

# Estrogenic Activity Assessment of Environmental Chemicals Using *in Vitro* Assays: Identification of Two New Estrogenic Compounds

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Environmental chemicals with estrogenic activities have been suggested to be associated with deleterious effects in animals and humans. To characterize estrogenic chemicals and their mechanisms of action, we established *in vitro* and cell culture assays that detect human estrogen receptor  $\alpha$  (hER $\alpha$ )-mediated estrogenicity. First, we assayed chemicals to determine their ability to modulate direct interaction between the hER $\alpha$  and the steroid receptor coactivator-1 (SRC-1) and in a competition binding assay to displace 17 $\beta$ -estradiol (E<sub>2</sub>). Second, we tested the chemicals for estrogen-associated transcriptional activity in the yeast estrogen screen and in the estrogen-responsive MCF-7 human breast cancer cell line. The chemicals investigated in this study were *o,p'*-DDT (racemic mixture and enantiomers), nonylphenol mixture (NPM), and two poorly analyzed compounds in the environment, namely, tris-4-(chlorophenyl)methane (Tris-H) and tris-4-(chlorophenyl)methanol (Tris-OH). In both yeast and MCF-7 cells, we determined estrogenic activity via the estrogen receptor (ER) for *o,p'*-DDT, NPM, and for the very first time, Tris-H and Tris-OH. However, unlike estrogens, none of these xenobiotics seemed to be able to induce ER/SRC-1 interactions, most likely because the conformation of the activated receptor would not allow direct contacts with this coactivator. However, these compounds were able to inhibit [<sup>3</sup>H]-E<sub>2</sub> binding to hER, which reveals a direct interaction with the receptor. In conclusion, the test compounds are estrogen mimics, but their molecular mechanism of action appears to be different from that of the natural hormone as revealed by the receptor/coactivator interaction analysis. **Key words:** coactivator SRC-1, environmental chemicals, estrogen receptor  $\alpha$ , MCF-7 cells, transcriptional activity, xenoestrogen, yeast. *Environ Health Perspect* 108:621–629 (2000). [Online 26 May 2000]

<http://ehpnet1.niehs.nih.gov/docs/2000/108p621-629lascombe/abstract.html>

Estrogens influence the growth, differentiation, and functions of many target organs, such as those of the female and male reproductive systems including mammary gland, uterus, vagina, ovary, testis, epididymis, and prostate (1,2). These steroid hormones also play an important role in bone maintenance, in the central nervous system, and in the cardiovascular system where estrogens have certain cardioprotective effects (1–3). The initial step in their mechanisms of action is their binding to an intracellular estrogen receptor (ER). There are two estrogen receptor isotypes,  $\alpha$  and  $\beta$ . Although ER $\alpha$  (NR3A1) (4) is well characterized, only recently has ER $\beta$  (NR3A2) (4) been discovered in the rat (5), mouse (6), and human (7). The two isotypes differ in the C-terminal ligand binding domain and in the N-terminal transactivation domain. The binding of the ligand causes a conformational change in the receptor that enables the estrogen/estrogen receptor complex to bind as a homodimer ( $\alpha/\alpha$  or  $\beta/\beta$ ) or heterodimer ( $\alpha/\beta$ ) (8) to specific sites on the DNA, the estrogen response element (ERE) (9,10). Once bound to DNA, the estrogen/estrogen receptor complex modulates the transcription of target genes (11,12) through which it exerts its effects. Other types of action of steroid hormones exist; for

estrogen, plasma membrane receptors have been described, and many of the actions of 17 $\beta$ -estradiol (E<sub>2</sub>)-like compounds still remain unanswered (13).

During the past 50 years or more, a large number of diverse synthetic chemicals (xenobiotics) have been released into the environment because of efforts to increase agricultural productivity or because modern industrial processes produce industrial waste. Thus, each year, vast quantities of pesticides, insecticides, fungicides, herbicides, solvents, detergents, styrenes, polychlorinated biphenyls, and penta- to nonylphenols are released into the ecosystem and accumulate in the air, water, and food chain (14,15). As many of these chemicals and industrial waste products have steroidlike activity, scientists and health officials have raised concerns about such environmental compounds, including natural products (e.g., coumestrol and genistein), pesticides and fungicides (DDT, lindane, methoxychlor, and vinclozolin), as well as other commercial chemicals such as bisphenol A and *p*-nonylphenol (16–21). By acting as estrogen mimics (xenoestrogens) they can disrupt normal endocrine function, possibly leading to reproductive failure in wildlife and humans, and can also induce tumors in estrogen-sensitive tissues (22,23).

The molecular structure of exogenous natural and synthetic estrogens may be very similar to, or strikingly different from, the natural hormone E<sub>2</sub> (24–27). Despite their structural diversity, all of the exogenous estrogens, when ingested either as natural compounds (phytoestrogens, mycoestrogens) or contaminants (xenoestrogens), have the capacity to bind to the ER at a given concentration in target cells of the body and can initiate (agonist) or inhibit (antagonist) estrogen-like actions (16,28). In doing so, estrogen mimics have the potential to alter, either in a beneficial or harmful manner, the growth, development, and function of estrogen target tissues. 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 to identify chemicals with estrogenic activity (25). Given that prediction of estrogenic potency derived from structural information alone is not yet possible, robust and reliable assays capable of screening chemicals for estrogenic activity are required.

We developed a yeast estrogen screen (YES) by expressing the human estrogen receptor  $\alpha$  (hER $\alpha$ ) in cells that have a target gene with one copy of the ERE linked to the *lacZ* gene. Normally, yeast cells do not contain nuclear receptors for steroids, but they do possess proteins that are homologous to mammalian cells necessary for controlling transcription. Thus, the identification of chemicals that induce hER transcriptional activity is possible in the modified cells (29). Furthermore, to examine the activities of environmental chemicals in human cells, we performed an estrogen-responsive reporter

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We thank J. Diezi and P. Kucera for stimulating discussions and P. Balaguer for providing the MCF-7 stable transfected cell line.

This work was supported by grants from the University of Lausanne, the Swiss Federal Institute of Technology, and the Swiss Agency for the Environment, Forests and Landscape.

Received 23 December 1999; accepted 2 March 2000.

gene assay in MCF-7 cells stably transfected with an ERE-luciferase plasmid. Finally, we optimized and validated a novel *in vitro* ligand detector assay, the coactivator-dependent receptor ligand assay (CARLA), previously developed for the screening of peroxisome proliferator-activated receptor (PPAR) ligands (30), for  $E_2$  and used this assay to screen putative estrogenic chemicals. This assay is based on the direct ligand-dependent interaction between the ER and the transcriptional steroid receptor coactivator-1 (SRC-1) after activation of the receptor, which results from a ligand-induced conformational change.

