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Interaction of Methoxychlor and Related Compounds with Estrogen Receptor α and β, and Androgen Receptor: Structure-Activity Studies

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ABSTRACT

We previously demonstrated differential interactions of the methoxychlor metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) with estrogen receptor α (ERα), ERβ, and the androgen receptor (AR). In this study, we characterize the ERα, ERβ, and AR activity of structurally related methoxychlor metabolites. Human hepatoma cells (HepG2) were transiently transfected with human ERα, ERβ, and AR plus an appropriate steroid-responsive luciferase reporter vector. After transfection, cells were treated with various concentrations of HPTE or structurally related compounds in the presence (for detecting antagonism) and absence (for detecting agonism) of 17β-estradiol and dihydrotestosterone. The monohydroxy analog of methoxychlor, as well as monohydroxy and dihydroxy analogs of 2,2-bis(*p*-hydroxyphenyl)-1,1-dichloroethylene, had ERα agonist activity and ERβ and AR antagonist activity similar to HPTE. The trihydroxy metabolite of methoxychlor displayed only weak ERα agonist activity and did not alter ERβ or AR activities. Replacement of the trichloroethane or dichloroethylene group with a methyl group resulted in a compound with ERα and ERβ agonist activity that retained antiandrogenic activities. This study identifies some of the structural requirements for ERα and ERβ activity and demonstrates the complexity involved in determining the mechanism of action of endocrine-active chemicals that simultaneously act as agonists or antagonists through one or more hormone receptors.

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] is a chlorinated hydrocarbon pesticide structurally similar to dichlorodiphenyltrichloroethane [DDT; 1,1,1-trichloro-2,2-bis(chlorophenyl)ethane]. Like *o,p′*-DDT, methoxychlor is estrogenic in vivo (Bulger et al., 1978; Gray et al., 1989; Alm et al., 1996; Chapin et al., 1997; Cummings, 1997; Hall et al., 1997). However, methoxychlor has low affinity for the estrogen receptor (ER) and the in vivo estrogenic activity is caused by metabolism to phenolic estrogenic metabolites. The primary estrogenic metabolite of methoxychlor is 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). HPTE competes with estradiol for binding to ER, induces ornithine decarboxylase and uterotrophic activity in ovariectomized rats, and is approximately 100-fold more active than methoxychlor (Bulger et al., 1978; Ousterhout et al., 1981; Shelby et al., 1996).

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Estrogenic responses are mediated through two separate estrogen receptors, ERα and ERβ. These two receptors have homologous DNA and ligand binding regions (Kuiper and Gustafsson, 1997; Tremblay et al., 1997; Ogawa et al., 1998), and most compounds have similar binding affinities and transcriptional activities with ERα and ERβ (Kuiper et al., 1996, 1998; Mosselman et al., 1996; Tremblay et al., 1997).

We previously demonstrated that HPTE is an ERα agonist and an ERβ antagonist in HepG2 human hepatoma cells transfected with estrogen-responsive reporter constructs (Gaido et al., 1999). This unique activity of HPTE makes it an ideal compound with which to evaluate the in vitro and in vivo differences in ER-subtype dependent responses. We have also shown that HPTE is an androgen receptor (AR) antagonist in vitro (Maness et al., 1998). The differential activity of HPTE with ERα, ERβ, and AR may explain why some of the responses induced by methoxychlor in vivo differ from those induced by estradiol. For example, the ability of

ABBREVIATIONS: DDT, dichlorodiphenyltrichloroethane; ER, estrogen receptor; AR, androgen receptor; TLC, thin-layer chromatography; E2, 17β-estradiol; GC-MS, gas chromatography-mass spectrometry; *p,p′*-DDE, 2,2-bis(*p*-hydroxyphenyl)-1,1-dichloroethylene; C3, complement 3; Luc, luciferase; CMV, cytomegalovirus; CPRG, chlorophenol red-β-D-galactopyranoside; HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; DHT, dihydrotestosterone; THC, tetrahydrochrysene.

methoxychlor to act as an ER antagonist in the ovary (Hall et al., 1997) may be caused by the high level of ERβ expression relative to ERα in this tissue (Saunders et al., 1997).

