Articles

Assessing Environmental Chemicals for Estrogenicity Using a Combination of *In Vitro* and *In Vivo* Assays

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Because of rampant concern that estrogenic chemicals in the environment may be adversely affecting the health of humans and wildlife, reliable methods for detecting and characterizing estrogenic chemicals are needed. It is important that general agreement be reached on which tests to use and that these tests then be applied to the testing of both man-made and naturally occurring chemicals. As a step toward developing a comprehensive approach to screening chemicals for estrogenic activity, three assays for detecting estrogenicity were conducted on 10 chemicals with known or suspected estrogenic activity. The assays were 1) competitive binding with the mouse uterine estrogen receptor, 2) transcriptional activation in HeLa cells transfected with plasmids containing an estrogen receptor and a response element, and 3) the uterotropic assay in mice. The chemicals studied were 17β-estradiol, diethylstilbestrol, tamoxifen, 4-hydroxytamoxifen, methoxychlor, the methoxychlor metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), endosulfan, nonylphenol, *o,p'*-DDT, and kepone. These studies were conducted to assess the utility of this three-assay combination in the routine screening of chemicals, or combinations of chemicals, for estrogenic activity. Results were consistent among the three assays with respect to what is known about the estrogenic activities of the chemicals tested and their requirements for metabolic activation. By providing information on three levels of hormonal activity (receptor binding, transcriptional activation, and an *in vivo* effect in an estrogen-responsive tissue), an informative profile of estrogenic activity is obtained with a reasonable investment of resources. *Key words*: competitive binding, endocrine disruptors, estrogen receptor, transfection, uterotropic assay. *Environ Health Perspect* 104:1296–1300 (1996)

Estrus is that period of the reproductive cycle in mammalian females in which ovulation occurs and the female is typically most receptive to mating. In rodents, the most commonly used laboratory model for studies of reproduction, estrus follows the surge of estrogen produced by the maturing ovarian follicles during proestrus. Thus, chemicals that induce estrus, or a biological response associated with estrus, are traditionally defined as estrogens (*1*). The capacity of a substance to induce such effects is termed estrogenicity.

With growing concern that estrogenic chemicals in the environment, either naturally occurring or man-made, may adversely affect the health of humans, domestic animals, and wildlife (*2,3*), the need for meaningful, standardized, and widely accepted methods for reliably detecting and characterizing estrogenic chemicals has gained importance. Many assays for estrogenicity have been proposed and several are in broad use. Descriptions of many of these are found in the proceedings of the conference on Estrogens in the Environment, III: Global Health Implications (*4*).

Because of the multiple biological effects of estrogens and the influence of absorption, metabolism, distribution, and excretion on the manifestation of their estrogenic activity, any single assay can provide only limited information on those effects. *In vitro* assays can provide valuable insights on mechanisms of action but are restricted in their capacity to mimic whole animal metabolism and distribution. *In vivo* assays permit the detection of effects resulting from multiple mechanisms but may give indications only of gross effects and reveal little about mechanisms of activity.

In an initial effort to assess the utility of using a combination of *in vitro* and *in vivo* assays to screen for estrogenicity, 10 known or alleged estrogenic chemicals were tested using assays that assess estrogenic activity at three different levels of action. The first assay, competitive binding with the estrogen receptor (ER), uses a cell-free system to determine the extent to which the test chemical binds to the ER, as reflected by its effect on the binding of 17β-estradiol (*5*). Second, if a chemical is estrogenic, the consequence of its binding to the ER should be transcriptional activation of estrogen responsive genes. This effect has been determined using an assay that employs human HeLa cells transfected with an ER and an estrogen response element (ERE) linked to a chloramphenicol acetyl transferase (CAT) reporter gene. Transcriptional activation is determined by measuring the amount of CAT protein produced by the cells following treatment with the test chemical (*6*). The third assay was used to determine effects on an estrogen-responsive tissue in an intact animal. The weanling mouse uterotropic assay, one of the most well-established and widely used methods to detect estrogenicity, was employed to determine if exposure to the test chemical led to increased uterine wet weight (*7*).