We studied two poorly characterized compounds present in the environment, tris-4-(chlorophenyl)methane (Tris-H) and tris-4-(chlorophenyl)methanol (Tris-OH), and compared their activities to the organochlorinated pesticide *o,p'*-DDT (the racemic mixture and both enantiomers) and a mixture of nonylphenols (NPM; released from polystyrene), which have already been identified to have estrogenic activity. Tris-H and Tris-OH have been detected as microcontaminants in the marine environment (31). There is a lack of knowledge with respect to the origin and toxicology of these compounds. The possible sources of Tris-OH include synthetic (optically active) high polymers, agrochemicals, and dye production (32). Another study suggested that Tris-OH may also be produced by degradation of Tris-H in the environment, which is thought to be a by-product in the manufacture of technical grade DDT (33).

In this report, we present a study of the mechanisms of action of the estrogen mimetics *o,p'*-DDT and NPM. Furthermore, we have also determined for the first time that Tris-H and Tris-OH have estrogenic activity in cellular tests. In the CARLA test, none of the xenobiotics studied was able to induce interaction between ER and SRC-1; this is similar to results with the antiestrogens ICI 182,780 and hydroxytamoxifen. We investigated the ability of some compounds to compete with  $E_2$  for ER binding sites by using  $E_2$  binding assays in MCF-7 cells in culture.

## Materials and Methods

**Chemicals reagents and culture media.** We purchased  $E_2$  and diethylstilbestrol (DES) from Sigma (St. Louis, MO). The *o,p'*-DDT was a gift from D. Ehrenstorfer (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland); the nonylphenol mixture (NPM; ring and chain isomers; purity 90%) was a gift from P. Balaguer (INSERM, Montpellier, France); and the ICI 182,780 was a gift from A. Waking (ICI Pharmaceuticals, Alderley Park, Macclesfield, UK). Tris-H and Tris-OH were synthesized as described below with a purity

of 94%. Stock solutions ( $10^{-2}$  M) of test compounds were prepared in ethanol. Ethanol concentration in the culture medium never exceeded 0.1% (v/v) for both the yeast and the mammalian cell cultures. Yeast medium components were purchased from Difco (Basel, Switzerland) and Sigma. We purchased the culture media for stably transfected mammalian cells (DMEM and DMEM/F-12) from Gibco-BRL (Basel, Switzerland).

**Synthesis of Tris-H and Tris-OH.** We synthesized Tris-OH by adding 4-chlorobromobenzene to 4,4'-dichlorobenzophenone by a Grignard reaction. In a reactor under a nitrogen atmosphere, we added 1.34 g magnesium to 15 mL dry diethyl ether. The solution was stirred and heated gently to a reflux, and then 9.74 mg (0.05 M) 4-chlorobromobenzene was added. After 45 min, the reaction mixture was cooled to room temperature. Under continuous stirring, we added 15 mL dry ether and 8.41 g (0.03 M) 4,4'-dichlorobenzophenone. The solution was reheated until reflux. After 15 min, we poured the reaction mixture slowly into an ice-cooled beaker containing 20 mL 3 M sulfuric acid. The aqueous solution was extracted three times with ether. Ether phases were pooled and dried with sodium sulfate. Ether was evaporated under reduced pressure to obtain an oily orange solution. This mixture was chromatographed through a 40 g Silica gel 60 column (Merck, Darmstadt, Germany) using petroleum ether as the solvent; after evaporation, we obtained a white solid composed of 93.5% Tris-OH and 6.5% 4,4'-dichlorobenzophenone. Further purification was performed on the same Silica gel 60 column using ether/hexane (1:3 v/v). After evaporation of solvent, we isolated the pure Tris-OH as a white crystalline compound with a composition of 100% Tris-OH.

Tris-H was synthesized by reduction of Tris-OH with sodium borohydride. We dissolved 1.005 g Tris-OH (96.5% purity) in 22 mL trifluoroacetic acid (stirred and cooled to 0°C).  $\text{NaBH}_4$  (1.054 g) was added slowly, and an exergonic reaction was observed at each addition. The solvent was evaporated under reduced pressure, and residues were dissolved with 25 mL  $\text{H}_2\text{O}$  and 25 mL dichloromethane, giving two clear phases. This mixture was washed with 100 mL of a 2% (wt/v) sodium carbonate solution and extracted three times with 100 mL chloroform. Chloroform phases were pooled, dried with sodium sulfate, and evaporated under reduced pressure. The residue was chromatographed through a 20 g Silica gel 60 column using petroleum ether as the eluate. After evaporation, the residue was recrystallized from methanol giving crystalline Tris-H of high purity. We determined purity and structure by gas chromatography-electron

capture detection (GC-ECD) and gas chromatography-mass spectrometry (GC-MS).

**Plasmids.** The pGEX GST-hER and pSG5 SRC-1 plasmids (34) were a gift from M. Parker (Cancer Research Fund, London, UK). The p2HG-hER expression vector (35), the p2HG plasmid (36), and the pLGERE reporter plasmid (36) were a gift from M. Tsai-Pflugfelder (Institut Suisse de Recherches Expérimentales sur le Cancer, Lausanne, Switzerland).

**Yeast strain.** The yeast strain used in this study was the YRG-2 competent cell line (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 lys2::UAS<sub>GAL1</sub>-TATA<sub>GAL1</sub>-HIS3 URA3::UAS<sub>GAL4</sub><sup>17mers</sup>(x3)-TATA<sub>CYC1</sub>-Lac2) provided by Stratagene (Basel, Switzerland). Yeast cells were transformed using the lithium acetate method (37) either by the p2HG-hER expression vector and the pLGERE reporter plasmid, which contains one ERE linked to the *lacZ* gene, or by the empty p2HG vector with the same reporter plasmid. Double transformants, with the expression vector (or the empty vector) and the reporter plasmid, were selected by growth on minimal plates deficient in uracil and histidine.

**$\beta$ -galactosidase assay in yeast cells.** Transformed yeast cells were grown in synthetic drop-out medium without uracil and histidine (37) and supplemented with 2% (wt/v) glucose, 3% (v/v) glycerol, and 2% (wt/v) lactate. At the late log phase, the cultures were diluted (1:50) into the same medium without glucose, and the growth continued for 24 hr. Galactose was added to a final concentration of 2% (wt/v) to induce the yeast GAL1 promoter. Test compounds ( $E_2$ , synthetic estrogens, antagonists, and xenobiotics) were added as indicated during overnight incubation. After treatment, the cells were harvested by centrifugation (2,000 rpm for 5 min), resuspended in 1 mL of Z buffer (60 mM  $\text{Na}_2\text{HPO}_4$ , 40 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM KCl, 1 mM  $\text{MgSO}_4$ , and 35 mM 2-mercaptoethanol, pH 7), permeabilized by the addition of 8.5  $\mu\text{L}$  chloroform and 5.7  $\mu\text{L}$  0.1% (wt/v) SDS, and mixed for 10 sec at  $12,000 \times g$ . The reactions were incubated at 30°C with 200  $\mu\text{L}$  *o*-nitrophenyl  $\beta$ -D-galactopyranoside (4 mg/mL in Z buffer) and were terminated by the addition of 500  $\mu\text{L}$  1M  $\text{Na}_2\text{CO}_3$ . We removed the cell debris by centrifuging for 10 min in a microfuge at  $12,000 \times g$  and discarding the pellets. We measured the absorbance at 420 nm and determined the  $\beta$ -galactosidase activity by the formula [optical density (OD)<sub>420</sub>/OD<sub>600</sub> of assayed culture  $\times$  volume (milliliters) assayed  $\times$  time (min)] (37).

