Pure Appl. Chem., Vol. 75, Nos. 11–12, pp. 2069–2079, 2003.

Topic 3.8

Modification of endocrine active potential by mixtures*

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Abstract: Wildlife and humans are exposed to a complex mixture of endocrine active chemicals. The activity of a specific chemical in any mixture can be modified through interactions with other components of the mixture. The toxic equivalency factor (TEF) approach for risk assessment was developed for chemicals such as halogenated aromatics that induce their effects through ligand-activated receptors. For persistent halogenated aromatic AhR agonists, this approach has some utility. However, the use of the TEF approach for endocrine active compounds is confounded by the unique tissue- and response-specific activities of these structurally diverse compounds. The term "selective receptor modulator" describes the ability of a natural or synthetic receptor ligand to manifest agonist activity in one tissue or for one response and antagonist activity in other tissues or for another response in the same tissue. Thus, it is possible for chemicals in a mixture to behave in an additive manner for one response and an antagonist manner for another response. A mechanisms-based hazard risk assessment of endocrine active chemical mixtures must account for these multiple variables.

INTRODUCTION

Risk assessment of chemicals has primarily focused on individual compounds where overall risks are estimated from human exposure data and toxicity data obtained in laboratory animal studies. In reality, wildlife and humans are exposed to complex mixtures of endocrine active chemicals that include endogenous steroid hormones, environmental contaminants such as pesticides and plasticizers, and natural substances such as phytoestrogens and fungal metabolites. These endocrine active chemicals act through multiple pathways, and the activity of a specific chemical in any mixture can be modified through interactions with other components of the mixture [1]. Figure 1 illustrates examples of different environmental contaminants that bind directly to receptors and activate intracellular signaling pathways; these include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an aryl hydrocarbon receptor (AhR) agonist [2]; 2,2-bis(*p*-chlorophenyl)(*o*-chlorophenyl)-1,1,1-trichloroethane (*o,p′*-DDT), an estrogen receptor (ER) agonist [3]; 1,1-bis(*p*-chlorophenyl)-1,1-dichloroethylene (DDE), an androgen receptor antagonist [4]; 2,3,3′,4′,5′,6-hexachlorobiphenyl (HCB), an agonist for constitutive androstane receptor (CAR) [5]; diethylhexylphthalate (DEHP), an agonist for the pregnane X receptor (PXR) [6]; and chlordane, an estrogen receptor-related-α (ERRα) antagonist [7].

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* **75**, 1617–2615 (2003).

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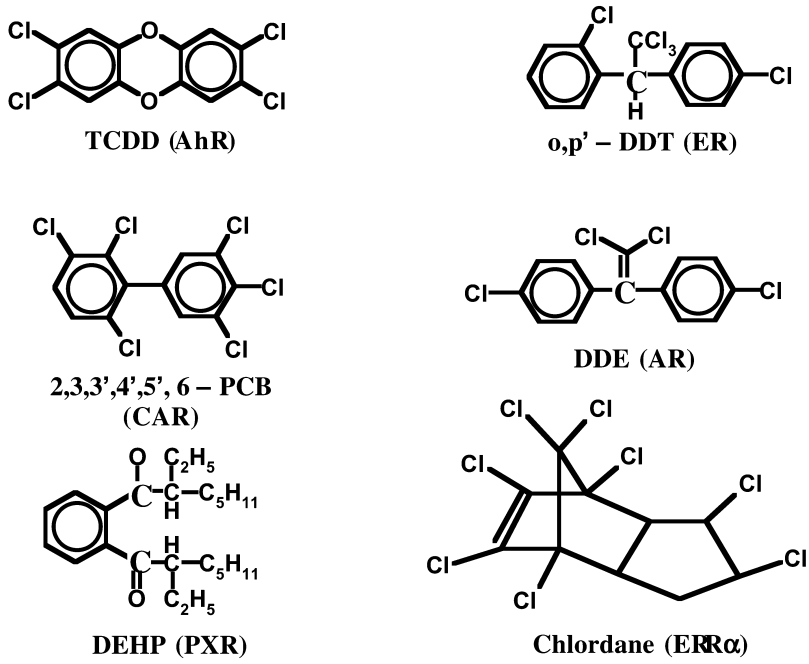

Fig. 1 Environmental contaminants that activate multiple receptor-mediated signaling pathways.