The physiological consequences of a chemical that is an ERα agonist, an ERβ antagonist, and an AR antagonist are unknown, and HPTE can serve as a model for investigating the effects of an agent that modulates multiple endocrine pathways. Additional studies with HPTE and structural analogs may lead to further insights on ligand specificity for ERα, ERβ, and AR. Therefore we compared the ERα, ERβ, and AR activity of HPTE and structural analogs and show that some chemicals similar in structure to HPTE also demonstrate unique ERα, ERβ, and AR activity.

Materials and Methods

Chemicals. HPTE was synthesized by dissolving 1 g of methoxychlor (Aldrich Chemical Co., Milwaukee, WI) in 100 ml of methylene chloride and then treating with excess boron tribromide in methylene chloride (Aldrich) for 24 h. Water (5 ml) was carefully added, and crude HPTE was isolated in methylene chloride. The residue (0.8 g) was purified by preparative thin-layer chromatography (TLC). The resulting HPTE was >97% pure as determined by gas-liquid chromatography.

Monohydroxymethoxychlor was synthesized by dissolving 1.0 g of methoxychlor in methylene chloride. Approximately 1.5 mol equivalents of boron dibromide in methylene chloride was slowly added over a period of 1 to 2 h. The progress of demethylation was monitored by TLC. The monohydroxymethoxychlor metabolite was isolated by preparative TLC using hexane/acetone (92:8) as solvent. Yields of 250 to 300 mg were obtained and the products were greater than 98% pure as determined by gas chromatography-mass spectrometry (GC-MS).

Trihydroxymethoxychlor and the corresponding trimethoxymethoxychlor were synthesized by ChemSyn Labs (Lenexa, KS). Dimethoxy-DDE was synthesized by dissolving 1.0 g of methoxychlor in dimethyl sulfoxide. Anhydrous sodium bicarbonate (3.0 g) was added and the mixture was heated at 140°C for 1 h. The mixture was diluted with water and the dimethoxy-DDE product was isolated by extraction with chloroform. The crystalline residue from the chloroform extract (0.75 g) was greater than 98% pure as determined by GC-MS. Dihydroxy-DDE was prepared from 2,2-bis(*p*-hydroxyphenyl)-1,1-dichloroethylene (*p,p′*-DDE) following the same procedure as described above for HPTE. Dihydroxy-DDE was greater than 98% pure as determined by GC-MS. Monohydroxy-DDE was prepared from *p,p′*-DDE following the same procedure as described above for monohydroxymethoxychlor. Monohydroxy-DDE was greater than 98% pure as determined by GC-MS. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were ≥97% pure.

Plating and Transfection. Transfection experiments were performed as described previously (Maness et al., 1998; Gaido et al., 1999). HepG2 human hepatoma cells (ATCC, Rockville, MD) were plated in triplicate in 24-well plates (Falcon Plastics, Oxnard, CA) at a density of 10^5 cells/well in complete medium consisting of phenol red-free Eagle's minimal essential medium (GIBCO/BRL, Grand Island, NY) supplemented with 10% resin-stripped fetal bovine serum (Hyclone, Logan, UT), 2% L-glutamine, and 0.1% sodium pyruvate. Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2/air and then transfected after the Superfect procedure (Qiagen, Valencia, CA) with three plasmids. For detection of ERα activity, cells were transfected with human ERα expression plasmid, plus an estrogen-responsive complement 3-luciferase (C3-Luc) reporter plasmid, and a constitutively active cytomegalovirus (CMV)-β-galactosidase reporter plasmid (transfection and toxicity control) (Tzukerman et al., 1994; Gaido et al., 1999). For detection of ERβ activity, cells were transfected with a human ERβ expression plasmid, a C3-Luc reporter plasmid, and CMV-β-galactosidase reporter plasmid (Gaido et al., 1999; Hall and McDonnell, 1999). For detection of AR activity, cells were transfected with a human AR expression plasmid, plus an androgen-responsive MMTV-Luc reporter plasmid, and CMV-β-galactosidase reporter plasmid (Maness et al., 1998). Transfected cells were rinsed with PBS and dosed with various concentrations of test chemical and dimethyl sulfoxide (vehicle control; Sigma) in complete medium. After a 24 h incubation, cells were rinsed with PBS and lysed with 65 μl of lysing buffer (25 mM Tris-phosphate, pH 7.8, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, 0.5% Triton X-100, 2 mM dithiothreitol). Lysate was divided into two 96-well plates for luciferase and β-galactosidase determination.