**CARLA pull-downs.** The protocol for the CARLA has been previously described (30). Briefly, fusion proteins of glutathione

S-transferase (GST) and the ligand binding domain (LBD) of the ER were bacterially expressed and partially purified on glutathione–Sephadex beads (Pharmacia, Piscataway, NJ). Beads were incubated with the test compounds ( $E_2$ , DES, xenobiotic) and radiolabeled SRC-1 (produced *in vitro* using a coupled transcription/translation rabbit reticulocyte lysate system (TNT; Promega, Madison, WI). The reaction was incubated at 4°C with constant rotation, and beads were collected by centrifugation and washed. The glutathione–Sephadex-bound proteins were dried under vacuum for 30 min before being resuspended in loading buffer [62.5 mM Tris, 2% (wt/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, pH 6.8] with bromophenol blue and subjected to SDS-PAGE. Before being dried and exposed to autoradiography, the polyacrylamide gels were stained with Coomassie Brilliant Blue to determine that equal amounts of fusion proteins were used in each reaction. The amounts of retained SRC-1 were determined by densitometry.

**Transcriptional activation assay in stably transfected MCF-7 cells.** The MELN41 cells (derived from MCF-7 cells) were stably transfected with the reporter plasmid ERE-luc (38). Cultures were maintained in DMEM containing phenol red and supplemented with 5% (v/v) fetal calf serum (FCS) and 1 mg/mL geneticin G418 (Sigma) in a 95% air/5% CO<sub>2</sub> environment at 37°C. Cells were grown as a monolayer under these conditions in accordance with routine cell-culture procedures. Three days before plating in Falcon 24-well-tissue-culture plates (Becton Dickinson, Franklin Lakes, NJ) cells were incubated in phenol-red free DMEM/F-12 medium supplemented with 3% (v/v) dextran/charcoal stripped serum to decrease the estradiol content and the background signal. After this period, cells were harvested by trypsinisation [0.1% (wt/v) trypsin in 0.02% (wt/v) EDTA-Hank's balanced salt solution] and plated at a concentration of  $2 \times 10^5$  cells/well in 24-well plates in the same medium as above for 24 hr. At 80% confluency, cells were treated with various concentrations of the test compounds for 12 hr and were harvested to measure luciferase activity. The Bradford protein assay (39) was performed on an aliquot of cell homogenate according to the instructions for the microassay procedure (Bio-Rad, London, UK). The experimental values are expressed as arbitrary luminescence units per microgram of protein. Basal activity corresponds to the value obtained with vehicle alone.

**Determination of environmental chemical-binding in whole cells.** We determined binding properties of test compounds using the MCF-7 cell line growing in monolayer culture (40). Briefly, MCF-7 cells were seeded in 24-well plates until confluency in

phenol-red free DMEM/F-12 medium supplemented with 2% (v/v) dextran/charcoal stripped serum. We removed growth medium and added 0.1 nM [<sup>3</sup>H]- $E_2$  to wells in quadruplicate in 0.5 mL DMEM/F-12 medium plus 0.1% (wt/v) bovine serum albumin (BSA) with or without unlabeled  $E_2$  or test compounds to assess nonspecific binding. After incubation for 1 hr at 37°C (when binding was at the maximum), the medium was removed and the cells were washed with phosphate buffer [5 mM sodium phosphate, 0.25 M sucrose, and 10% (v/v) glycerol, pH 7.4]. Cell-bound radioactivity was extracted from the cells with ethanol and determined by scintillation counting. The data were plotted as percentage of control (specific binding in the absence of competitor) versus the molar excess of competitor.

**Statistical analysis.** We analyzed the data using Student's *t*-test. Results were considered significant when  $p < 0.05$ . The data are presented as mean and standard deviation of the mean.

## Results

**CARLA assay.** A novel ligand assay for the rapid screening of a large number of compounds has been developed to characterize synthetic and natural PPAR ligands (30). This assay is based on the ligand-induced binding of SRC-1 to nuclear hormone receptors (34). In the case of xenoestrogens, we hypothesized that potential ligands for ER $\alpha$  would induce ER/SRC-1 interactions only in cases where a direct and specific binding of compounds to the LBD of the ER occurred (Figure 1A).

SRC-1 was expressed and labeled with [<sup>35</sup>S]-methionine, and the LBD of ER was expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase (GST). To optimize and validate the interaction assay, we performed GST pull-down assays in the presence of  $E_2$  at concentrations ranging from  $10^{-11}$  M to  $10^{-6}$  M. We observed no interaction of [<sup>35</sup>S]-SRC-1 with GST-ER LBD in the absence of hormone. As shown in Figure 1B,  $E_2$  induced interactions of the receptor with SRC-1 in a concentration-dependent manner. Similar results were obtained with DES (data not shown). In contrast, none of the four environmental chemicals tested (*o,p'*-DDT, NPm, Tris-H, and Tris-OH) nor the antiestrogens ICI 162,780 and tamoxifen (data not shown) was active in this test at concentrations up to  $10^{-4}$  M, which was the solubility limit for most of the compounds (Figure 1C).

**Receptor binding studies.** To screen environmental pollutants for possible interactions with ER, we measured displacement by xenobiotics of [<sup>3</sup>H]- $E_2$  bound to ER in MCF-7 cells growing in monolayer culture. Cells were incubated with 0.1 nM [<sup>3</sup>H]- $E_2$  in the

presence or absence of varying concentrations of nonlabeled chemicals. Figure 2 shows that specific [<sup>3</sup>H]- $E_2$  binding was completely inhibited by a 100-fold molar excess ( $10^{-8}$  M) of unlabeled  $E_2$ , with a concentration necessary to inhibit the binding of [<sup>3</sup>H]- $E_2$  by 50% (IC<sub>50</sub>) at approximately 4-fold excess, that is, at  $0.4 \times 10^{-9}$  M. All test compounds were able to displace [<sup>3</sup>H]- $E_2$  from the hER. Unlabeled (-) *o,p'*-DDT inhibited binding by approximately 86% at  $10^{-4}$  M. At the same concentration, (+) *o,p'*-DDT inhibited the binding by approximately 73%. Tris-OH and Tris-H caused a decrease in [<sup>3</sup>H]- $E_2$  binding by approximately 80% and 75%, respectively, at a concentration of  $10^{-4}$  M. The IC<sub>50</sub> value was approximately  $10^{-7}$  M for both compounds. These results demonstrate that these compounds may act by a direct interaction with hER.

