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o,p'-DDT and its metabolites inhibit progesterone-dependent responses in yeast and human cells

Diane M. Klotz [a], Beth L. Ladlie [a], Peter M. Vonier [a,d], John A. McLachlan [a,b,c,d], Steven F. Arnold [a,c,d,*]

[a] *Tulane-Xavier Center for Bioenvironmental Research, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA*
[b] *Department of Pharmacology, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA*
[c] *Department of Environmental Health Sciences, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA*
[d] *Molecular and Cellular Biology Program, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA*

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Abstract

Using a combination of in vitro assays we have evaluated whether DDT metabolites can interact with the progesterone receptor pathway in yeast expressing human progesterone receptor (hPR) and in T47D human breast cancer cells which express endogenous hPR. In transactivation assays using both yeast and T47D cells, *o,p'*-DDT and the metabolites *p,p'*-DDT, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE, *p,p'*-DDE, *p,p'*-DDA, and DDOH inhibited progesterone-induced reporter gene activity in a dose-dependent manner. None of the DDT metabolites functioned as hPR agonists. Whole cell competition binding assays using T47D cells indicated that the inhibitory effects of DDT metabolites on progesterone-dependent activites may occur through both hPR-dependent and hPR-independent pathways. Our results and previous reports of DDT metabolites interacting with estrogen and androgen receptors suggests that this class of environmental chemicals may interact with numerous hormone receptor signaling pathways. © 1997 Elsevier Science Ireland Ltd.

Keywords: Progesterone; Antiprogestin; Human progesterone receptor; *o,p'*-DDT; Environmental chemicals

1. Introduction

In 1968 the pesticide *o,p'*-DDT was shown to act as an estrogen by increasing the uterine weight and uterine ornithine decarboxylase activity of rats which were administered the chemical [1]. The feminization of male gull embryos upon exposure to *o,p'*-DDT or a combination of *p,p'*-DDE and *p,p'*-DDT (two metabolites of *o,p'*-DDT) or to 17β-estradiol provides further evidence for the estrogen-like effects produced by DDT metabolites [2]. In addition, Soto et al. have demonstrated the ability of *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDE, and *p,p'*-DDE to increase the rate of proliferation of MCF-7 human breast cancer cells [3]. Competition binding studies have demonstrated that the in vivo estrogen-like effects of *o,p'*-DDT and some DDT metabolites appear to be mediated by these chemicals binding to the estrogen receptor (ER) [4–6]. The observation that environmental chemicals may function through ER has led numerous laboratories to develop ER-based in vitro assays to characterize the (anti)-estrogenic activity of environmental chemicals. We have used human ER (hER) expressed in yeast, luciferase assays in estrogen-responsive mammalian cells, and competition binding assays with recombinant hER to identify the mechanism of action of several environmental estrogens [7]. For example, the DDT metabolites *o,p'*-DDD and *p,p'*-DDD were observed to interact with hER and induce the transcription of an estrogen-responsive reporter gene in MCF-7 human breast cancer cells.

* Corresponding author.

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In addition to reports identifying the interaction of DDT with the ER signaling pathway, other studies have investigated the interaction of DDT metabolites with other steroid hormone receptor signalling pathways. In a study by Kelce et al. the DDT metabolite *p,p'*-DDE was shown to act as an antiandrogen in both in vivo and in vitro assays [8]. Additional reports have described the interaction of DDT metabolites with the progesterone-response pathway. These include the inhibition of progesterone-induced enzymes in rats administered *p,p'*-DDE [9] and the decreased frequency of ova implantation upon exposure of mice to *o,p'*-DDT [10]. The binding of *o,p'*-DDE and *p,p'*-DDE to duck, hen, and rabbit progesterone receptor has also been demonstrated [11]. In addition, we have reported the limited interaction of DDT metabolites with alligator PR [12].

To date, no reports have directly examined the functional interaction of DDT metabolites with the human progesterone receptor (hPR). We have modified our original yeast assays with hER to express human PR and a progesterone-sensitive reporter in yeast (hPR-PRE) and have developed a progesterone-sensitive luciferase assay utilizing T47D human breast cancer cells. The hPR-based in vitro assays were used for the characterization of chemicals that induce or inhibit hPR activity.

The experiments reported here demonstrate that the DDT metabolites do not function as hPR agonists in yeast or T47D cells. However, the DDT metabolites inhibited the activity of progesterone in both the yeast and T47D cells. Based on the results from competition binding studies, the inhibitory activity of the DDT metabolites appeared to be mediated through both hPR-dependent and hPR-independent pathways.

2. Materials and methods

2.1. Materials

[^{3}H]R5020 (85.00 Ci/mmol) and radioinert R5020 were purchased from DuPont NEN Products (Boston, MA). 17β-estradiol, dexamethasone, testosterone, and *o,p'*-DDT (1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane) were purchased from Sigma (St. Louis, MO). *p,p'*-DDT (1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane), *o,p'*-DDD (1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane), *p,p'*-DDD (2,2-bis(4-chlorophenyl)-1,1-dichloroethane), *o,p'*-DDE (1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethylene), *p,p'*-DDE (2,2-bis(4-chlorophenyl)-1,1-dichloroethylene), *p,p'*-DDA (2,2-bis(*p*-chlorophenyl)-acetic acid), and DDOH (2,2-bis(*p*-chlorophenyl)-ethanol) were purchased from Aldrich (Milwaukee, WI).