Thus, 10 chemicals were assessed for evidence of estrogenicity at three levels of activity. The results indicate that this combination of tests may provide an efficient and effective approach to screening environmental chemicals for estrogenic activity.

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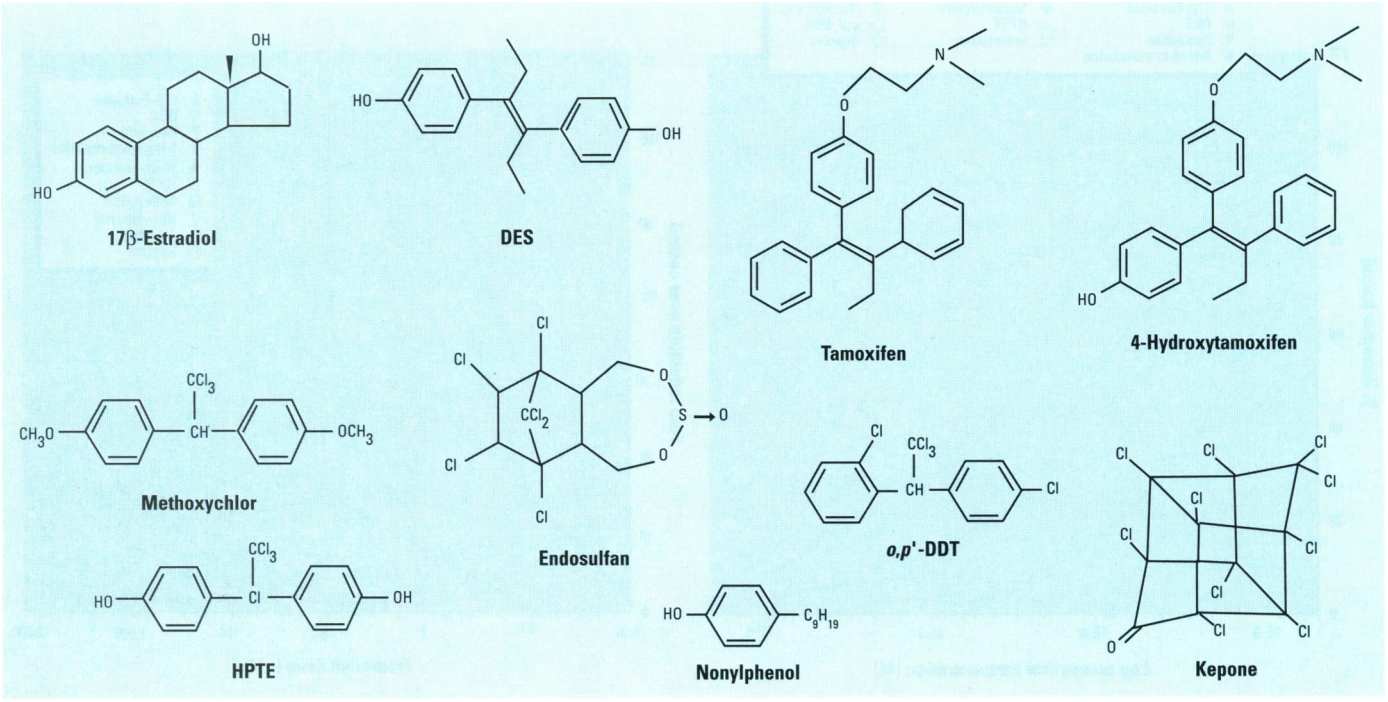

Figure 1. Chemical structures of the 10 chemicals studied. Abbreviations: DES, diethylstilbestrol; HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; *o,p'*-DDT, dichlorodiphenyltrichloroethane.

Materials and Methods

Chemicals. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): diethylstilbestrol (DES; 99+% pure); 17β-estradiol (E_2; 99+% pure); and tamoxifen (99% pure). 4-Hydroxytamoxifen (99+% pure) was a gift from ICI Pharmaceuticals (Wilmington, DE). Other chemicals were obtained as follows: methoxychlor (98% pure) from Drexel Chemical Co. (Memphis, TN); 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE; 99+% pure) from Cedra Corp. (Austin, TX); a,b-endosulfan (98% pure; a = 78%, b = 20%) and kepone (98% pure) from Chem Service (West Chester, PA); nonylphenol (99.5% pure) from Schenectady International (Schenectady, NY); and *o,p'*-DDT (99+% pure) from Lancaster Synthesis (Windham, NH). The structures of these chemicals are presented in Figure 1.