**Effects of xenobiotics on the transcriptional activity of hER in yeast.** We used a yeast system that expresses hER to screen the estrogenic potential of the environmental pollutants. The YRG-2 yeast strain contains an estrogen-responsive reporter gene built with one copy of the consensus ERE linked to the yeast CYC 1 promoter located upstream of the *E. coli* gene for  $\beta$ -galactosidase (*lacZ*). Xenobiotics belonging to various classes of compounds, i.e., pesticides and plasticizers, were tested. Yeast cells were grown overnight at 30°C in liquid culture in the absence or presence of increasing concentrations of  $E_2$  or a xenobiotic. We assessed sensitivity and reproducibility of the assay by measuring the response to  $E_2$ .

Figure 3 illustrates the concentration-dependent effects of  $10^{-11}$ – $10^{-6}$  M  $E_2$  and  $10^{-10}$ – $10^{-6}$  M DES in the YRG-2 yeast strain. The  $\beta$ -galactosidase activity was dose dependent up to concentrations of  $10^{-7}$ – $10^{-6}$  M and then reached a plateau for both ER agonists. DES was less efficacious than  $E_2$  at inducing  $\beta$ -galactosidase activity (Figure 3A). We tested a range of chemical concentrations from  $10^{-8}$  M to  $10^{-5}$  M to validate our assay and to assess if it detects estrogen-like responsiveness to known xenoestrogens. Thus, the  $\beta$ -galactosidase activity was significantly induced by *o,p'*-DDT and the other known xenoestrogen, NPm, at each concentration tested in a dose-dependent manner and with a maximal effect at  $10^{-5}$  M (Figure 3B). At  $10^{-5}$  M, racemic *o,p'*-DDT increased  $\beta$ -galactosidase activity by 3.2-fold, corresponding to 41% of the activity induced by  $10^{-7}$  M  $E_2$ . NPm at  $10^{-5}$  M induced  $\beta$ -galactosidase activity to a level similar to that of *o,p'*-DDT. These results demonstrate that our system is responsive to xenoestrogens.

Figure 4 provides evidence that Tris-H and Tris-OH are estrogenic. In the yeast assay, this effect was studied at high concentrations

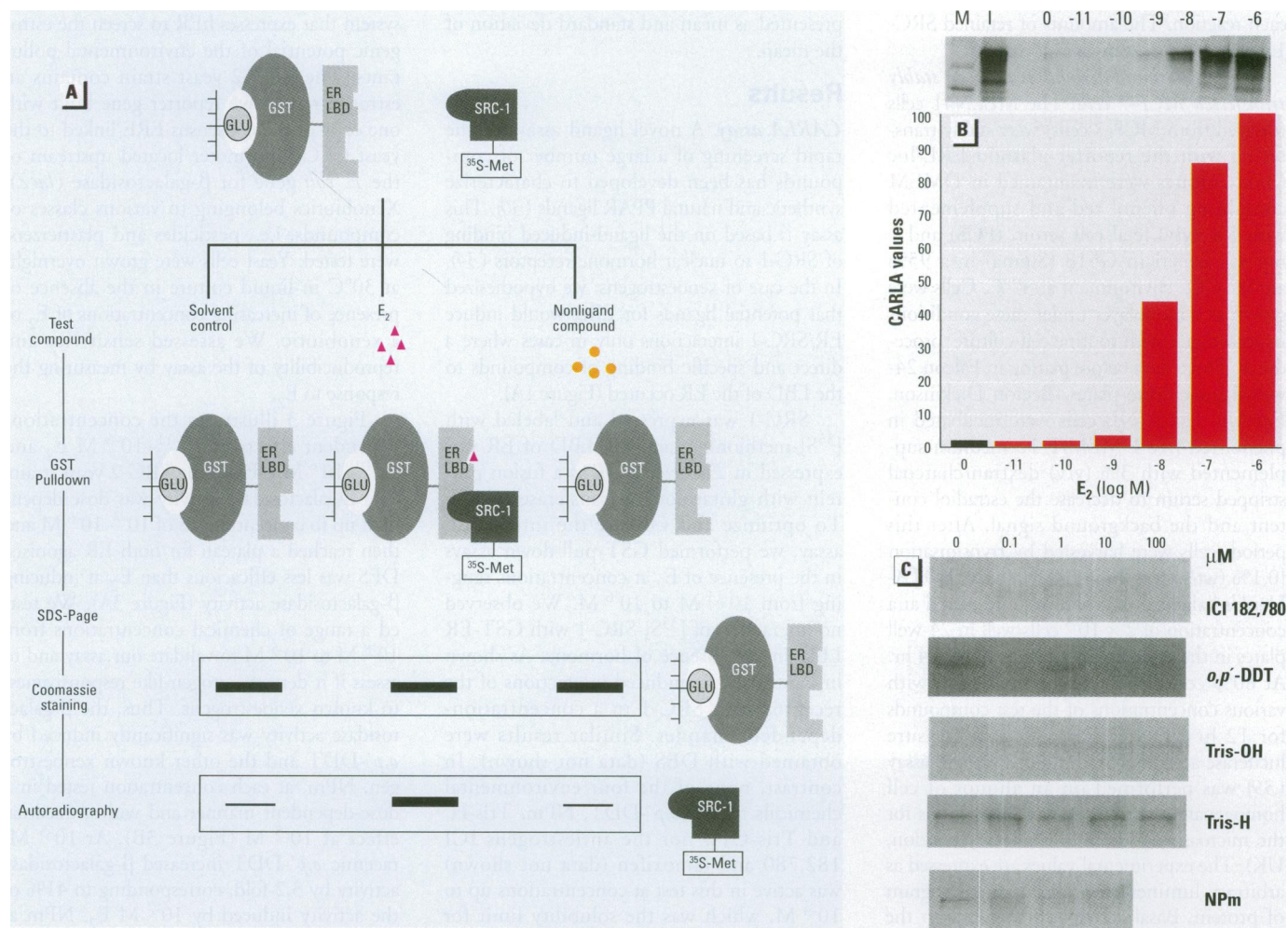
ranging from  $10^{-6}$  M to  $10^{-4}$  M. Both compounds increased  $\beta$ -galactosidase activity to reach 70% of the activity with  $E_2$ , although the activity decreased with  $10^{-4}$  M Tris-OH for unknown reasons. This indicates partial agonistic activity in this test. The same compounds were also tested using the YRG-2 yeast strain, which does not express hER (Figure 4). There was no induction of the *lacZ* gene in the presence of  $E_2$  or the test compounds. Taken together, these results indicate that the estrogenic activity detected in our yeast system that expresses hER is the result of an interaction between the receptor protein and the xenobiotics or their metabolites, and not a consequence of an unspecific activation of the basal transcriptional machinery.

Further control experiments (Figure 5) revealed that in the YRG-2 strain, the potent antiestrogen ICI 182,780 was not able to antagonize  $E_2$  activity even when a 100-fold

molar excess was used. More interestingly, when tested alone, ICI 182,780 exhibited full agonist activity. This result is in agreement with the findings of Kohno et al. (41) with a different yeast strain. Tamoxifen also had an agonist activity in this assay (data not shown). These latter results prompted us to use a mammalian cellular system, in which this antiestrogen behaves as a pure antagonist (42–44).