TOXIC EQUIVALENCY FACTOR APPROACH FOR RISK ASSESSMENT OF HALOGENATED AROMATICS

The toxic equivalency factor (TEF) approach for hazard and risk assessment can be used for chemical mixtures that act through a common pathway, where the toxic equivalents (TEQs) of a mixture are equal to the sum of the concentrations of the individual compounds (C_i) times their fractional potency (TEF_i) relative to a common standard.

$$TEQ = \sum [C_i \cdot TEF_i]$$

The TEF approach has been used for TCDD and related 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) where TCDD is assigned a potency of 1 [8–11]. All of these compounds bind and activate the AhR and induce a broad spectrum of toxic responses, including a wasting syndrome, developmental and reproductive toxicity, thymic atrophy, impaired immune responses, chloracne, hepatic responses and porphyria, carcinogenic and anticarcinogenic responses, and induction of CYP1A1 and other drug-metabolizing enzymes. Development of TEFs for individual PCDD and PCDF congeners used data from multiple quantitative structure–activity studies that reported response-specific potencies of congeners relative to TCDD [11]. For each compound, a range of TEFs was obtained, and scientific judgment was used to select individual TEF values for PCDD and PCDF congeners (Table 1).

However, environmental samples containing PCDDs and PCDFs also contain higher levels (>100-fold) of polychlorinated biphenyl (PCB) mixtures [12–14]. Two structural classes of PCB congeners also bind the AhR and induce prototypical AhR-mediated biochemical and toxic responses [15,16]. Coplanar or non-ortho substituted PCBs that include 3,3′,4,4′-tetrachlorobiphenyl (tetraCB), 3,4,4′,5-tetraCB, 3,3′,4,4′,5-pentaCB (PCB 126), and 3,3′,4,4′,5,5′-hexaCB are the most active AhR agonists, and their mono-ortho derivatives containing one ortho-chloro substituent also exhibit weak AhR agonist activities. TEFs have been assigned to the most environmentally relevant non-ortho and mono-

ortho substituted PCB congeners (Table 1) [17]. These values have been extensively used by scientists to calculate TEQs in environmental samples, and, in many samples, the TEQs-PCBs are comparable or higher than the TEQs-PCDDs/PCDFs [14,18].

Table 1 Toxic equivalency factors for the 2,3,7,8-substituted PCDDs and PCDFs and selected polychlorinated biphenyl congeners [37,50].

Congener		TEF
PCDD	2,3,7,8-TCDD	1.0
	1,2,3,7,8-PentaCDD	0.5
	1,2,3,4,7,8-HexaCDD	0.1
	1,2,3,6,7,8-HexaCDD	0.1
	1,2,3,7,8,9-HexaCDD	0.1
	1,2,3,4,6,7,8-HeptaCDD	0.01
	OCDD	0.001
PCDF	2,3,7,8-TCDF	0.1
	2,3,4,7,8-PentaCDF	0.5
	1,2,3,,7,8-PentaCDF	0.1/0.05
	1,2,3,4,7,8-HexaCDF	0.1
	2,3,4,6,7,8-HexaCDF	0.1
	1,2,3,6,7,8-HexaCDF	0.1
	1,2,3,7,8,9-HexaCDF	0.1
	1,2,3,4,6,7,8-HeptaCDF	0.01
	1,2,3,4,7,8,9-HeptaCDF	0.01
	OCDF	0.001
PCB	3,3′,4,4′,5-PentaCB	0.1
	3,3′,4,4′,5,5′-HexaCB	0.01
	3,3′,4,4′-TetraCB	0.0005
	2,3,3′,4,4′-PentaCB	0.0001
	2,3,3′,4,4′,5-HexaCB	0.0005
	2,3,4,4′,5-PentaCB	0.0001
	2,3,3′,4,4′,5′-HexaCB	0.0005
	2′,3,4,4′,5-PentaCB	0.0001
	2,3,4,4′,5-PentaCB	0.0005

Several studies report non-additive antagonistic interactions between AhR-active PCBs, PCDDs, PCDFs, and AhR-independent PCB congeners such as 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153) [19–24]. For example, in cells or animals treated with an effective dose of TCDD or 3,3′,4,4′,5-pentaCB, cotreatment with 2,2′,4,4′,5,5′-hexaCB inhibits induction of CYP1A1 and related activities, porphyria, teratogenicity (murine fetal cleft palate and hydronephrosis), chick embryotoxicity, malformations, liver lesions, and edema. Moreover, the ratios of 2,2′,4,4′,5,5′-hexaCB/TCDD (or TEQ) required for non-additive antagonist interactions are in the range of those observed in environmental samples.