2.2. Yeast assays

The yeast strain DY150 (MAT a. *ura* 3–1, *leu* 2–3, 112 *trp* 1–1, *his* 3–11, 15 *ade* 2–1, *can* 1–100) was transformed with the YEPhPR-B expression plasmid, to express the B isoform of hPR, and the reporter plasmid YEPEG2LEU2 [13], which contains two progesterone-response elements (PREs) linked to the *lac*Z gene, to create yeast strain hPR-PRE. A single yeast colony was grown in synthetic medium (without leucine or tryptophan) overnight at 30°C. The next day the overnight culture was diluted so that the absorbance at 600 nm (A_{600}) equaled 0.2. Typically, 25 μl of the overnight culture was diluted into 975 μl fresh medium and grown overnight in the presence of the test chemicals. All chemicals were prepared in dimethyl sulphoxide (DMSO) or ethanol (EtOH) and added to the medium so the concentration of DMSO/EtOH did not exceed 1%. 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 $MgSO_4$, 35 mM β-mercaptoethanol). The cells were permeabilized by the addition of 25 μl $CHCl_3$ and 25 μl 0.1% SDS followed by vortexing for 25 s. The reactions were equilibrated at 30°C for 10 min, then 160 μl ONPG (4 mg/ml in Z-buffer) was added and the reactions returned to 30°C. The reactions were terminated by the addition of 400 μl 1M Na_2CO_3, the cell debris were removed by centrifugation at 15 000 × *g* for 6 min, and the absorbance of the samples at 420 nm (A_{420}) was measured. β-Galactosidase activity was expressed in Miller units, determined using the following formula: [A_{420}/(A_{600} of 1/10 dilution of cells × volume of culture × length of incubation)] × 1000. The β-galactosidase activity stimulated by the chemicals divided by the β-galactosidase activity stimulated by 25 nM progesterone and multiplied by 100 was used to calculate per cent progesterone activity. The data are representative of two independent experiments of three replicates each.

2.3. Tissue culture

T47D human breast cancer cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD), BME and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin (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 150 cm^2 culture flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

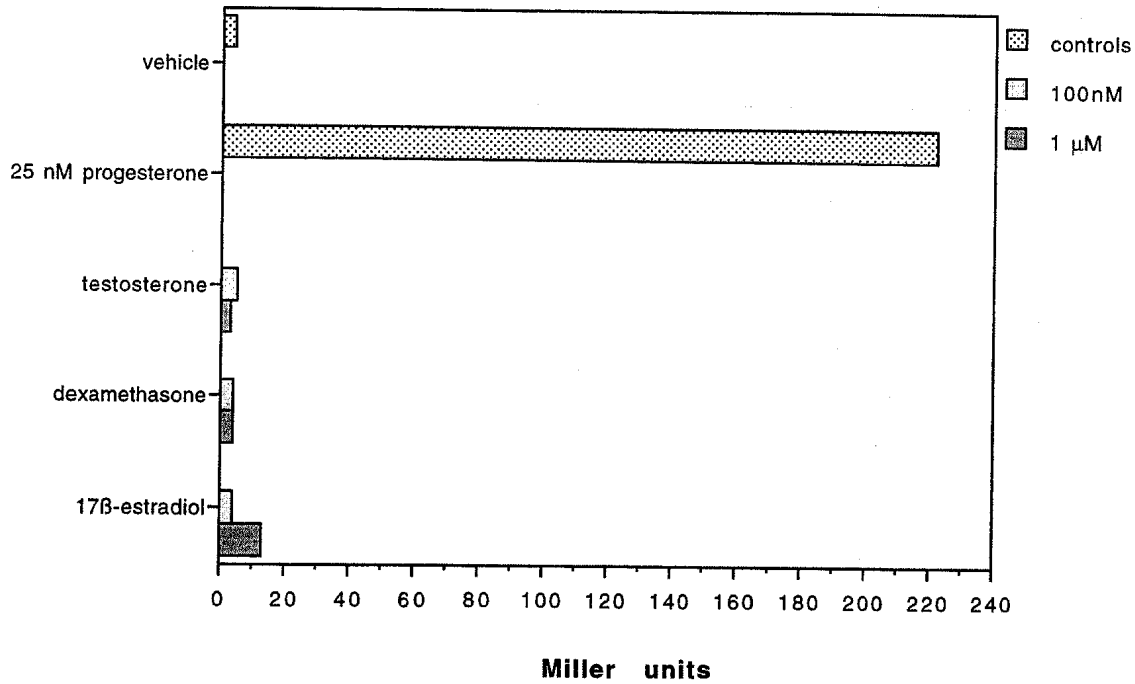

Fig. 1. Steroid specificity of yeast strain hPR-PRE. Yeast expressing hPR and a progesterone-inducible *lacZ* gene were grown in the presence of various steroid hormones and β-galactosidase activity was measured. Data are graphed as Miller units and are representative of two independent experiments with three replicates each.