Competitive binding assay. [2,4,6,7-^{3}H] E_2, 110 Ci/mmol, with >98% radiochemical purity was obtained from Du Pont-New England Nuclear (Boston, MA). The competitive binding assay was carried out according to the previously described procedure (*8*). Estrogen receptors were obtained from female CD-1 [ICR] BR mice from Charles River Breeding Laboratories (Raleigh, NC). Females were ovariectomized at 10–12 weeks of age and sacrificed 2 weeks later. Animals were kept in a controlled environment with 12 hr of light and 12 hr of dark. The autoclavable NIH-31 open formula, natural ingredient rodent diet (Agway, Inc., St. Mary, OH) and reverse osmosis/deionized (RO/DI) water were given *ad libitum*. After sacrifice by cervical dislocation at 8–10 weeks of age, the uteri were removed and frozen on dry ice. The frozen uteri were then placed in ice-cold TEGM buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, pH 7.6) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) for 15 sec at speed setting 6.5 at a ratio of 50:1 (milligrams of tissue weight/per milliliter of buffer). The homogenate was filtered through 100–125 mm mesh Nitex filtering media and centrifuged at 1,000*g* for 10 min; the supernatant was then decanted and centrifuged at 45,000 rpm for 50 min. The 105,000*g* supernatant was used for cytosol receptor binding assays.

Aliquots of 100 µl cytosol were incubated with 5 nM [^{3}H]E_2 and increasing concentrations of unlabeled competitors (0.5 nM–5 µM). The mixtures were incubated at 4°C for 18 hr, and then 250 µl of 60% HAP (hydroxyapatite) in TEGM buffer was added to each tube. Tubes were centrifuged at 1,000*g* for 10 min and the resulting HAP pellet was washed twice with 3 ml TEGM buffer then suspended in scintillation cocktail. The radioactivity was measured using a Beckman CS 9800 scintillation counter (Beckman, Fullerton, CA). The binding affinities were determined using Ligand Competition Analysis Software by Lundon Software (Chagrin Falls, OH).

Transcriptional activation assay in ER-transfected HeLa cells. HeLa cells were used because of their estrogen responsiveness in the presence of ER and estrogens, allowing for detection of weak estrogens. In addition, they can be treated in serum-free media to ensure that the estrogen background is null. Testing with and without the ER proves that any response is through direct interactions with the ER. The mouse ER was used in these experiments for consistency among the three assays.

The estrogen responsive reporter vector, ERET81CAT, and the pRSV vector containing the mouse ER cDNA (without the neomycin resistance cassette) was constructed as previously described (*9*). HeLa cells, which do not contain ER, were cotransfected with both vectors or with only the ERET81CAT vector to determine if observed activity was ER dependent. The cells were grown in DMEM/F12 medium (1:1) without phenol red (Sigma), supplemented with 5% fetal bovine serum and penicillin-streptomycin. Cells were electroporated and treated as previously described (*6*). During and after transfection, the cells were maintained in DMEM/F12 medium plus insulin-transferrin-sodium selenite (Sigma). All procedures using serum-free conditions were performed with Falcon plastics (Becton Dickinson, Franklin Lakes,