Another serious problem associated with the TEF approach for risk assessment of halogenated aromatic compounds is the effects of concurrent exposures to AhR-active phytochemicals. The TEF approach was initially developed at a time when only a limited number of synthetic industrial compounds or combustion by-products were characterized as AhR agonists. However, more recent studies have demonstrated that structurally diverse phytochemicals and endogenous biochemicals also interact with the AhR; these include flavonoids, indole-3-carbinol and related heteroaromatics, carotenoids, 7-ketocholesterol, compounds in herbal extracts, bilirubin, biliverdin, and resveratrol [25–34]. It is estimated that serum levels of "natural" AhR agonists/antagonists (e.g., flavonoids) are in the nM to low μM range [26]. In contrast, serum levels of TEQs (for TCDD and related compounds) are in the subpicomolar range suggesting that ratios of phytochemical AhR agonists/TEQs are 10^4 to 10^6. In most studies with

phytochemicals that exhibit AhR antagonist activity, their inhibitory effects would be observed at antagonist/agonist ratios of 10^4 to 10^6, whereas their levels in serum are below concentrations for agonist-induced responses. Thus, the TEF approach for hazard and risk assessment of halogenated aromatics has significant deficiencies due to interactions of "TEQ-compounds" (Table 1) with PCBs and phytochemicals that inhibit AhR-mediated responses.

USING THE TEF APPROACH FOR ENDOCRINE ACTIVE CHEMICALS

The U.S. Environmental Protection Agency's (USEPA's) Endocrine Disruptor Screening Program (EDSP) has outlined several in vitro and in vivo bioassays for identifying endocrine active chemicals (EACs). These assays can provide data on relative activity for mixtures [35–38]. For example, we have used multiple bioassays to show that the estrogen equivalents (EQs) in 200 milliliters (ml) of red wine are at least 1000 times higher than the EQs for the average daily intake of a mixture of known estrogenic pesticides in the diet (Table 2) [38]. The use of individual bioassays and EQs is comparable to the TEF/TEQ method for hazard and risk assessment of TCDD and related halogenated aromatics and is based on their common mechanism of action through binding to common steroid hormone receptors.

Table 2 Estimated daily intake of estrogen equivalents.

	Estrogen equivalents (μg)
Wine (200 ml)	1.87
Pesticide mix (2.44 μg*)	0.00021

*Estimated daily intake of organochlorine pesticides determined in a 1995–1996 Food and Drug Administration market survey for contaminants in food.
From [38]

However, results of ongoing studies indicate that risk assessment of AhR agonists and other EACs using an additive approach may be too simplistic [1,39–42]. Studies in our laboratories have demonstrated that structurally diverse synthetic and naturally occurring ER agonists differentially activate various estrogen-responsive constructs in cancer cell lines cotransfected with wild-type or variant forms of ERα [43–47].

LIGAND-DEPENDENT REGULATION OF STEROID HORMONE RECEPTOR STRUCTURE AND FUNCTION

Selective receptor modulator (SRM) describes the ability of a ligand to manifest receptor agonist activity in some tissues but block receptor activity in other tissues [48]. The properties of selective receptor modulators are due in part to unique ligand-induced conformational changes in the steroid hormone receptor that affect the subsequent tissue-specific recruitment of other nuclear factors required for ligand-induced gene expression [49,50]. The antiestrogenic drug tamoxifen is an example of a selective estrogen receptor modulator (SERM). Tamoxifen functions as an estrogen receptor antagonist in breast cancer cells, but behaves as an agonist in the uterus and bone [51–56]. This tissue-specific estrogen receptor activity of tamoxifen is likely related to its differential interactions, relative to estradiol, with domains of the estrogen receptor [57].

Figure 2 illustrates structurally diverse synthetic and natural estrogenic compounds used in our studies to investigate the effects of ligand structure on estrogen receptor function [43–46]. We hypothesized that the diversity in structure of these compounds would likely result in induction of unique conformations of the estrogen receptor that would ultimately affect estrogen receptor function in a unique gene- and tissue-specific manner similar to the pharmaceutical SRMs. Results of studies in HepG2