**ERE-luciferase reporter gene in stably transfected MCF-7 cells.** To examine the ability of the environmental chemicals to trigger hER-mediated transcriptional activation in mammalian cells, we used MCF-7 human breast cancer cells that were stably transfected with a plasmid containing one ERE copy linked to the luciferase gene. The cells were incubated in the presence or absence of increasing concentrations of  $E_2$  or the environmental chemicals for 12 hr; extracts were

then assayed for luciferase activity. Figure 6 illustrates the concentration response curve for the range from  $10^{-12}$  M to  $10^{-6}$  M  $E_2$ . The median effective concentration ( $EC_{50}$ ) value for  $E_2$ -induced response was approximately  $10^{-10}$  M. The maximal luciferase induction was observed at a concentration of  $10^{-8}$  M. Thus,  $10^{-8}$  M  $E_2$  was included as a positive control in further experiments to serve as a reference for comparison with other chemicals. As shown in Figure 7A, both the racemic mixture and the enantiomers of the organochlorinated compound *o,p'*-DDT were effective at inducing luciferase activity. In each case, the maximal induction was observed at the highest concentration tested ( $10^{-5}$  M). A concentration of  $10^{-4}$  M was toxic (data not shown). The L-enantiomer of *o,p'*-DDT was the most efficacious compound at  $10^{-5}$  M, even more than  $E_2$  at  $10^{-8}$  M (124% of  $E_2$  effect), indicating that it



**Figure 1.** Ligand-dependent interaction *in vitro* between ER $\alpha$  and SRC-1. Abbreviations: I (input), amount of [ $^{35}$ S]-SRC-1 used in each reaction; M, molecular weight marker. (A) CARLA assay protocol. See "Materials and Methods" for details. The binding of a ligand enhances the interaction between the nuclear receptor LBD and the SRC-1, and thus, the amount of pulled SRC-1 in the presence of a ligand was higher than in the absence of ligand (solvent or nonligand compound). (B) *In vitro* translated and [ $^{35}$ S]-labeled SRC-1 was incubated with GST-ER in the presence of various concentrations of  $E_2$ . The relative CARLA value of 100 for  $E_2$  corresponds to the amount of SRC-1 retained by ER LBD in the presence of  $10^{-8}$  M  $E_2$ . (C) *In vitro* translated [ $^{35}$ S]-labeled SRC-1 was not retained on GST-fusion proteins of the ER $\alpha$  LBD in the presence of environmental chemicals. ICI 182,780, an antiestrogen, was used as a negative control. NPm was used at concentrations ranging from 100 nM to 10  $\mu$ M and the other compounds at concentrations ranging from 100 nM to 100  $\mu$ M according to their solubility.

is a full agonist of hER-mediated transactivation. As in the yeast system, NPm, Tris-H, and Tris-OH also exhibited hER-mediated estrogenicity in MCF-7 cells (Figure 7B). However, the two latter chemicals did not yield hyperbolic dose–response curves. Tris-H and Tris-OH showed a statistically significant weak effect from a concentration of  $10^{-8}$  M, but they were much less potent and efficacious than  $E_2$  and the other test compounds. NPm ( $10^{-5}$  M) induced luciferase activity to an extent similar to  $E_2$ , indicating that it might also be a full agonist of the hER (Figure 7B).

To demonstrate that the effects of the chemicals were mediated by the hER, we incubated MCF-7 cells in the presence of environmental chemicals alone or together with  $10^{-6}$  M ICI 182,780, a pure ER antagonist in these cells. The luciferase activity induced by all of the chemicals tested was abolished completely in the presence of ICI 182,780 (Figure 8), demonstrating that the chemicals interact in a specific manner with the hER in this cellular assay. We also observed that after pretreatment with 3% dextran/charcoal stripped serum, the MCF-7 stably transfected cells were not completely devoid of  $E_2$  because ICI 182,780 was able to decrease the solvent value. This could be due to residual estrogens present in the serum, as we have observed the capability of these cells to respond to lower  $E_2$  levels. Alternatively, there was a weak  $E_2$ -independent activity of the ER that possibly resulted from hormone-independent signaling as described previously (45).

## Discussion

Several natural and man-made chemicals have been labeled as endocrine disruptors, with most of them exhibiting estrogen-like activity (46,47). As a result, there are numerous examples of reproductive anomalies in wildlife in areas contaminated with chemicals that display hormone-like activity (15). Essential for the understanding of potential hazards is the determination of whether these chemicals interact directly with steroid receptors, such as the estrogen receptors. Several *in vitro* assays have been developed to screen chemicals for estrogenic activity, including yeast-based screens (29), the MCF-7 cell proliferation assay (48), estrogen-responsive reporter gene assays (49), and ER binding assays (50,51).

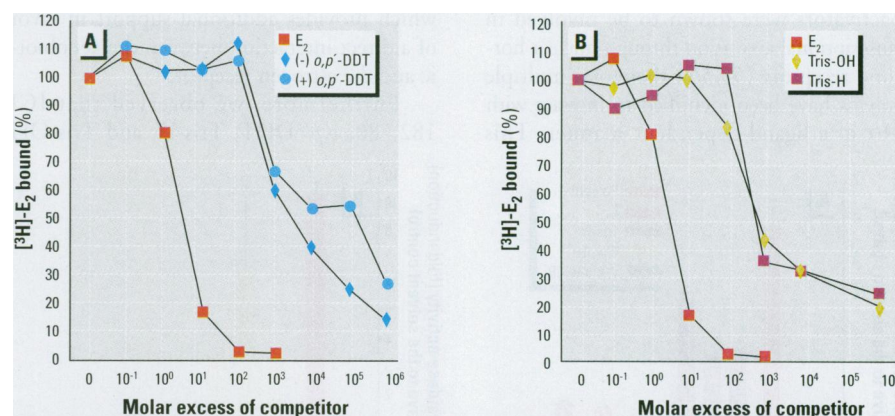
**In vitro test systems: advantages and drawbacks.** We used a combination of complementary assays to study the estrogenic activity of different environmental pollutants. This combination of *in vitro* techniques includes the GST-pulldown assay (CARLA test), which detects interactions between ER and a coactivator; a competition binding assay that assesses ligand interactions

with the receptor; a yeast-based estrogen receptor assay (YES); and a luciferase reporter assay in cultured MCF-7 cells that reveals transcriptional activation.