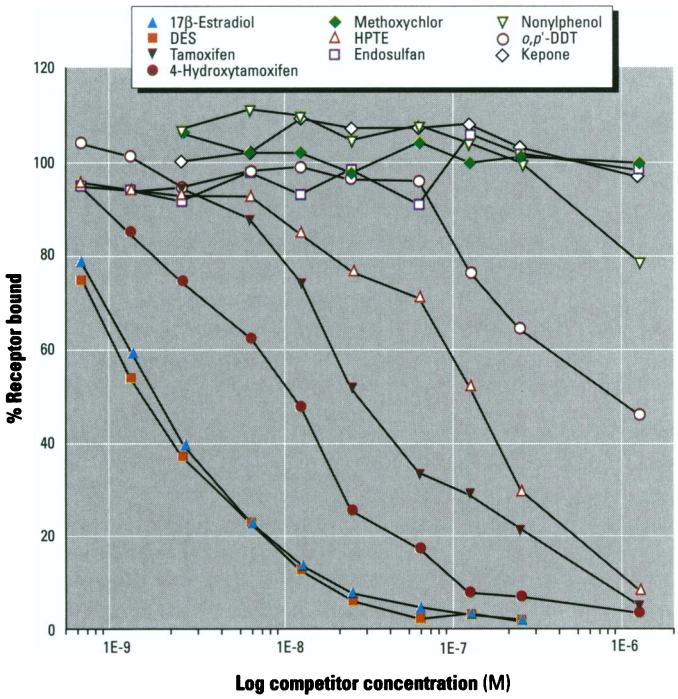

Figure 2. Competitive binding assay. Abbreviations: DES, diethylstilbestrol; HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane. Mouse uterine cytosol was incubated with 5 nM of [^{3}H]E_2, and 0.5 nM–5 μM of unlabeled test chemicals. After incubation for 18 hr at 4°C, the receptor was precipitated by addition of 60% hydroxyapatite. The precipitate was washed with buffer and the radioactivity was counted as described in Materials and Methods. Results are representative of two separate experiments performed in duplicate.

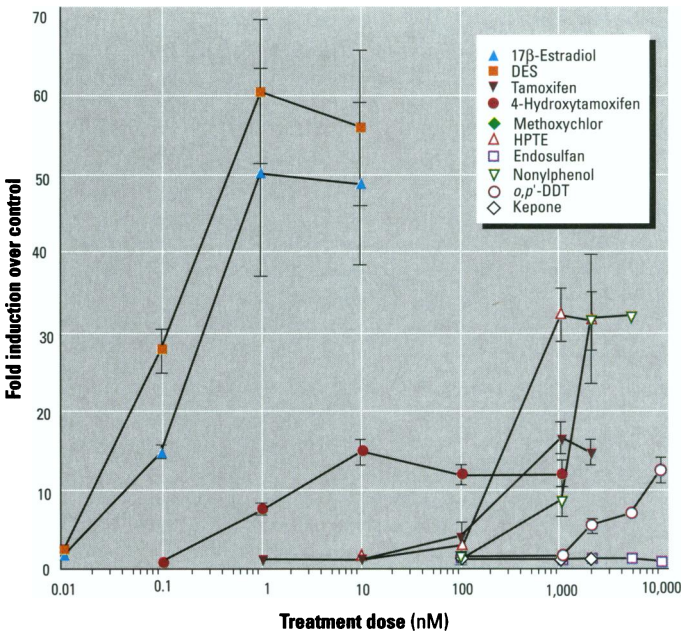

Figure 3. Transfection assay. Abbreviations: DES, diethylstilbestrol; HPTE, 2-2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane. Dose curves represent estrogenic activation of a CAT reporter gene in HeLa cells transfected with the mouse estrogen receptor (mER). Fold induction of CAT protein levels was determined from basal CAT levels (vehicle only) in HeLa cells transfected with the mouse ER expression and ERET81CAT reporter vectors. The data represent the mean ± SE. Dose curves were determined from at least three separate transfection experiments.