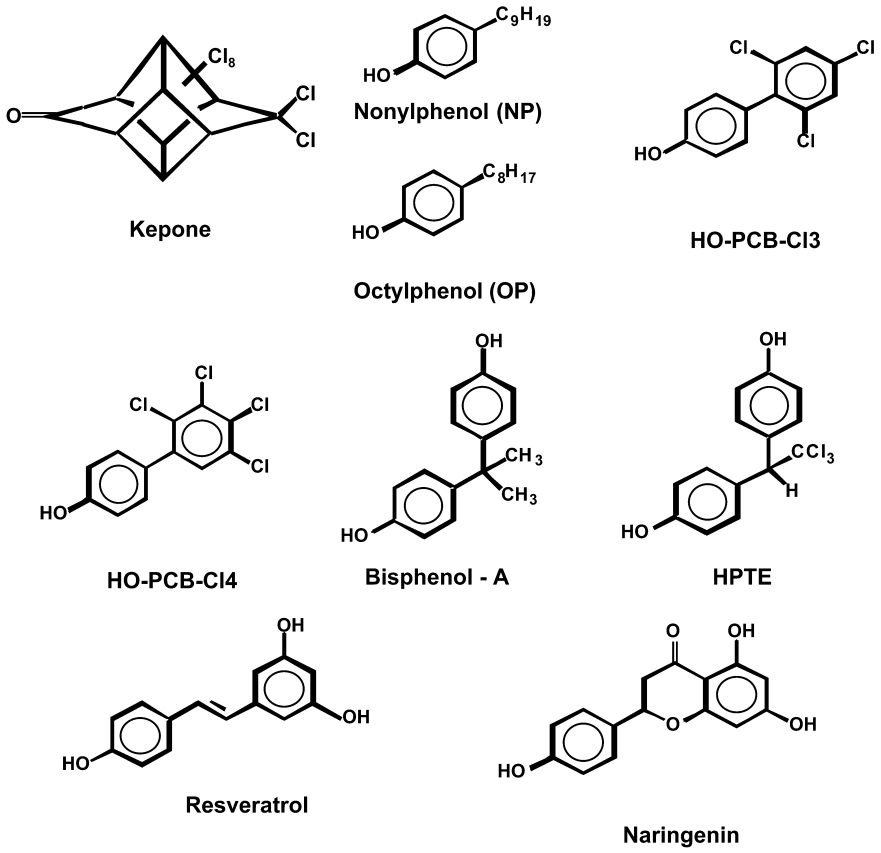

Fig. 2 Structures of naturally occurring and synthetic compounds.

human hepatoma cells confirmed our hypotheses and showed that the phenolic compounds (mono- and dihydroxy) gave similar but not identical patterns of induced gene expression that were clearly different from those observed for the phytoestrogens naringenin and resveratrol and the chlorinated hydrocarbon kepone [43,45].

Cell context is also an important determinant. For example, resveratrol induced reporter gene activity in U2 human osteogenic sarcoma cells transfected with hERα-AF1 while naringenin was inactive, whereas these activities were reversed in HepG2 cells [45]. In HepG2 cells cotreated with estradiol plus synthetic and natural estrogens, bisphenol A (BPA) and naringenin exhibited partial antiestrogenic activity with one or more forms of wild type or variant hERα [47,58]. BPA also exhibited SERM-like activity in an estrogen-responsive human endometrial carcinoma cell line [59]. In this cell line, estradiol induced both progesterone receptor expression and cell proliferation, whereas BPA induced only progesterone receptor expression. In combination studies, BPA inhibited the induction of cell proliferation by estradiol. These results indicate that BPA can act as both an estrogen and an antiestrogen in the same cell, depending on the response being examined. The inhibitory effects of BPA have also been observed in vivo [47]. Ongoing studies using wild-type and variant forms of hERα in HepG2, U2, and MDA-MB-231 breast cancer cells show that activation of receptor activity by natural and synthetic estrogens depends on ligand structure, cell context, and form of hERα [43–45,47]. Moreover, most of the test compounds exhibit antiestrogenic activity in one or more of these assays.

The pattern of ERα activation by BPA and 2,2′-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) in HepG2 cells was similar; however, results obtained using U2 and MDA-MBA-231 cells

clearly distinguish between the two 4,4′-dihydroxydiphenylmethane analogs that differ only in their methylene bridge substituents (Fig. 2). Recent studies in our laboratories also show that BPA, not HPTE, activates ERß (Table 3) [44,46]. In contrast, HPTE acts as an ERß antagonist and an androgen receptor (AR) antagonist. BPA does not interact with the AR.

Table 3 Differential interaction of structural analogs with ERα, ERß, and AR.

	ERα	ERß	AR
HPTE	+++	– – –	– – –
Bisphenol A	++	++	
p,p′-DDE			– – –
Di-hydroxy DDE	+++	– – –	– – –

From [44]

Together, these results indicate that structurally diverse natural and synthetic compounds can have selective receptor modulating activity capable of having additive or antagonistic properties when combined, making it difficult, if not impossible, to predict the activity of a mixture of these chemicals in target tissues in vivo.