Luciferase activity was determined by adding 100 μl of Luc assay reagent (Promega, Madison, WI) to the first 96-well plate containing 20 μl of lysate. Luminescence was determined immediately using a ML3000 microtiter plate luminometer (Dynatech Laboratories, Chantilly, VA).

β-Galactosidase activity was determined by adding 20 μl of β-galactosidase assay reagent to 30 μl of lysate in the second 96-well plate. β-Galactosidase assay reagent consisted of a 4 mg/ml solution of chlorophenol red-β-D-galactopyranoside (CPRG; Sigma) in 150 μl of CPRG buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.8) Absorbance at 570 nm was determined over a 30 min period using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA).

HepG2 cells lack detectable levels of endogenous ERα, ERβ, and AR and in the absence of transfected receptor, Luc activity remains below the level of detection (data not shown). Background activity after receptor transfection averaged 150 ± 56 normalized Luc units with ERα, 31 ± 6 normalized Luc units with ERβ, and 5 ± 1 normalized Luc units with AR. We have previously confirmed by Western analysis that ERα and ERβ are expressed at equal concentrations under the conditions of our assay (Hall and McDonnell, 1999).

Statistical Analysis. Unless otherwise noted, values presented in this study represent the means ± S.E. resulting from at least three separate experiments with triplicate wells for each treatment dose. Dose-response data were analyzed using the sigmoidal dose-response function of the graphical and statistical program Prism (GraphPad, San Diego, CA).

Results

We compared the activity of HPTE and structural analogs in HepG2 cells transfected with expression vectors for human ERα, ERβ, and AR along with the appropriate reporter plasmid (Fig. 1). HepG2 cells were dosed with set concentrations of chemical alone and in combination with an inducing dose of either 17β-estradiol (E2) or dihydrotestosterone (DHT) for determining antagonistic activity with ERα/β and AR, respectively. HPTE (Fig. 1C) exhibited ERα agonist, and ERβ and AR antagonist activity as described previously (Maness et al., 1998; Gaido et al., 1999). HPTE does display some partial ERβ agonist activity of approximately 13% of that obtained with a maximally inducing dose of estradiol (Maness et al., 1998). The monohydroxy metabolite of methoxychlor, as well as the mono- and dihydroxy analogs of *p,p′*-DDE (Fig. 1, B, H, and I), also had ERα agonist and ERβ and AR antagonist activity. Bisphenol A exhibited ERα and ERβ agonist activity but did not have antiandrogenic activity (Fig. 1K). Replacement of the trichloromethyl of HPTE or dichloromethylene group of dihydroxy-DDE results in a conversion from ERβ antagonist activity to full ERβ agonist activity but retains ERα agonist and AR antagonist activity (Fig. 1, C and