2.4. Transient transfections and luciferase assays

T47D cells were transferred to phenol red-free Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS) for 48 h prior to plating. Cells, 2×10^7, were plated out in a 75 cm^2 tissue culture flask and allowed to attach overnight. The next day the cells were transfected with 5 μg PRE-tk-luciferase vector and 25 μg pBluescript (Stratagene, La Jolla, CA) as filler DNA, using Lipofectamine (Life Technologies, Bethesda, MD) as previously described [4]. Cells were then plated into 96-well plates at a density of 1×10^5 cells/well and allowed to attach overnight. Cells were treated with vehicle, progesterone, DDT metabolites, or progesterone and DDT metabolites for 18 h at 37°C. For luciferase assays the medium was removed and the cells were incubated in 1X lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) for 15 min at 25°C. Luciferase activity was determined in a monolight 9600 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) using Luciferase Assay Substrate (Promega, Madison, WI). Luciferase activity of samples treated with chemicals divided by the luciferase activity of those treated with progesterone and multiplied by 100 was used to determine per cent progesterone-induced luciferase activity. The data shown are representative of at least two independent experiments with six replicates each.

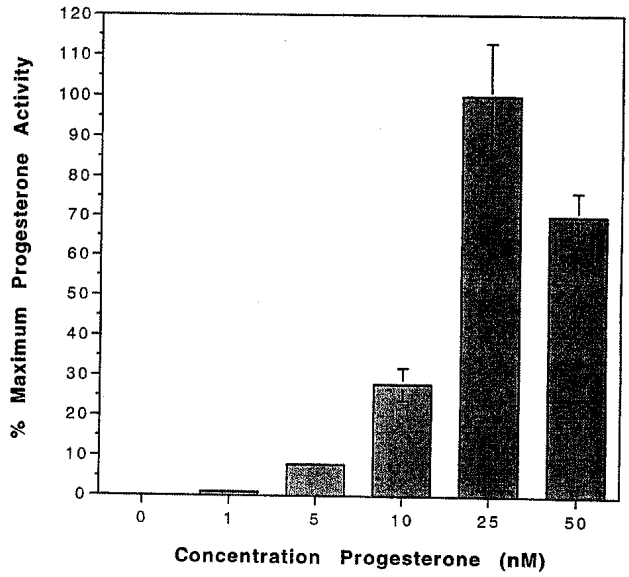

Fig. 2. Induction of progesterone-sensitve β-galactosidase activity by progesterone in yeast strain hPR-PRE. Yeast strain hPR-PRE was grown in the presence of increasing concentrations of progesterone. Data are graphed as Miller units and are representative of two independent experiments with three replicates each.

2.5. Competition binding assays

T47D cells were transferred to phenol red-free DMEM supplemented with 5% DCC-FBS for 72 h