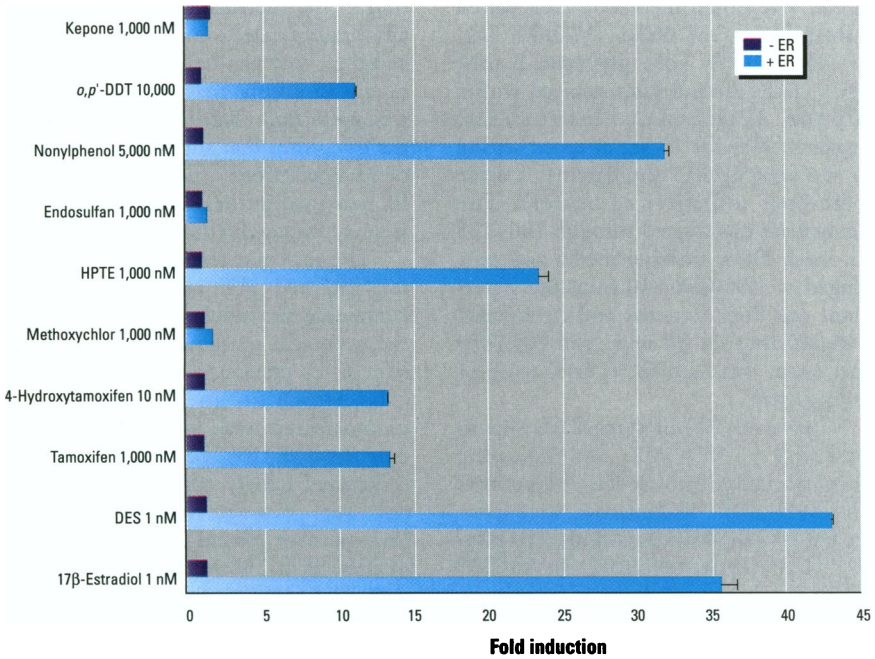

Figure 4. Transfection assay. Abbreviations: HPTE, 2-2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; DES, diethylstilbestrol. Estrogen receptor (ER)-dependent activation of an estrogen-responsive CAT reporter gene. Fold induction of CAT protein levels was determined from basal CAT levels (vehicle only) in HeLa cells transfected with the mouse ER expression and ERET81CAT reporter vectors (+ ER) or the ERET81CAT reporter vector only (- ER). Cells were treated in triplicate and the data represent the mean ± SE.

NJ). Triplicate samples for each hormone concentration were harvested at 28 hours post-transfection and assayed for CAT protein using the CAT-ELISA kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Mouse uterotropic bioassay. Female CD-1 mice [Crl:CD-1(ICR)] (Charles River Breeding Laboratories) were bred to males of the same strain at NIEHS. Timed pregnant females delivered their young on day 19 of gestation. At delivery, all litters were randomly standardized to 10 female pups per dam. All animals were housed in a temperature-controlled room (22 ± 1°C) with a 12 hr light and 12 hr dark cycle. Mice were provided with RO/DI water and fed autoclavable NIH-31 rodent diet *ad libitum*. All animal care procedures were preapproved by the NIEHS Animal Care and Use Committee and complied with the procedures in the *Guide for the Care and Use of Laboratory Animals*. Female pups were weaned on day 17 and housed five per cage. Starting on day 17, the immature mice were subcutaneously injected for 3 consecutive days with varying doses of each test compound dissolved in corn oil and were sacrificed on the morning of the fourth day (20 days old). High doses were selected based on a maximum of 1,000,000

