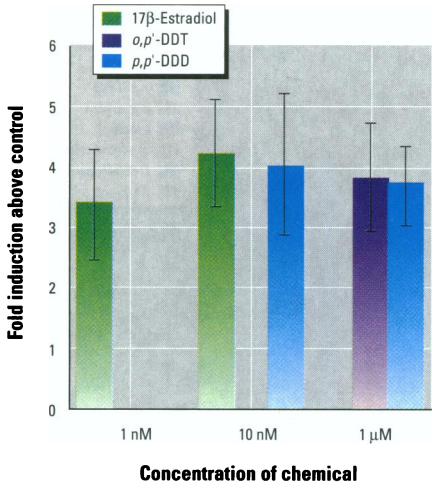

Figure 5. Induction of luciferase activity in MCF-7 cells in 96-well plates. MCF-7 cells were transiently transfected in 96-well plates with pEREluc (see Materials and Methods for description of vector) and then treated for 18 hr with increasing concentrations of 17β-estradiol, *o,p'*-DDT, *p,p'*-DDD, or vehicle. Cell extracts were measured for luciferase activity. Data (mean ± SE) are representative of two independent experiments of six replicates. Luciferase activity induced by 17β-estradiol was not assayed at 1 μM, and the luciferase activity induced by *o,p'*-DDT and *p,p'*-DDD was not assayed at 1 nM. *o,p'*-DDT did not induce luciferase activity at 10 nM.

combination of *in vitro* techniques included YES, the competition binding assay, and the MCF-7 cell luciferase assay. In these assays, *o,p'*-DDT and *p,p'*-DDD displayed the greatest estrogenic activity of the chemicals tested. Alachlor, *o,p'*-DDD, *cis*-nonachlor and *trans*-nonachlor demonstrated weak estrogenic activity, while benomyl had no estrogenic activity.

o,p'-DDT has previously been shown to have estrogenic activity in the rat (*20,21*), while other DDT metabolites such as *p,p'*-DDD and *o,p'*-DDD (mitotane) have been shown to be nonestrogenic or only weakly estrogenic in the rat (*20*). Alachlor is the active ingredient in many trade name herbicides, as well as being one of the most widely used herbicides in the United States (*22*). Previous studies have indicated that administration of alachlor to rats has no adverse reproductive effects (*23*), and Soto et al. (*17*) have shown that alachlor is not estrogenic in the E-SCREEN assay.

Our data on the estrogenicity of *p,p'*-DDD are in contrast with previous whole animal studies. Such studies have shown that, in the rat, *p,p'*-DDD does not function as an estrogen when measured by the uterine glycogen response or induction of uterine ornithine decarboxylase (*20,21*). To be relevant to the human, these studies sought to examine the estrogenic activity of the DDT metabolites using hER. The data herein show that *p,p'*-DDD activated estrogen-dependent β-galactosidase activity in yeast, inhibited the binding of [^{3}H]17β-estradiol to hER, and activated an estrogen-responsive reporter gene in MCF-7 cells. In fact, *p,p'*-DDD appears to be a full agonist of hER because it was able to activate an estrogen-responsive gene in MCF-7 cells to the same level as 17β-estradiol.

The reasons for differences between our studies and previous reports in whole animals may be severalfold. *p,p'*-DDD may still function as an estrogen in the rat; however, due to its low affinity for ER, the time course of response may be delayed compared to estradiol. Studies have shown that, in the rat, chlordecone (Kepone), also a chlorinated insecticide, elicits the same degree of estrogenic response as estradiol when measured by redistribution of ER to the nucleus, uterine weight gain, and synthesis of progesterone receptor (*24*). The uterine responses to chlordecone occurred over a matter of days, whereas they occurred within several hours in response to estradiol. Species differences may also play a role in the differential ability of environmental chemicals to act as estrogens in separate systems. In different animals or tissues, the dose of environmental chemical necessary to elicit a response may vary, as may the time of treatment necessary to detect the response. The multiassay approach presented here, however, provides investigators with insight into the mechanism of action of these environmental compounds with respect to whether ER is mediating the estrogenic response of the chemical.