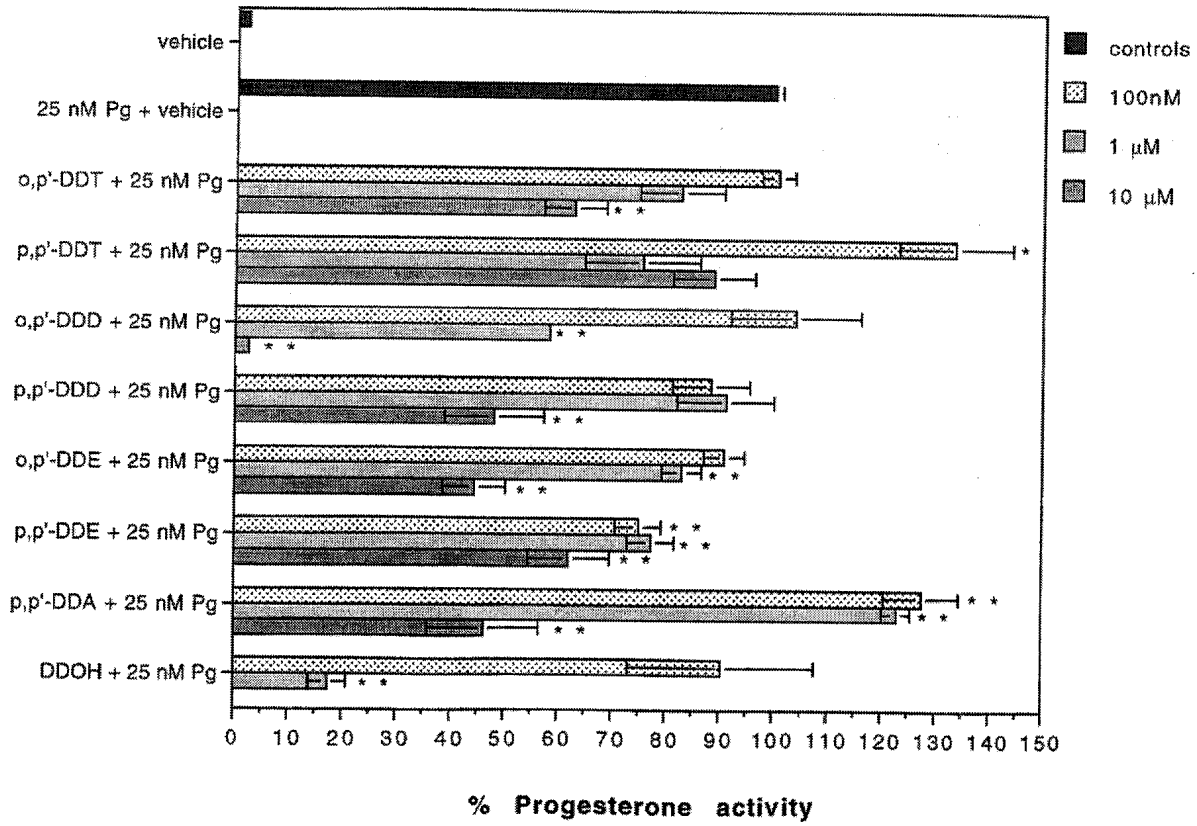

Fig. 3. Inhibition of progesterone-induced β-galactosidase activity by DDT metabolites in yeast strain hPR-PRE. Yeast strain hPR-PRE was grown in the presence of progesterone and various concentrations of DDT metabolites. Data are graphed as per cent progesterone-induced β-galactosidase activity remaining and are representative of two independent experiments with three replicates each. DDOH (10 μM) was toxic to the yeast. $^{*}P < 0.05$, $^{**}P < 0.01$ represents a significant decrease in progesterone-induced β-galactosidase activity in the presence of the DDT metabolites.

prior to the assay. Cells were rinsed with serum-free, phenol red-free DMEM without supplements and resuspended in binding medium (serum-free, phenol red-free DMEM without supplements but containing 15 mM HEPES). Cells, 1×10^6, were transferred to microcentrifuge tubes, and the volume of binding medium was brought up to 1 ml total. [^{3}H]R5020 (1 nM) was added to the T47D cells in the presence or absence of vehicle, 100-fold, 1000-fold, or 10 000-fold molar excess radioinert R5020 or DDT metabolites. Samples were incubated at 37°C with gentle shaking for 1 h. Samples were then centrifuged at $5000 \times g$ for 3 min, binding buffer removed, and cells washed three times in ice-cold phosphate-buffered saline (PBS). Final cell pellets were resuspended in 100 μl PBS, and bound radiolabeled hormone was determined by scintillation counting. The data shown are representative of two independent experiments with three replicates each.

2.6. Statistical analyses

Data were analyzed using a one-way analysis of variance (Microsoft Excel Version 5.0, Microsoft Corporation).

3. Results

3.1. Determination of the steroid specificity of yeast strain hPR-PRE

Yeast strain hPR-PRE was created to express hPR and a progesterone-responsive *lac*Z gene as described in Section 2. To examine the steroid specificity of yeast strain hPR-PRE, the yeast were treated with vehicle, progesterone, or various other steroid hormones. Progesterone induced β-galactosidase activity (Fig. 1). 17β-Estradiol, dexamethasone, and testosterone did not induce β-galactosidase activity.

To determine the concentration of progesterone required to induce maximal β-galactosidase expression, yeast strain hPR-PRE was treated with varying concentrations of progesterone and the β-galactosidase activity was measured. Maximal β-galactosidase activity was stimulated by 25 nM progesterone (Fig. 2). This is consistent with previous reports in which hPR was expressed in yeast [13].

Table 1
Comparison between IC_{50} values for transactivation assays and competition binding assays

Chemical	Luciferase IC_{50} (μM)	β-galactosidase IC_{50} (μM)	hPR binding IC_{50} (μM)
o,p'-DDT	0.110	>10	3.5
p,p'-DDT	0.110	>10	>10
o,p'-DDD	0.450	1.5	>10
p,p'-DDD	0.170	9.0	>10
o,p'-DDE	0.400	7.0	5.5
p,p'-DDE	0.250	>10	>10
p,p'-DDA	5.5	9.0	>10
DDOH	0.350	0.350	4.0

Concentrations of chemicals required to achieve 50% inhibition of progeterone-induced luciferase activity in T47D cells, progestrogene-induced β-galactosidase activity in yeast, and [^{3}H]R5020 binding in T47D cells. Concentrations of DDTs greater than 10 μM were toxic to T47D cells.

3.2. Inhibition of progesterone-induced β-galactosidase activity in yeast by DDT metabolites

To test the capacity of DDT metabolites to function as hPR agonists, yeast strain hPR-PRE was incubated with 100 nM, 1 μM, or 10 μM of the various DDT metabolites. None of the DDT metabolites induced β-galactosidase expression at the concentrations tested (data not shown).