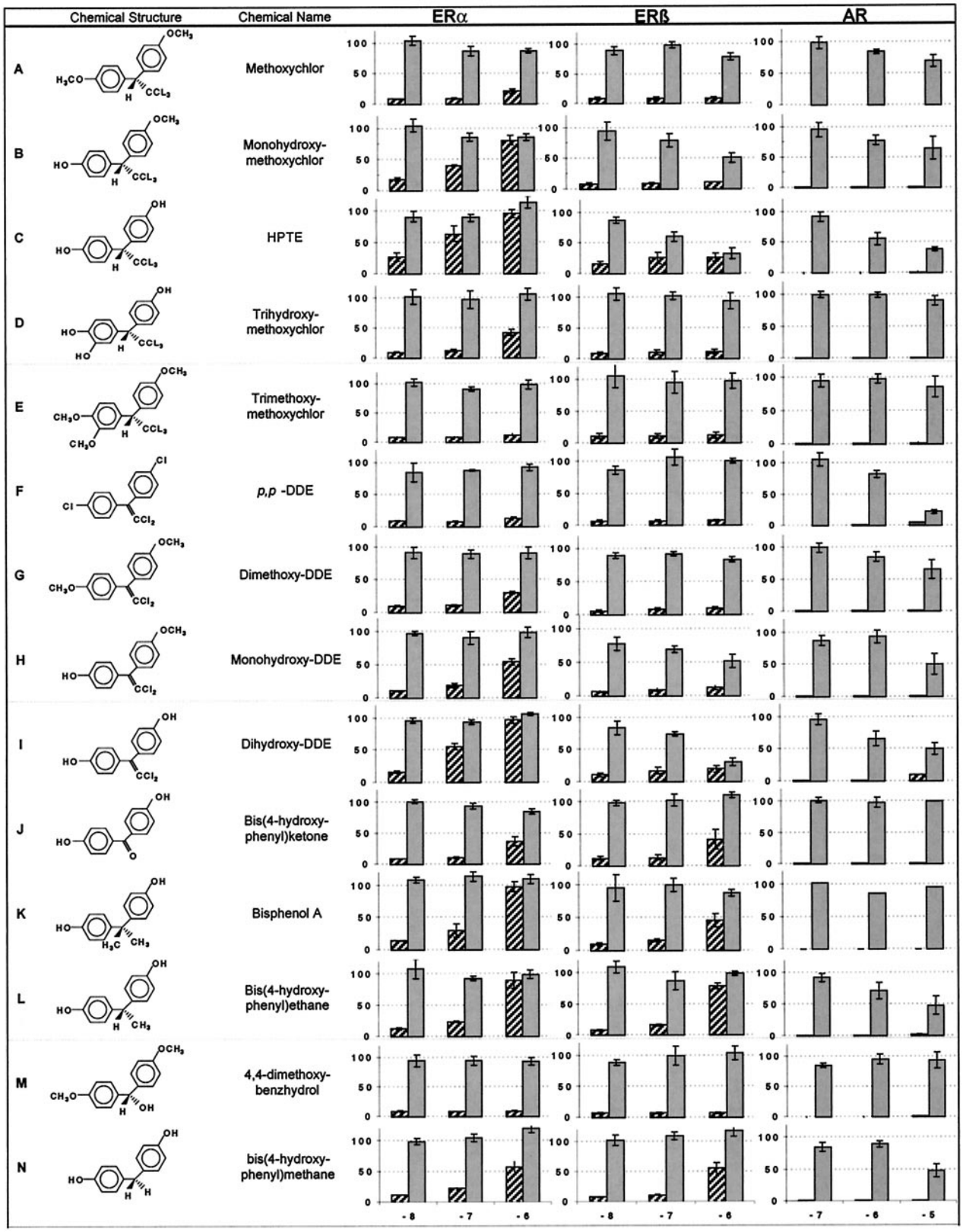

Fig. 1. ERα, ERβ, and AR activity of methoxychlor analogs. HepG2 cells were transiently transfected with expression plasmids for human ERα, ERβ, and AR plus C3-Luc and a constitutively active β-galactosidase expression plasmid (transfection and toxicity control). Cells were treated with increasing concentrations of methoxychlor analogs alone for detecting agonist activity (hatched bars) and with an inducing dose of either estradiol or DHT for detecting antagonist activity (shaded bars). Luciferase activity was normalized to β-galactosidase activity. Values represent the means ± S.E. of three separate experiments and are presented as percentage response, with 100% activity defined as the activity achieved with 10^{-7} M estradiol for ERα and ERβ, and 10^{-7} M DHT for AR. The abscissa represents log molar concentration. Agonists cause an increase in percentage response (rising hatched bars) with increasing concentration, whereas antagonists cause a decrease in percentage response (declining shaded bars) with increasing concentration.

I versus L). Trimethoxy and trihydroxy ring substituted compounds (Fig. 1, D and E) exhibited minimal ERα agonist activity and did not affect ERβ or AR-dependent responses.

Concentration-response curves for selected ERα and ERβ agonists are presented in Fig. 2, A and B. EC_{50} values for ERα and ERβ agonist activity are presented in Table 1. HPTE and dihydroxy-DDE were most potent as ERα agonists and were approximately 17- and 25-fold less potent, respectively, than estradiol. HPTE and dihydroxy-DDE were followed in ERα agonist potency by monohydroxy methoxychlor, bisphenol A, monohydroxy-DDE, bishydroxyphenylmethane, and bishydroxyphenylethane. Bishydroxyphenylmethane and bishydroxyphenylethane were equally potent as ERβ agonists and were approximately 285-fold less potent than estradiol.