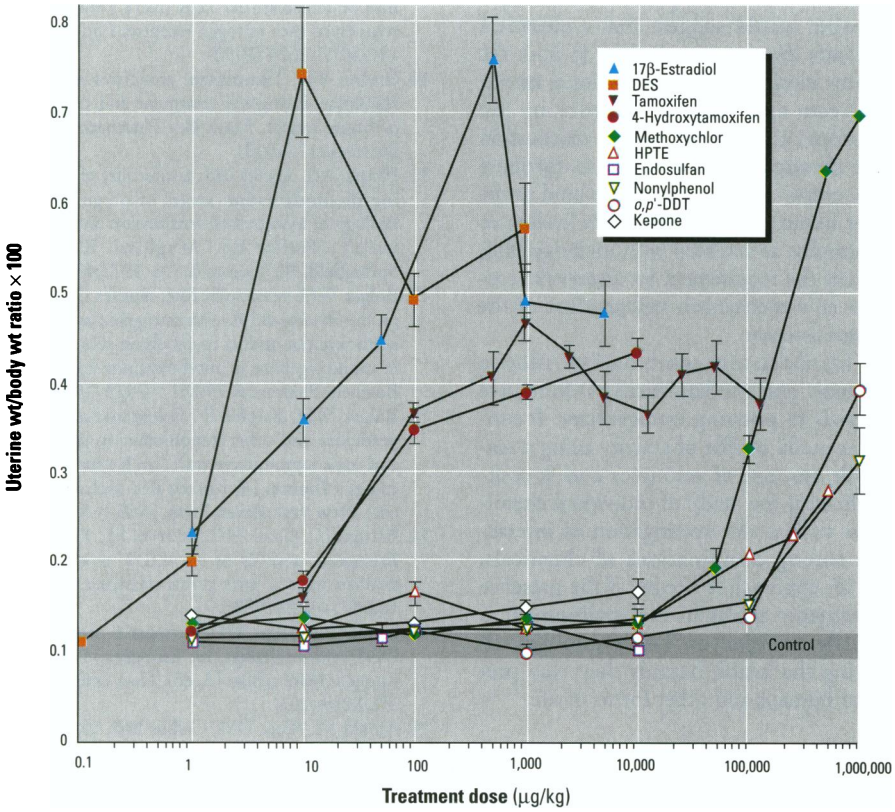

Figure 5. Uterotropic assay. Abbreviations: DES, diethylstilbestrol; HPTE, 2-2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane. Immature CD-1 mice were treated subcutaneously with various concentrations of chemicals dissolved in corn oil on postnatal days 17, 18, and 19. Animals were sacrificed on the morning of the day following the last injection (day 20). A ratio of uterine weights to body weights was plotted. Numbers are the mean ± the SE. Each data point represents results from a minimum of five animals.

Table 1. Summary of estrogenicity test results

Chemical	Competitive binding	Transcriptional activation	Uterotropic assay	Comments
17β-Estradiol	+++	+++	+++	Positive control
DES	+++	+++	+++	Positive control
Tamoxifen	++	++	++	Intrinsic estrogenic activity
4-Hydroxytamoxifen	++	++	++	Metabolite shows greater activity *in vitro*
Methoxychlor	–	–	++	Requires metabolic activation
HPTE	++	++	++	Estrogenic metabolite of methoxychlor
Endosulfan	–	–	–	No evidence of estrogenicity in these three tests
p-Nonylphenol	+	++	+	Confirms intrinsic and *in vivo* estrogenic activity
o,p'-DDT	+	+	+	Confirms intrinsic and *in vivo* estrogenic activity
Kepone	–	–	+	Weak *in vivo* effect

Abbreviations: DES, diethylstilbestrol; HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane.
+ = Positive test results; relative levels of effects are indicated by number of +s. – = Negative test results.

µg/kg or mortality in range-finding studies. No deaths were observed in any of the high-dose groups reported here. Body weights and uterine wet weights were determined. Uterine tissues were fixed in Bouin's fixative and processed for future histological examination. A minimum of five mice per dose point were used.

Results and Discussion

Results of the competitive binding (Fig. 2), transcriptional activation (Fig. 3,4), and uterotropic assays (Fig. 5) are presented. E_2 and DES were included as standards in the current study to monitor and assure the performance of the test systems and to provide reference data against which to compare the results of the other eight chemicals. Both produced the effects expected, exhibiting substantially greater estrogenic activity in all three assays than any of the other eight chemicals.

The breast cancer adjuvant therapeutic agent tamoxifen and its metabolite, 4-hydroxytamoxifen, exhibit both estrogenic and antiestrogenic activities (*10,11*). Tamoxifen and 4-hydroxytamoxifen both exhibited potent dose-related effects in all three assays. While these two chemicals gave nearly identical effects in the uterotropic assay (Fig. 5), the metabolite consistently exhibited greater activity in the transfection assay (Fig. 3) and the receptor binding assay (Fig. 2), which do not employ an exogenous source of metabolic activation. These results show the inherent estrogenic activity of tamoxifen and demonstrate that this activity is enhanced by hydroxylation.