To examine the ability of the DDT metabolites to inhibit the activity of progesterone, yeast strain hPR-PRE was treated with 100 nM, 1 μM, or 10 μM of the DDT metabolites in the presence of 25 nM progesterone, the concentration of progesterone which was shown to induce maximal β-galactosidase expression. The DDT metabolites inhibited progesterone-induced β-galactosidase expression in yeast strain hPR-PRE (Fig. 3).

The most effective DDT metabolite at inhibiting progesterone-induced β-galactosidase activity was DDOH, with an IC_{50} (concentration of chemical necessary to inhibit progesterone-induced β-galactosidase activity by 50%) of 350 nM (Table 1, Fig. 3). The next most effective DDT metabolite was *o,p'*-DDD with an IC_{50} of 1.5 μM. The IC_{50} for *o,p'*-DDE was approximately 7 μM, and the IC_{50} for both *p,p'*-DDD and *p,p'*-DDA was 9 μM. *o,p'*-DDT, *p,p'*-DDT, and *p,p'*-DDE did not inhibit progesterone-induced β-galactosidase activity by 50% in the range of concentrations tested.

In the yeast transactivation assays, the *o,p'*-isomers of the DDT metabolites were more effective at inhibiting progesterone-induced β-galactosidase activity in yeast than their *p,p'*-isomer counterparts. For example, *o,p'*-DDD had an IC_{50} of 1.5 μM while the IC_{50} for *p,p'*-DDD was 9 μM. Interestingly, *p,p'*-DDT at 100 nM or *p,p'*-DDA at both 100 nM and 1 μM, in combination with 25 nM progesterone, significantly increased β-galactosidase activity above that induced by progesterone alone (Fig. 3).

3.3. Inhibition of progesterone-induced luciferase activity in T47D human breast cancer cells by DDT metabolites

To examine the effects of DDT metabolites on hPR activity in mammalian cells, T47D human breast cancer cells were used. T47D cells were transiently transfected with a progesterone-responsive luciferase reporter vector, as described in Section 2, and treated with 100 nM, 1 μM, or 10 μM of various DDT metabolites alone or in the presence of 1 nM progesterone (the concentration of progesterone which induced maximal luciferase activity). The DDT metabolites alone did not induce progesterone-responsive luciferase activity in T47D cells at any of the concentrations tested (Fig. 4). Similar to the results observed in the yeast assays, the DDT metabolites inhibited progesterone-induced reporter gene activity (Fig. 5). In T47D cells, the IC_{50} values for all DDT metabolites, except *p,p'*-DDA, were lower than 1 μM (Table 1). The IC_{50} for *p,p'*-DDA was 5.5 μM. In contrast to the yeast assays, the *o,p'*-isomers did not appear to be more effective at inhibiting progesterone-induced reporter gene activity in the T47D cells than their respective *p,p'*-isomers.

3.4. Inhibition of [^{3}H]R5020 binding to hPR in T47D cells by DDT metabolites

To examine whether the anti-progestin effects of DDT metabolites in T47D resulted from the displacement of progesterone from hPR, whole cell competition binding assays were utilized. T47D cells were incubated with [^{3}H]R5020 in the presence or absence of 100-, 1000-, or 10 000-fold molar excess of DDT metabolites as described in Section 2. As expected, radioinert R5020 effectively competed with [^{3}H]R5020 for binding to hPR in T47D cells (Fig. 5). The only DDT metabolites to inhibit [^{3}H]R5020 binding by 50% were *o,p'*-DDT, *o,p'*-DDE, and DDOH. The IC_{50} (concentration of chemical to inhibit the binding of [^{3}H]R5020 by 50%) for *o,p'*-DDT was 3.5 μM, the IC_{50} for *o,p'*-DDE was