Whether the concentrations of environmental chemicals necessary to induce estrogenic activity are too high to be physiologically relevant is an important consideration. In competition binding studies, the IC_{50} values for *o,p'*-DDT and *p,p'*-DDD were 1 μM and 11 μM, respectively. Luciferase assays, however, demonstrated that nanomolar concentrations of some environmental chemicals such as *o,p'*-DDT (100 nM–1 μM) and *p,p'*-DDD (100 nM), are sufficient for full agonistic activity. These differences indicate that competition binding assays do not necessarily reflect the effective concentration of a chemical and that luciferase assays may be more sensitive at determining the concentration at which an environmental chemical exerts estrogenic activity. Competition binding assays are also limited in that they can not distinguish between an ER agonist and an ER antagonist, whereas functional assays, such as the luciferase assays, are able to do so.

Discrepancies between competition binding data and luciferase data for *o,p'*-DDT and *p,p'*-DDD may be based on the fact that, in luciferase assays, the environmental chemicals bind to unliganded ER as opposed to competition binding assays in which the environmental chemicals must compete with 17β-estradiol for binding to ER. In competition binding assays, the structure of *o,p'*-DDT, compared to that of *p,p'*-DDD, may make it better able to compete with 17β-estradiol for binding to hER. In contrast, *p,p'*-DDD may, upon binding to hER, create a more potent transcriptionally active complex than does the binding of *o,p'*-DDT to hER, allowing greater transcription of the luciferase reporter gene.

In terms of comparable levels of environmental estrogens that may occur in the environment, one investigation has determined the concentrations of some environmental chemicals, including *trans*-nonachlor, to be as high as 200 nM in alligator eggs from Lake Apopka, Florida (*25*), demonstrating that it is possible for local concentrations of environmental chemicals to reach the concentrations that could elicit a biological response.

Finally, we were interested in determining whether the ability of environmental chemicals to induce the expression of the luciferase reporter gene could be determined in a microassay. By using the microassay, a large number of chemicals can be evaluated. However, the decreased induction of luciferase activity observed using the 96-well plate procedure compared to the induction observed with the 35-mm well transfection protocol limits this assay to identifying only a positive or negative response with respect to the ability of an environmental chemical to induce estrogen-responsive reporter gene activity. At the present time, in order to determine whether a chemical acts as a partial or full agonist, a more sensitive assay such as the 35-mm well transfection procedure will be necessary.

Proliferation assays were not included in these studies. The ability to bind to hER and regulate the transcription of estrogen-regulated genes may have effects in addition to the proliferation of the breast cancer cell. For example, the induction of estrogen-regulated gene transcription may result in the production of growth factors or other proteins that act to regulate the growth of cells surrounding the breast cancer cell. These surrounding cells may ultimately produce other factors that affect the breast cancer cell. Recent studies have also shown that different MCF-7 stocks respond uniquely to both estradiol and environmental estrogens in proliferation assays (*26,27*), possibly adding to discrep-

ancies among data from different laboratories. For example, alachlor was not identified as an estrogen by the E-SCREEN assay (*17*), but it did display estrogenic effects in our laboratory. In another example, *o,p'*-DDT was shown to be estrogenic in the E-SCREEN (*17*), and it also exhibited estrogenic activity in our assays. Whether the differences between our data on alachlor and those of Soto et al. (*17*) are due to the use of separate MCF-7 stocks or other phenomena is unclear.

The *in vitro* assays used here were not selected to definitively determine whether an environmental chemical is an estrogen (as the definition of a true estrogen is still the focus of much debate) or to replace the testing of environmental chemicals for estrogenic activity in animals. Rather, this approach was designed as a multifaceted procedure to examine several different aspects of estrogenic activity, namely, the ability to bind to ER and the capacity to activate estrogen-responsive genes through ER. The ability of a chemical to elicit any of these responses may indicate it will be estrogenic *in vivo*.

This three-tiered approach will aid in the characterization of large numbers of chemicals for estrogenic activity and, more importantly, provide insight into the mechanisms involved in the mediation of the estrogenic activity of environmental chemicals. We suggest, therefore, that these *in vitro* assays are an important addition to other biological assays, including whole animal studies, which examine the estrogenicity of environmental chemicals, as well as an effective method for beginning to dissect the molecular mechanisms involved in the estrogenic responses elicited by many environmental chemicals.

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