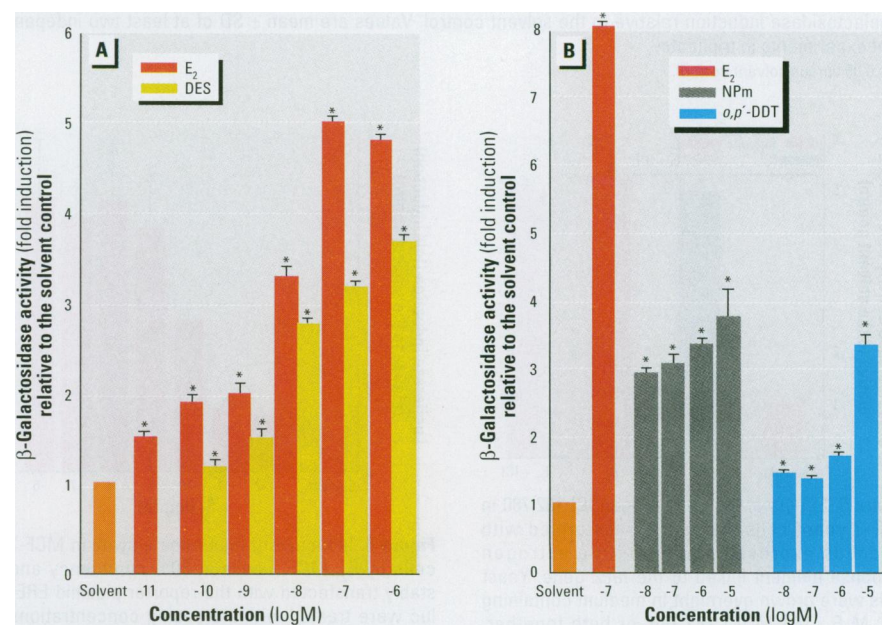
The CARLA test and the competitive binding assay are not able to discriminate between estrogenic and antiestrogenic properties of environmental chemicals, but they can give useful and important information about the interaction of these chemicals with the ER and the molecular mechanism of their action.

The advantages of the YES assay for assessing chemical interactions with ER are its

specificity and its ease of manipulation. Indeed, it is a simple eukaryotic system in which estrogenic responsiveness is primarily due to the ligand binding properties of the ER and its ability to stimulate the basal transcription machinery (52). However, an important drawback to this assay is its responsiveness to antiestrogens (41,53); another disadvantage is that the thick cell wall of the yeast limits permeability of substances (54). In early experiments, we used the GA 24 yeast strain, which had a high estrogen sensitivity but was unresponsive to xenobiotics (data not shown). Therefore, we chose the YRG-2



**Figure 2.** Inhibition of  $[^3\text{H}]\text{-E}_2$  binding by environmental chemicals. Some compounds tested in the CARLA assay were measured for their ability to inhibit the binding of  $[^3\text{H}]\text{-E}_2$  to hER by displacing it in competition binding assays. MCF-7 cells growing in monolayer culture were incubated with 0.1 nM  $[^3\text{H}]\text{-E}_2$  for 1 hr at  $37^\circ\text{C}$  in the absence or presence of increasing concentrations of radioinert  $E_2$ , (-) *o,p'*-DDT, (+) *o,p'*-DDT, Tris-H, or Tris-OH. The data are plotted as the percentage of control in which specific binding in the absence of competitor is equal to 100%.



**Figure 3.** Effect of  $E_2$ , DES, and established estrogenic compounds on the transcriptional activity of hER in YRG-2 yeast cells, which express hER and contain the reporter plasmid pLGERE. Yeast cells were grown in medium containing increasing concentrations of  $E_2$ , DES, *o,p'*-DDT, NPm, or vehicle (EtOH). Following overnight incubation the cultures were assayed for  $\beta$ -galactosidase activity (see “Materials and Methods” for details). Values represent the  $\beta$ -galactosidase induction relative to the solvent control. Values are mean  $\pm$  SD of at least two independent experiments in triplicate.

\* $p < 0.05$  versus solvent control.

strain that demonstrated responsiveness to both estrogens and xenoestrogens. Animal cells are much more sensitive to environmental conditions as compared to yeast and allow screening of substances for both estrogenic and antiestrogenic properties.

We concentrated our research on two poorly studied compounds, Tris-H and Tris-OH, and compared their activity with the already known xenoestrogens *o,p'*-DDT and NPM. For *o,p'*-DDT, we analyzed both the racemic mixture and the two enantiomers.

**Screening by CARLA.** The CARLA provided information on the interactions between the ligand and the receptor. Recently, several coactivators were shown to be involved in transcriptional activation through nuclear hormone receptors (55,56); therefore, multiple proteins have been identified to interact with ER $\alpha$  in a ligand-dependent manner. This

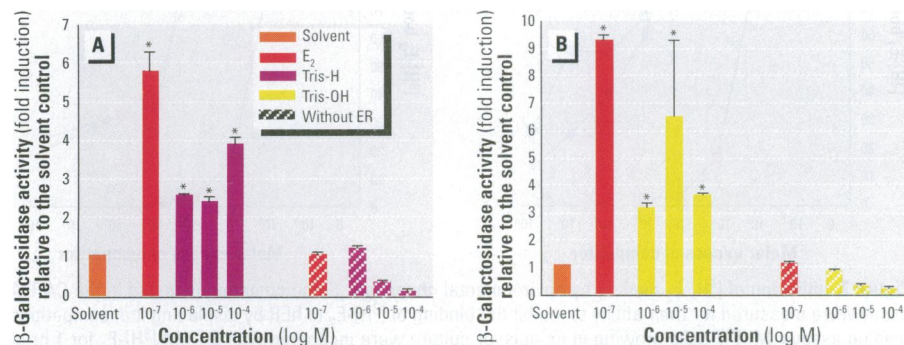
property was used in the CARLA to identify potential ER ligands with a specific interaction between the coactivator SRC-1 and the fusion protein GST-ER $\alpha$  LBD.

Our results demonstrate that, in contrast to E<sub>2</sub>, none of the environmental pollutants tested was able to induce an interaction between SRC-1 and the ER $\alpha$  LBD. However, all of the chemicals investigated stimulated the ER-mediated transcriptional activity of either the reporter *lacZ* gene in yeast or the luciferase reporter gene in MCF-7 cells. Furthermore, *o,p'*-DDT (racemic mixture and enantiomers), Tris-H, and Tris-OH were able to displace E<sub>2</sub> from its binding site, which provides additional support in favor of a direct interaction between the xenobiotic and the estrogen receptor.

Furthermore, we observed that ICI 182,780, *o,p'*-DDT, Tris-H, and Tris-OH

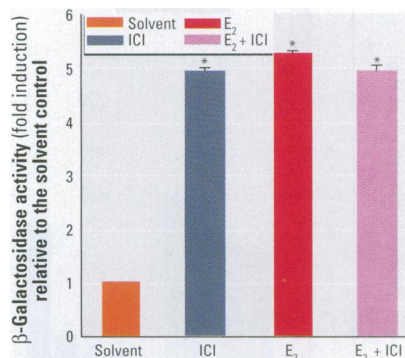
were able to inhibit E<sub>2</sub>-dependent interaction of SRC-1 with the ER LBD. However, the extent of inhibition, although always observed, varied from one experiment to the other for as yet unknown reasons. By contrast, the addition of the thyroid hormone triiodothyronine, which is not a ligand of ER, had no effect on this E<sub>2</sub>-dependent interaction. These results also suggest that the pollutants tested are able to bind directly to the ER. However, xenobiotics would not allow an interaction between the nuclear receptor and the coactivator SRC-1. It is likely that different molecules influence interactions of ER with coactivators other than SRC-1. Thus, each chemical or group of chemicals may use a distinct coactivator to regulate ER-mediated transcription. Using the yeast-two hybrid assay (GAL4 DBD-ER LBD and GAL4 AD-coactivator fusion proteins), Nishikawa et al. (57) showed that 10<sup>-6</sup> M NPM was inactive with respect to SRC-1, which is in agreement with our results, but appeared to activate the ER via TIF-2. In our case, it seems likely that the chemicals tested recruit coactivators distinct from SRC-1 to interact with the ER. It is also possible to explain these results by different ligand selectivity of ER for coactivator binding. The absence of an effect of the compounds investigated in the CARLA merely excludes an action of these compounds via SRC-1, but does not rule out an activation of ER. The results obtained in the CARLA provided evidence for a molecular mechanism of action of the test compounds via hER that is distinct from that of E<sub>2</sub>.