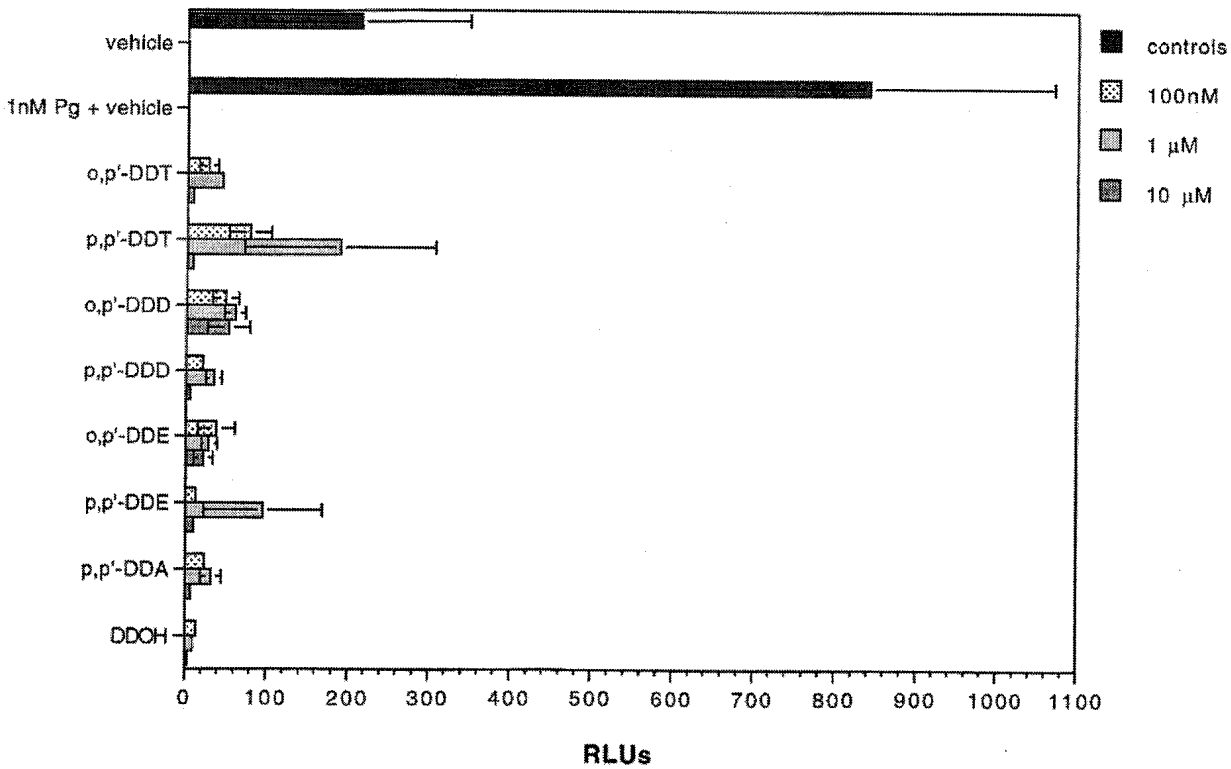

Fig. 4. Induction of progesterone-responsive luciferase gene activty in T47D human breast cancer cells by DDT metabolites. T47D human breast cancer cells were transfected with a progesterone-inducible luciferase reporter gene and cultured in the presence of 1 nM progesterone or various concentrations of DDT metabolites. Data are graphed as relative light units (RLUs) and are representative of two independent experiments with six replicates each. Concentrations of the DDT metabolites greater than 10 μM were toxic to the T47D cells.

4.1 μM, and the IC_{50} for DDOH was 5.5 μM (Table 1, Fig. 5). None of the other DDT metabolites was able to inhibit the binding of [^{3}H]R5020 to hPR by 50% at any of the concentrations tested. However, 10 μM *o,p'*-DDD or *p,p'*-DDD inhibited [^{3}H]R5020 binding to hPR by 44% and 42%, respectively. Ten μM *p,p'*-DDE inhibited [^{3}H]R5020 binding to hPR by 34%. Concentrations of greater than 10 μM for some of the DDT metabolites were toxic to T47D cells and were not used in the whole cell competition binding studies.

The *o,p'*-isomers of the DDT metabolites were more effective at inhibiting the binding of [^{3}H]R5020 to hPR than the respective *p,p'*-isomers (Fig. 6). For example, the IC_{50} values for *o,p'*-DDT and *o,p'*-DDE were 3.5 μM and 5.5 μM, respectively, while the IC_{50} values for *p,p'*-DDT and *p,p'*-DDE were not reached (Table 1). This is consistent with the increased ability of the *o,p'*-isomers, compared with the respective *p,p'*-isomers, to inhibit reporter gene activity in the yeast assays.

4. Discussion

The research presented herein describes the ability of DDT and its metabolites to interact with hPR in yeast and human breast cancer cells. The DDT metabolites did not function as hPR agonists, but inhibited progesterone-induced reporter gene activity in both yeast and T47D human breast cancer cells. In addition, the inhibitory effects of DDT metabolites on progesterone activity partially correlated with their interactions with hPR in whole cell competition binding assays.

The concentration of the DDT metabolites required to inhibit progesterone-induced reporter gene activity in the yeast and T47D cells differed. For most of the DDT metabolites, the IC_{50} values were greater in the yeast than in the T47D cells. This discrepancy may be due to the difference in the concentration of progesterone necessary to stimulate maximal reporter gene activity in the yeast compared with the T47D cells. The concentration of progesterone used to test the inhibitory activities of the DDT metabolites in the T47D cells was 1 nM, whereas in the yeast 25 nM progesterone was used. The reason the yeast require a higher concentration of progesterone for maximal stimulation of reporter gene activity is not known. It has been reported, however, that the affinity of hPR expressed in yeast for progesterone is similar to that of hPR in mammalian cells [13].