We characterized the ERβ antagonist activity of selected compounds in HepG2 cells by determining the effect of various concentrations across a complete estradiol dose-response range (Fig. 3, A–C). Each of the tested compounds caused parallel shifts in the estradiol dose-response curve, indicating competitive antagonism. Schild regression analyses were performed and equilibrium dissociation (K_B) values determined (Table 1). HPTE, monohydroxy methoxychlor, monohydroxy-DDE, and dihydroxy-DDE demonstrated relatively similar antagonist potencies.

Similar experiments were performed to characterize AR antagonist activity (Fig. 4, Table 1). HPTE, dihydroxy-DDE, and *p,p′*-DDE demonstrated similar AR antagonist potencies. Monohydroxy-DDE, bishydroxyphenylmethane, and bishydroxyphenylethane were approximately 3- to 5-fold less potent than HPTE, dihydroxy-DDE, and *p,p′*-DDE.

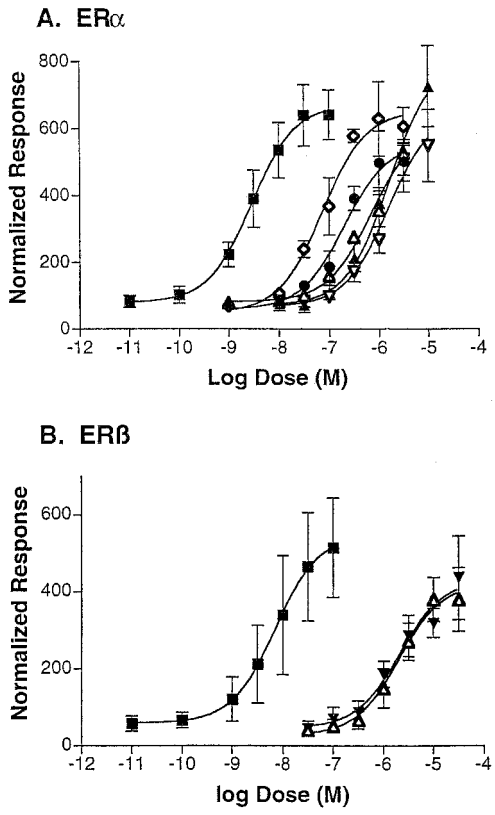

Fig. 2. Dose response of selected methoxychlor analogs with ERα (A) and ERβ (B). Experiments were performed as described in Fig. 1. Luciferase activity was normalized to β-galactosidase activity. Values represent the means ± S.E. of three separate experiments. ■, E2; ●, monohydroxy-methoxychlor; △, monohydroxy-DDE; ◇, dihydroxy-DDE; ▽, bishydroxy-phenyl methane; ▲, bishydroxyphenyl ethane.

Discussion

ERα and ERβ bind structurally diverse classes of chemicals, and it is difficult to compare structure-dependent receptor binding or transactivation between chemical classes. In contrast, structure-activity correlations within a single structural class such as the triphenylethanes can be used to design ER agonists and antagonists (e.g., tamoxifen) for clinical applications as selective estrogen receptor modulators. For a series of mono-, di-, and trihydroxyphenyl ethane/ethylene analogs structurally related to methoxychlor, the order of potency as ERα agonists was dihydroxyphenyl > monohydroxyphenyl > trihydroxyphenyl, suggesting that the optimal structure for ERα agonist activity contained two *p*-hydroxyphenyl groups substituted at a single ethane or ethylene carbon atom (i.e., bis-substitution). In contrast, the fully methylated metabolites (e.g., Fig. 1, A versus C; Fig. 1, G versus D) were significantly less active as ERα agonists; this is consistent with previous studies with methoxychlor and HPTE (Bulger et al., 1978; Ousterhout et al., 1981; Shelby et al., 1996). A similar pattern was observed for the substituted diphenylmethane analogs even though only a limited number of these compounds were tested (Fig. 1, J and N versus M).

With few exceptions (Fig. 1, K and L), the bishydroxy/methoxyphenyl ethane or ethylene analogs were not significant ERβ agonists, and only two bishydroxyphenylmethanes (Fig. 1, J and N) induced measurable ERβ-dependent reporter gene activity. Thus, our results demonstrate that this series of bishydroxyphenyl-substituted ethanes, ethylenes, and methanes are preferential ERα agonists and exhibit weak to nondetectable ERβ agonist activity. These results are unique because previous studies for ER subtype-dependent ligand binding and transactivation report similar ERα and ERβ activity for various structural classes of estrogenic compounds (Kuiper et al., 1996, 1998; Mosselman et al., 1996; Tremblay et al., 1997).