Evidence of the *in vivo* estrogenic activity of methoxychlor and its requirement for metabolic conversion to HPTE, the active estrogen, is reviewed by Bulger and Kupfer (*12,13*). In contrast to tamoxifen, methoxychlor exhibits no intrinsic estrogenic activity, and conversion to HPTE is required for activity. No activity was seen with methoxychlor in either *in vitro* assay, whereas HPTE was clearly active in both assays (Fig. 2,3). In contrast, methoxychlor induced large increases in uterine weights at high doses and, although HPTE was active in the same dose range, it produced much smaller increases in uterine weights (Fig. 5). These results suggest that subcutaneous exposure to HPTE results in lower target organ concentrations than does equal exposure to the parent compound. This may be due to greater reactivity of the active metabolite and more rapid inactivation or detoxification.

o,p'-DDT and nonylphenol are environmental contaminants with reported estrogenic activity (*14–19*). Both exhibited estrogenicity in the three assays reported here. In the competitive binding assay (Fig. 2), nonylphenol reduced estradiol binding by about 20% at the highest concentration tested. In the transfection assay (Fig. 3), transcription was induced by nonylphenol to a level greater than tamoxifen or 4-hydroxytamoxifen and equal to HPTE. An effect was also seen in the uterotropic assay, albeit at doses exceeding 10,000 µg/kg. These results extend the characterization of nonylphenol's estrogenic activity as reported by Soto et al. (*17*) using MCF7 cells and Lech et al. (*19*) using vitellogenin mRNA induction in trout liver.

Over the same dose range where effects were seen with nonylphenol, *o,p'*-DDT gave a greater response in the receptor binding assay (Fig. 2). Although *o,p'*-DDT gave a response in the transfection assay (Fig. 3), it was less active than nonylphenol. *o,*p'-DDT was uterotropic (Fig. 5), showing roughly the same level of effect in the same dose range as HPTE and nonylphenol.

In the present studies, the reported estrogenic chemicals endosulfan (*20*) and kepone (*13,21–24*) were both negative in the receptor binding assay (Fig. 2) and the transcriptional activation assay (Fig. 3), indicating no activity *in vitro*. These kepone results are in contrast to reports of binding to the estrogen receptors of animals other than mice. Consistent with these negative results *in vitro*, endosulfan was negative in the uterotropic assay (Fig. 5). However, kepone gave a small dose-related increase in uterine weights over a dose range of 100–10,000 µg/kg (Fig. 5). This result agrees with earlier reports of similar effects in birds and rats (*13*) and mice (*24*). In the uterotropic assay, test doses for both endosulfan and kepone were limited to 10,000 µg/kg by toxicity. Further studies on the estrogenic potential of these two chemicals are underway.

As seen in Table 1, results were the same in the two *in vitro* assays for all 10 chemicals. Because these two assays measure different endpoints, they should not, at this time, be considered redundant. Testing of a larger, more diverse set of chemicals may reveal differences in responses. This is likely to occur with estrogen antagonists that bind the receptor but do not activate transcription. Further, the lack of an exogenous source of metabolic activation is a current limitation to the *in vitro* assays, for which there is only partial compensation by the use of the uterotropic assays in the three-test combination.

Finally, in reaching judgments on the biological significance or hazard associated with estrogenic activity identified in tests such as those reported here, it is important to keep in mind that the activity of some chemicals is observed only at levels of exposure that are orders of magnitude higher than those where effects are seen with estrogens such as estradiol and DES.

In summary, the combination of three assays employed in this study provides a rational and informative approach to assessing the estrogenicity of chemicals. Consistency of results among all three assays, as seen with the tamoxifens, HPTE, and *o,p′*-DDT, offers assurance that the chemical under study is truly estrogenic or, with consistent negative results as with endosulfan, lacks meaningful estrogenic activity. Inconsistent results may also be informative, providing clues as to mechanism of action of the test chemical, e.g., binding to the ER and failure to elicit transcription or uterotropic responses would suggest that a chemical inactivates the receptor. Activity in both the receptor binding and transcriptional activation tests and negative results in the uterotropic assay would suggest inactivation of the chemical *in vivo* or failure to distribute to the uterus. Likewise, failure to bind to the ER or induce transcription while leading to uterotrophy, as was seen with methoxychlor, indicates the requirement for metabolic activation or a mechanism independent of the estrogen receptor.

This three-test combination offers a systematic and mechanistically informative approach to assessing estrogenicity. It provides a useful profile of activity using a reasonable amount of resources and is compatible with the study of individual chemicals as well as the investigation of interactions among combinations of chemicals. Such an approach is needed if the presence of estrogenic agents in the environment is to be determined as a first step toward assessing the health hazards they may present to humans and other forms of life.

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