The differences observed between the IC_{50} values obtained in the whole cell competition binding assays and the reporter gene assays in yeast and mammalian cells may be indicative of whether the DDT metabolites

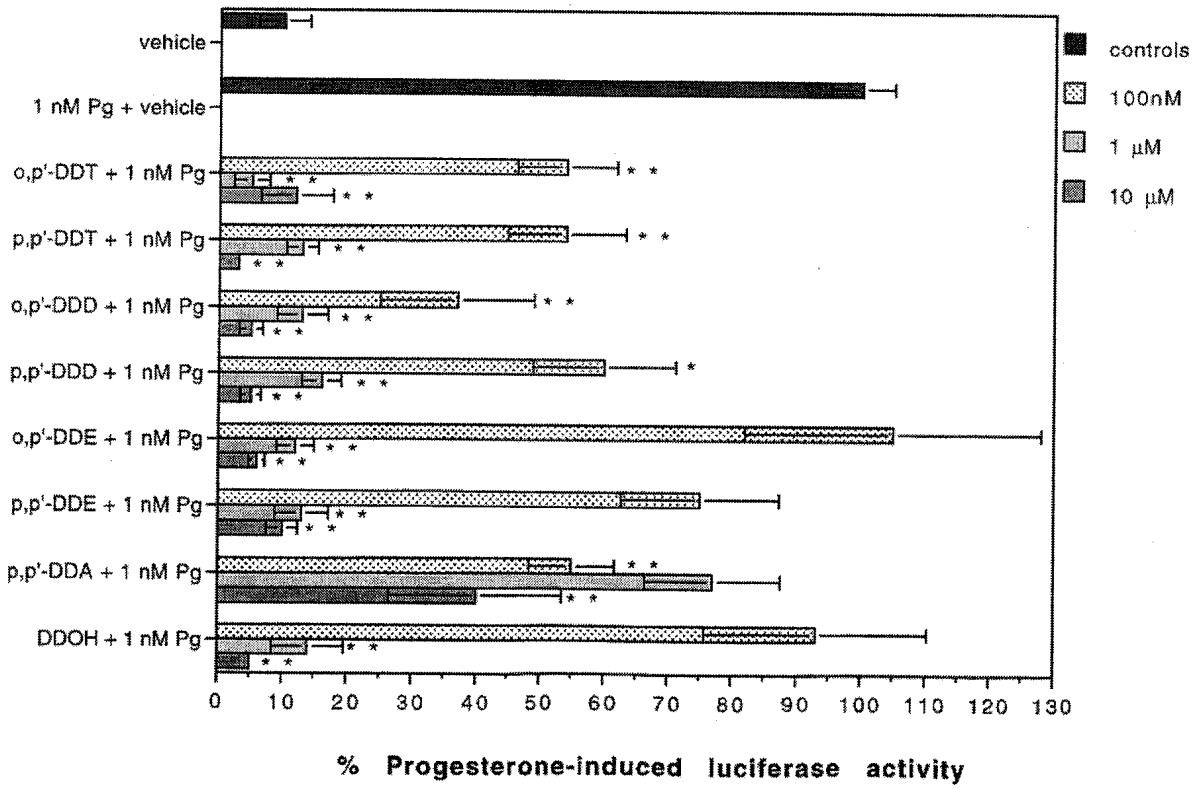

Fig. 5. Inhibition of progesterone-induced luciferase gene activity in T47D human breast cancer cells by DDT metabolites. T47D human breast cancer cells were transfected with a progesterone-inducible luciferase reporter gene and cultured in the presence of 1 nM progesterone and various concentrations of DDT metabolites. Data are graphed as per cent progesterone-induced luciferase activity remaining and are representative of two independent experiments with six replicates each. Concentrations of the DDT metabolites greater than 10 μM were toxic to the T47D cells. $^{*}P < 0.05$, $^{**}P < 0.01$ represents a significant decrease in progesterone-induced luciferase activity in the presence of the DDT metabolites.

inhibit the activity of progesterone through a hPR-dependent or hPR-independent pathway. One of the most effective inhibitors of progesterone-induced luciferase activity in T47D cells was *p,p'*-DDT with an IC_{50} of 100 nM. In competition binding studies, *p,p'*-DDT did not reduce [^{3}H]R5020 binding by 50% even at 10 μM. The failure of *p,p'*-DDT to displace [^{3}H]R5020 from hPR in binding studies is consistent with the weak inhibitory activity of *p,p'*-DDT in yeast reporter gene assays. Overall, the capacity for the DDTs to compete with [^{3}H]R5020 for binding to hPR more closely paralleled the abilities of the DDTs to inhibit progesterone-induced reporter gene activity in yeast than in T47D cells. The ability of the DDT metabolites to inhibit progesterone-induced gene transcription in the yeast may reflect the ability of the DDTs to compete with progesterone for binding to hPR. In contrast, the ability of the DDT metabolites to inhibit progesterone-induced gene transcription in the T47D cells may result from the interaction of the DDTs with other signaling pathways unique to mammalian cells. The hPR-independent pathways that may mediate the inhibitory activity of some DDT metabolites in T47D cells remain to be defined. Nonetheless, the use of yeast and mammalian cell assays and competition binding studies may provide a method to distinguish chemicals that directly inhibit hPR activity from chemicals that indirectly influence hPR activity.