The results of our studies also demonstrate that methoxychlor and structurally related analogs exhibit minimal ERα antagonist activity but that three compounds (Fig. 1, B, H,

TABLE 1
A comparison of the agonist and antagonist potencies of methoxychlor analogs with ERα, ERβ, and AR

Chemicals	ERα		ERβ		AR	
	EC_{50}	K_B	EC_{50}	K_B	EC_{50}	K_B
			$\times 10^{-8}$ *M*			
Estradiol	0.3		0.7			
DHT					1.0	
HPTE	5.1[a]			3.0[a]		31.6
Monohydroxy-methoxychlor	19.8			7.2		
Monohydroxy-DDE	67.0			8.5		100.0
Dihydroxy-DDE	7.4			5.1		29.4
Bishydroxyphenylmethane	150.0		200.0			142.0
Bishydroxyphenylethane	160.0		210.0			136.0
p,p′-DDE						36.9[b]
Bisphenol A	64.0[c]		89.0[c]			

[a] From Gaido et al. (1999).
[b] From Maness et al. (1998).
[c] From Gould et al. (1998).

and I) in addition to HPTE (Fig. 1C) are highly effective ERβ antagonists. Structural features required for this response include bis(4-hydroxyphenyl) or bis(4-hydroxyphenyl),(4-methoxyphenyl) groups attached to chlorine-substituted ethane/ethylene moieties. Additional compounds are required to more accurately define structural requirements for ERβ antagonist activities; however, our results clearly demonstrate remarkable structure-dependent differences among these compounds for activity as ERβ antagonists.

Several compounds investigated in this study exhibited antiandrogenic activity. Four analogs that were ERβ antagonists (Fig. 1, B, C, H, and I) were among the most active antiandrogens and exhibited activity similar to that observed for *p,p′*-DDE. Interestingly, both *p,p′*-DDE (Fig. 1F) and dihydroxy-DDE (Fig. 1I) exhibited similar antiandrogenic activities, and the interchange of two *p*-chloro and two *p*-hydroxyl substituents had minimal effects on this AR response. In contrast, the two *p*-hydroxyl groups (but not *p*-chloro substituents) conferred both ERα agonist and ERβ antagonist activity on dihydroxy-DDE, demonstrating that subtle substituent changes can affect some but not all ligand-activated hormone receptor action.