**Inhibition of [<sup>3</sup>H]-E<sub>2</sub> binding.** The results obtained in our displacement experiments using radiolabeled E<sub>2</sub> demonstrate a direct interaction between the test compounds and the hER, but the affinities were lower than those for unlabeled E<sub>2</sub>. Tris-H and Tris-OH were able to inhibit [<sup>3</sup>H]-E<sub>2</sub> binding to the hER at concentrations for which they were active in transactivation assays. The displacement obtained with 1,000–10,000-fold molar excess of Tris-H and Tris-OH is approximately 60–70%. This is a drastic decrease in comparison with the results obtained in the MCF-7 transactivation assay. Indeed, the luciferase activity was induced by 2- to 3-fold. Several explanations are possible. The compounds might effectively displace the natural hormone, but they have less affinity for the receptor than estradiol. Also, we cannot exclude that the conformation of the receptor after binding to the test compound is not appropriate to fully activate the luciferase reporter gene. Furthermore, the comparison of both *o,p'*-DDT enantiomers revealed that the L-enantiomer was more active in inhibiting [<sup>3</sup>H]-E<sub>2</sub> binding to the hER. This is in agreement with the results obtained in the



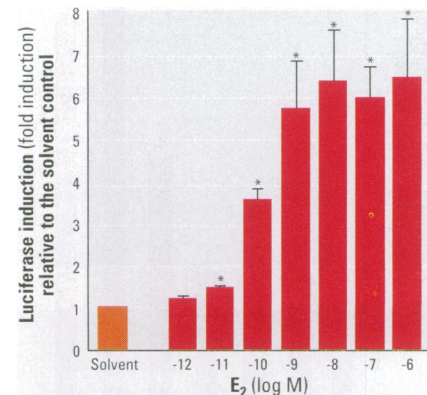
**Figure 4.** Response of YRG-2 yeast cells, which contain the reporter plasmid pLGERE and that express or do not express hER, to Tris-H (A) and Tris-OH (B). Following overnight incubation the cultures were assayed for the  $\beta$ -galactosidase activity (see "Materials and Methods" for details). Values represent the  $\beta$ -galactosidase induction relative to the solvent control. Values are mean  $\pm$  SD of at least two independent experiments in triplicate.

\* $p < 0.05$  versus solvent control.



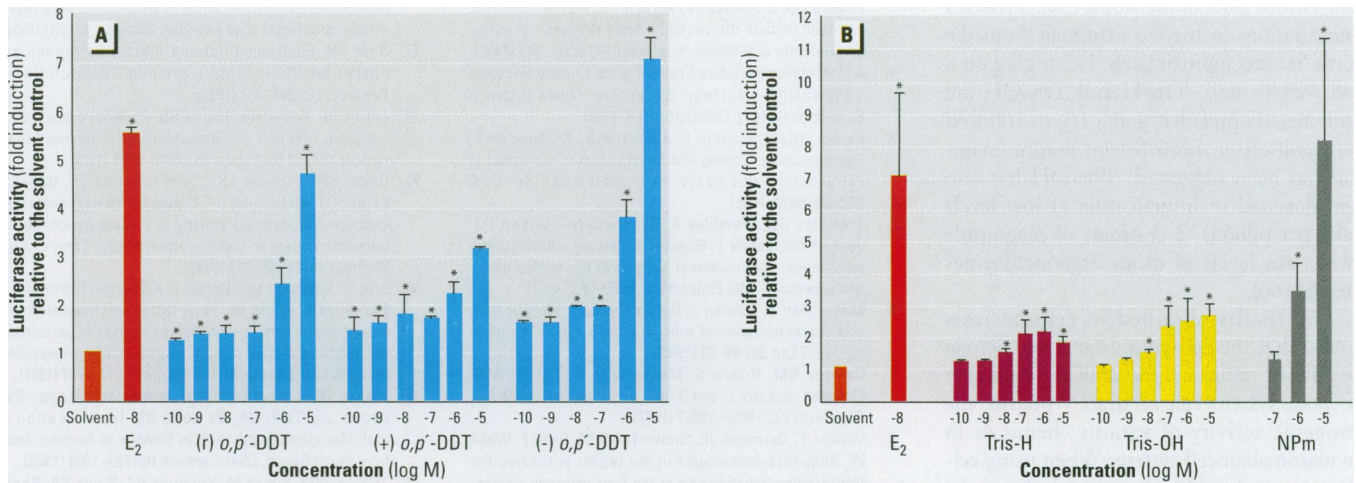
**Figure 5.** Effects of the antiestrogen ICI 182,780 in YRG-2 yeast cells that were transformed with plasmids expressing hER and the estrogen response element linked to the *lacZ* gene. Yeast cells were grown overnight in medium containing 10<sup>-8</sup> M E<sub>2</sub>, 10<sup>-6</sup> M ICI 182,780, or both together. Cells were harvested and assayed for  $\beta$ -galactosidase activity (see "Materials and Methods" for details). Values represent the  $\beta$ -galactosidase induction relative to the solvent control. Values are the mean  $\pm$  SD of at least two independent experiments in triplicate.

\* $p < 0.05$  versus solvent control.



**Figure 6.** Induction of luciferase activity in MCF-7 cells by E<sub>2</sub>. MCF-7 cells at 80% confluency and stably transfected with the reporter plasmid ERE-luc were treated with increasing concentrations of E<sub>2</sub>. Data are presented as mean  $\pm$  SD and represent three independent experiments representing six measurements.

\* $p < 0.05$  versus solvent control.