The ability of the DDT metabolites to function as anti-progestins may play an important role in their activity as estrogens. Metabolites of DDT are known to act as estrogens both in vitro and in vivo. Based on reports demonstrating that hPR can quench the activity of hER in mammalian cells [14], a chemical that functioned as both an estrogen and an antiprogestin would be expected to produce a stronger estrogenic response than a chemical that exclusively had estrogenic activity. In addition, T47D cells express hER as well as hPR, and the DDT metabolites would be expected to interact with hER in addition to their interaction with hPR. Communication between hER and hPR signaling pathways, either through direct interactions or indirect interactions involving second messengers, may account for the differential ability of the DDT metabolites to regulate hPR activity in the yeast and T47D cells.

In conclusion, the research presented here demonstrates that the DDT metabolites function as hPR antagonists in both yeast and T47D human breast cancer cells. In addition, the inhibitory activity of the DDT metabolites in T47D cells appears to involve both

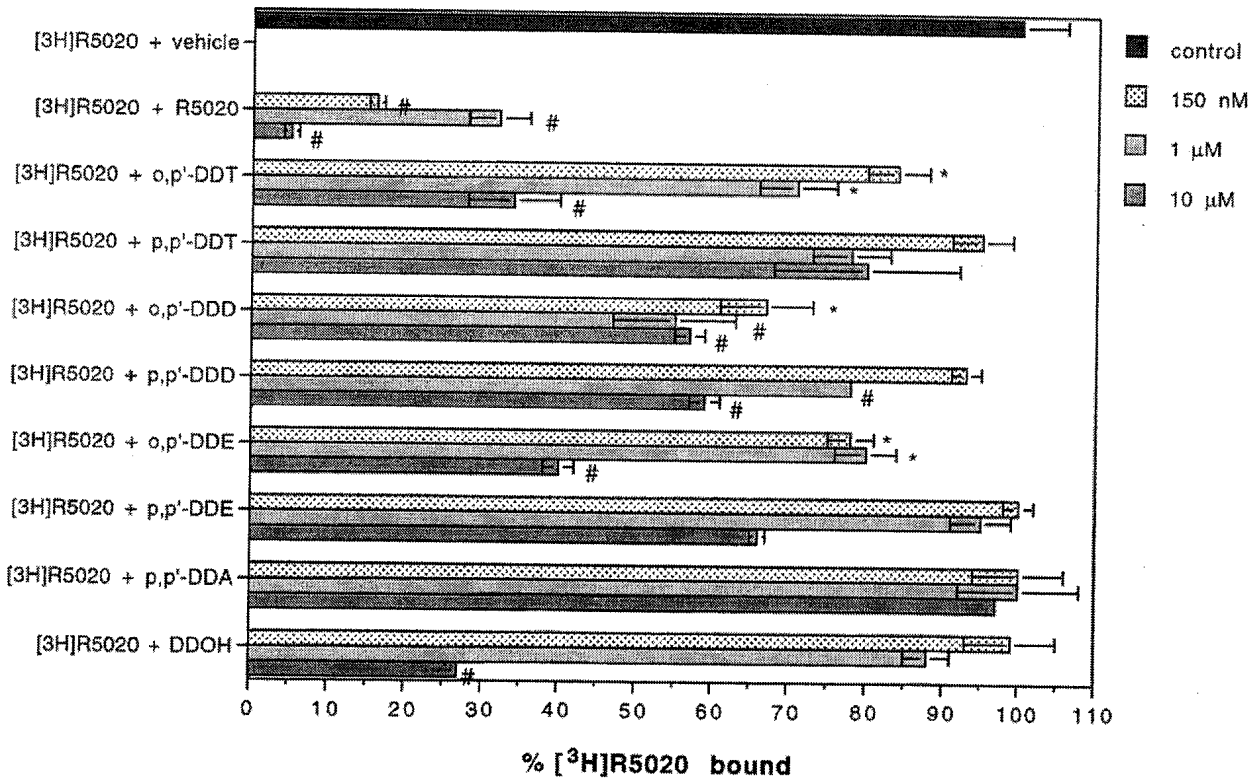

Fig. 6. Inhibition of [^{3}H]R5020 binding to hPR by DDT metabolites. T47D human breast cancer cells were exposed to 1 nM [3H]R5020 and 100-, 1000-, or 10 000-fold molar excess radioinert R5020 or DDT metabolites as described in Section 2. Data are graphed as per cent [^{3}H]R5020 bound after competition and are representative of two independent experiments of three replicates each. Concentrations of the DDT metabolites greater than 10 μM were toxic to the T47D cells. $*P < 0.05$, $**P < 0.01$ represents a significant decrease in [^{3}H]R5020 binding in the presence of competitor.

hPR-dependent and hPR-independent pathways. The broad receptor specificity of the DDT metabolites suggests that some classes of environmental chemicals may exhibit hormonal cross-talk between different hormone signaling pathways. Furthermore, the results herein indicate that the interaction of hormonally-active chemicals in the environment with diverse hormone receptor signaling pathways are complex.

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