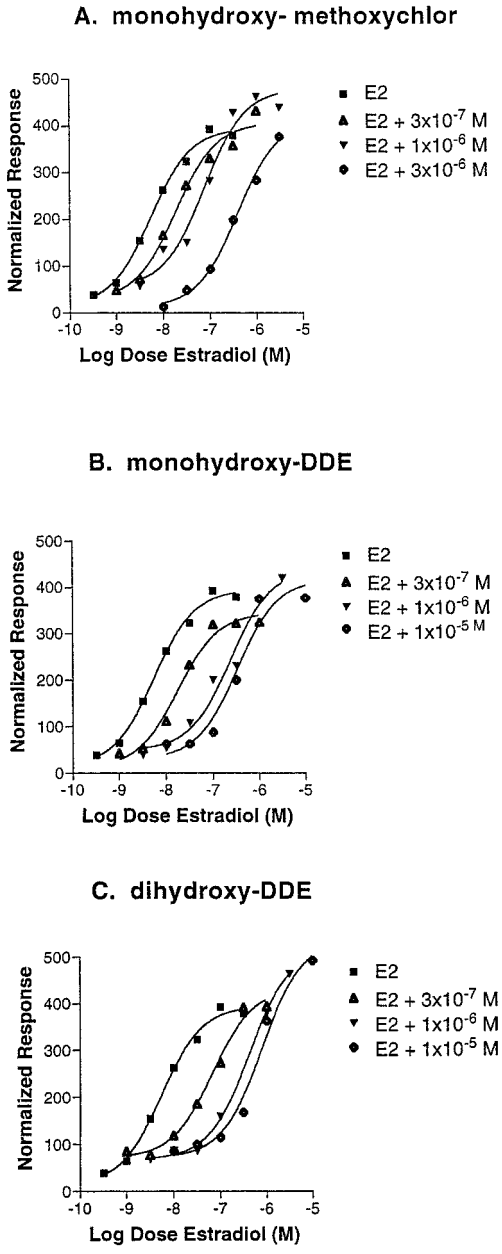

Fig. 3. Effect of various concentrations of methoxychlor analogs on an estradiol dose-response curve with ERβ. Experiments were performed as described in Fig. 1 with 10^{-10} to 10^{-5} M E2 either alone or in combination with the indicated concentrations of monohydroxy-methoxychlor (A), monohydroxy-DDE (B), and dihydroxy-DDE (C). Luciferase activity was normalized to β-galactosidase activity. Values represent the means of three separate experiments.

Methoxy-DDE and monohydroxy-DDE are impurities in technical grade methoxychlor (Bulger et al., 1985), and dihydroxy-DDE is formed during the metabolism of methoxychlor in mice (Kapoor et al., 1970). HPTE, monohydroxy-methoxychlor, monohydroxy-DDE, and dihydroxy-DDE have previously been shown to compete with estradiol for ER binding in vitro and demonstrate uterotropic activity in vivo (Bulger et al., 1978, 1985; Ousterhout et al., 1981). Thus, exposure to methoxychlor results in a complex interaction of multiple metabolites with different activities at ERα, ERβ, and AR.

The molecular mechanism by which a ligand can act as an ERα agonist and an ERβ antagonist is of both toxicological and pharmacological interest. The overall structure of the ERβ ligand-binding domain is very similar to that of ERα (Pike et al., 1999), and most compounds demonstrate similar binding affinities and transcriptional activities with ERα and ERβ (Kuiper et al., 1996, 1997; Mosselman et al., 1996; Tremblay et al., 1997). The helix 12 region present on both receptors plays an important role in the mechanism of ER action (Darimont et al., 1998). This region folds over the ligand binding pocket and exposes a region on both receptors involved in coactivator binding. ERα and ERβ antagonists such as raloxifene and hydroxytamoxifen contain bulky constituents that reposition helix 12 and block receptor interaction with coactivators (Brzozowski et al., 1997; Pike et al., 1999). HPTE analogs used in this study do not have substituents of the size and character of raloxifene and consequently less likely to physically reposition helix 12. However, X-ray crystallography and sequence analysis comparison of the ligand-binding domains of ERα and ERβ suggest that the agonist orientation of helix 12 in ERβ may be unstable and thus easier to antagonize than ERα (Pike et al., 1999). The ERα agonist/ERβ antagonists identified in this study may be able to stabilize helix 12 in the agonist orientation for ERα but not for ERβ. X-ray crystallographic studies are needed to confirm this hypothesis.

The *R,R*-enantiomer of tetrahydrochrysene (*R,R*-THC) has also recently been shown to have differential ERα and ERβ activity (Meyers et al., 1999; Sun et al., 1999). Like HPTE, *R,R*-THC behaves as an ERα agonist and an ERβ antagonist. In contrast, the *S,S*-enantiomer (*S,S*-THC) is an agonist with both ERα and ERβ. The equilibrium dissociation value (K_B) for *R,R*-THC has not been determined, and whether *R,R*-THC is an ERβ competitive antagonist remains to be demonstrated. THC compounds differ considerably in structure from the methoxychlor analogs presented in this study, and this class of compounds will provide additional information regarding the ligand specificity of ERα and ERβ binding and transactivation.

Less is known about the mechanism of AR antagonism by AR ligands. AR antagonists are generally thought to prevent