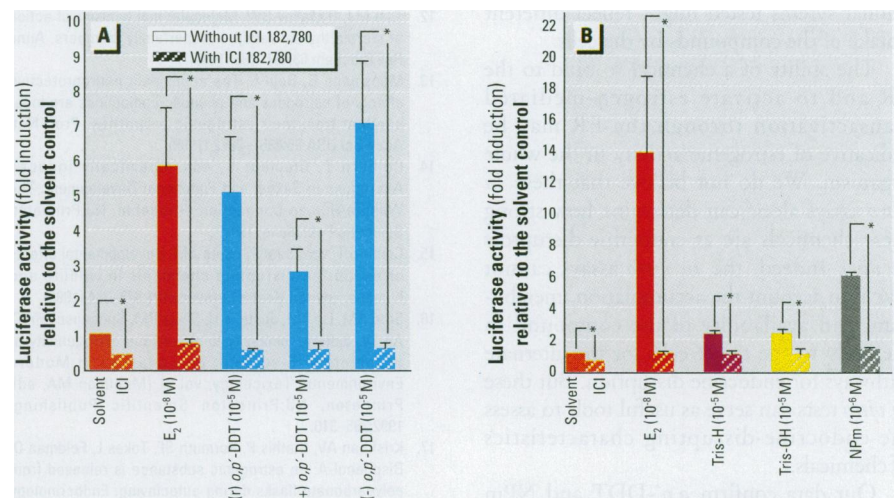


**Figure 7.** Induction of luciferase activity in MCF-7 cells by environmental chemicals. MCF-7 cells at 80% confluency and stably transfected with the reporter plasmid ERE-luc were treated with increasing concentrations of environmental chemicals or vehicle. (r) *o,p'*-DDT, racemic mixture. E<sub>2</sub> (10<sup>-8</sup> M) was used as reference (concentration required to obtain the highest activity). Data are presented as mean  $\pm$  SD and represent three independent experiments representing six measurements. \* $p$  < 0.05 versus solvent control.

transactivation assay performed in MCF-7 cells because it showed the greatest activity at this concentration.

**Cellular assays.** Our studies show that the L-enantiomer of *o,p'*-DDT is more potent than the D-enantiomer in regulating ER-mediated cellular response in MCF-7 human breast cancer cells. It displays full agonist activity on the ER, reaching the maximal effect at 10<sup>-5</sup> M. *o,p'*-DDT had a stronger effect in stably transfected MCF-7 cells than in yeast, suggesting a higher sensitivity of the mammalian cellular system. More interestingly, we showed that this pesticide could trigger hER-mediated transcriptional activation in MCF-7 cells at low concentrations (10<sup>-10</sup> M). *o,p'*-DDT has previously been shown to have estrogenic activity in the rat (58,59) and in *in vitro* assays (49,60). Most research on *o,p'*-DDT has been performed using the racemic mixture. Nevertheless, Mc Blain (61) showed that the estrogenic activity of *o,p'*-DDT resides essentially with the L-enantiomer.

As for *o,p'*-DDT, our results indicate that nonylphenol (an estrogenic xenobiotic released from modified polystyrene) displayed its greatest estrogenic activity in the *in vitro* cellular tests at a concentration of 10<sup>-5</sup> M. The comparison between both cellular assays revealed that 10<sup>-5</sup> M nonylphenol had much more estrogen-like activity on reporter gene expression in mammalian cells than in yeast. A statistically significant effect was obtained at 10<sup>-6</sup> M nonylphenol in MCF-7 cells and at 10<sup>-8</sup> M in yeast. Therefore, nonylphenol seems to be more efficacious in yeast than in mammalian cells. In MCF-7 cells, nonylphenol appeared to be a full agonist of hER-mediated transactivation because it increased luciferase activity to the same extent as E<sub>2</sub>, albeit at a higher concentration. Balaguer et



**Figure 8.** Inhibition of environmental chemical-induced luciferase activity by the antiestrogen ICI 182,780. MCF-7 cells stably transfected with pERE-luc were treated with vehicle, E<sub>2</sub>, or environmental chemicals in the presence or absence of 10<sup>-6</sup> M ICI 182,780. Data are presented as mean  $\pm$  SD and represent three independent experiments representing six measurements. \* $p$  < 0.05.

al. (62) obtained the greatest activity of NPm at this concentration, but in their case, the sensitivity of the MCF-7 stable transfectants to E<sub>2</sub> was higher. Differences between their results and our study could be attributable to the use of a different clone of stable transfectants or to the dextran/charcoal stripped serum prepared. Nonylphenol has been shown to induce proliferation of human breast cancer cells and to trigger mitotic activity in the rat endometrium (63). In a stably transfected ER-mediated luciferase reporter gene assay in the human T47D breast cancer cell line, this chemical was one of the most potent xenoestrogens tested, with an EC<sub>50</sub> of 260 nM (60). Nonylphenol has also been shown to inhibit [<sup>3</sup>H]-E<sub>2</sub> binding to the ER of rainbow trout and to stimulate vitellogenin

gene expression in this species (64). Danzo (51) demonstrated that 10<sup>-4</sup> M nonylphenol caused a dramatic decrease in [<sup>3</sup>H]-E<sub>2</sub> binding (75%), indicating that the effect is mediated via the ER.

So far, no data are available concerning the endocrine-disrupting effects of Tris-H and Tris-OH. The data presented here using *in vitro* cellular tests provide evidence for the first time of the ability of both compounds to modulate ER-mediated transactivation in yeast and in human breast cancer cells. In these two reporter gene assays, both chemicals acted as weak agonists of hER. The 10<sup>-4</sup> M concentration of Tris-H and Tris-OH could not be tested in MCF-7 cells because of cellular toxicity. Tris-H and Tris-OH have been found in fish, birds, and marine mammals

from various parts of the world (65). Tris-OH concentrations in marine mammals from the North Sea are approximately 1–2 mg/kg on a lipid weight basis. Tris-H and Tris-OH are highly bioaccumulative, and a 10- to 100-fold biomagnification from fish to marine mammals has been suggested. Tris-OH has also been detected in human milk at low levels (parts per billion), 2–3 orders of magnitude lower than levels of other organochlorines detected (66).

The results obtained in cellular tests showed that the compounds analyzed act via the ER. In addition, the data indicate that the yeast system can accurately predict the estrogenic activity of various chemicals in the mammalian cell system. When using cellular tests, it should be kept in mind that the permeability of the xenobiotics through the cell membrane can differ because of their distinct chemical structures. In other words, the differences observed between the two cellular systems tested might reflect different uptake of the compounds by the cells.

The ability of a chemical to bind to the ER and to activate estrogen-mediated transactivation through the ER may be indicative of estrogenic activity in the whole organism. We do not believe that these *in vitro* assays alone can determine how strong these chemicals are as endocrine disruptors *in vivo*. Indeed, the *in vitro* assays cannot take into account the accumulation, metabolism, and availability of the compound in the body to the target cells, or the alternate pathways for endocrine disruption. But these *in vitro* tests can serve as useful tools to assess the endocrine-disrupting characteristics of chemicals.

Our data confirm *o,p'*-DDT and NPM to be xenoestrogens and, more interestingly, for the first time, identify Tris-H and Tris-OH as estrogen mimics. Moreover we show that *o,p'*-DDT (racemic mixture and both enantiomers), as well as Tris-H and Tris-OH, act at low concentrations (nanomolar); this might have important consequences for living organisms in a contaminated environment. These chemicals act directly via the ER, but their mechanism of action seems to be different from that of E<sub>2</sub>. However, the impact of environmental Tris-H and Tris-OH on the development, reproduction, and health of wildlife and humans has yet to be determined.

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