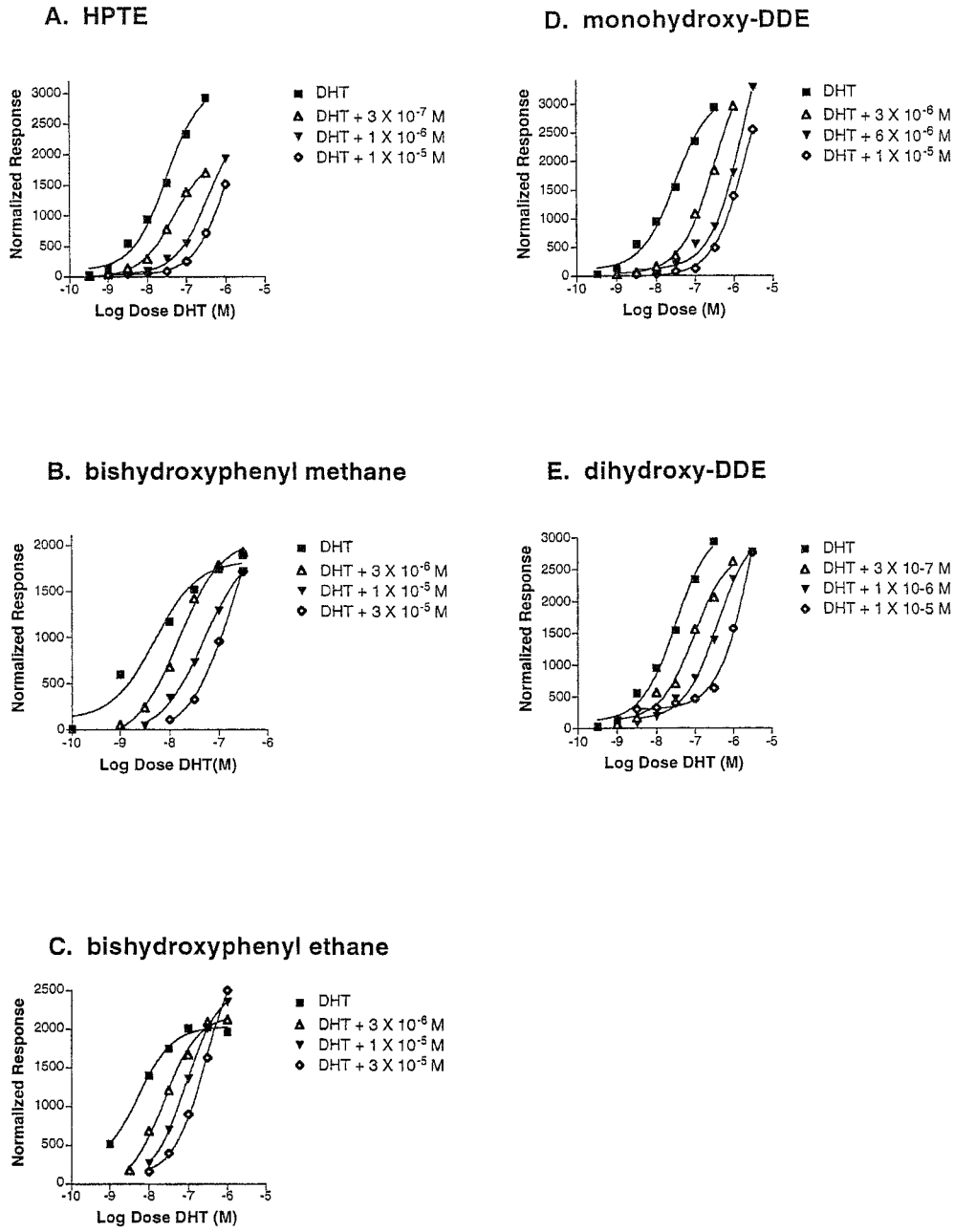

Fig. 4. Effect of various concentrations of methoxychlor analogs on a DHT dose-response curve with AR. Experiments were performed as described in Fig. 1 with 10^{-9} to 10^{-5} M DHT either alone or in combination with the indicated concentrations of HPTE (A), bishydroxyphenylmethane (B), bishydroxyphenylethane (C), monohydroxy-DDE (D), and dihydroxy-DDE (E). Luciferase activity was normalized to β-galactosidase activity. Values represent the means of three separate experiments.

or reduce binding of AR to DNA (Kelce et al., 1995, 1998). However, the specific mechanisms responsible for this inhibition of AR-DNA binding remain unknown.

Much still remains to be determined about the precise roles of ERα, ERβ, and AR in reproductive development and endocrine function, especially in humans; the physiological consequences of exposure to chemicals that are ERα agonists, ERβ antagonists, and AR antagonists are unknown. HPTE and its structural analogs give us further insights into the ligand specificity of ERα, ERβ, and AR and serve as model chemicals for investigating ERα, ERβ, and AR steroid hormone receptor interactions.

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