MODIFICATION OF ENDOCRINE ACTIVE POTENTIAL IN VIVO BY CHEMICAL MIXTURES

We investigated the combined effect of estradiol and HPTE on gene expression in the reproductive tissues of male and female mice [60]. Alone, estradiol and HPTE acted similarly on expression of most genes in the ovaries, uterus, testes, and prostate (Table 4). However, in each tissue, there were subsets of genes differentially regulated by these two compounds. In the uterus, progesterone receptor, ERα, AR, insulin-like growth factor 1, insulin-like growth factor binding protein 5, and clusterin mRNAs were significantly reduced with both E2 or HPTE treatments, whereas cathepsin B was induced. Conversely, induction of cathepsin B by E2 in the ovary was reversed after cotreatment with HPTE, and ERβ expression was induced by HPTE but not E2. In addition, E2 uniquely upregulated glutathione peroxidase 3, glutathione *S*-transferase, and cytochrome P450 17α-hydroxylase, with no effect of HPTE. In male mice, mast cell growth factor, clusterin, cyclin A2, and glutathione peroxidase 3 (GPX3) mRNAs were significantly induced with either E2 or HPTE treatments in the testes, whereas insulin-like growth factor 1A (IGF-IA) and UDP-glucuronosyltransferase mRNAs were decreased. In the prostate, IGF-IA, clusterin, and GPX3 mRNAs were induced by E2 and HPTE. IGF binding protein 3 was induced by E2 but not by HPTE in the testes, and this E2-specific induction was blocked by cotreatment with HPTE. Cytochrome P450 17α-hydroxylase mRNA was downregulated by E2, and AR was uniquely upregulated by HPTE in the testes. These results demonstrate that E2 and HPTE induce both common and also unique patterns of tissue-specific and receptor-dependent gene expression.

We also investigated the combined effect of genistein and methoxychlor, the parent compound of HPTE, on reproductive development in Sprague–Dawley rats [61]. Sprague–Dawley rats were exposed to the compounds either alone or in combinations through dietary administration during pregnancy and lactation; the offspring were exposed after lactation. Both compounds singly and in combination accelerated vaginal opening and altered estrous cyclicity in female offspring, and these estrogenic responses to genistein plus methoxychlor were additive. Methoxychlor but not genistein delayed preputial separation in male rats, an indication of antiandrogenic action. When administered in combination with methoxychlor, genistein enhanced the effects of methoxychlor (Fig. 3). While the estrogenic responses are supported by in vitro estrogen-receptor-based transcriptional activational assays, the potentiation of

Table 4 Effect of estradiol and HPTE on gene expression.

		Uterus[2]	Ovary[2]	Testis	Prostate
Estrogen receptor α	Estradiol	↓	NC	NC	
	HPTE	↓	NC	NC	
	Estradiol + HPTE	+	NC	NC	
Progesterone receptor	Estradiol	↓	↑		
	HPTE	↓	NC		
	Estradiol + HPTE	+	+		
Androgen receptor	Estradiol	↓	NC	NC	NC
	HPTE	↓	NC	↑	NC
	Estradiol + HPTE	+	NC	*	NC
Insulin-like growth factor 1A	Estradiol	↓	NC	↓	↓
	HPTE	↓	NC	↓	↓
	Estradiol + HPTE	+	NC	+	+
IGF binding protein	Estradiol	↓	↓	↑	↑
	HPTE	↓	NC	↑	NC
	Estradiol + HPTE	+	–	–	–
Clusterin	Estradiol	↓	NC		↑
	HPTE	↓	NC		↑
	Estradiol + HPTE	+	NC		+[1]
Glutathione peroxidase 3	Estradiol	↑	↑	↑	↑
	HPTE	NC	NC	↑	↑
	Estradiol + HPTE	–	–	+	+
Glutathione *S*-transferase	Estradiol	↓	↑	NC	NC
	HPTE	NC	NC	NC	NC
	Estradiol + HPTE	–	–	NC	NC
Cathepsin B	Estradiol	↑	↑		NC
	HPTE	↑	NC		NC
	Estradiol + HPTE	+	–[1]		NC
Cytochrome P45017	Estradiol		↓	↓	
	HPTE		↓	NC	
	Estradiol + HPTE		+[1]	+[1]	
Mast cell growth factor	Estradiol			↑	
	HPTE			↑	
	Estradiol + HPTE			+	
Cyclin A2	Estradiol			↑	NC
	HPTE			↑	NC
	Estradiol + HPTE			+[1]	NC

↑ Indicates an increase in gene expression.
↓ Indicates a decrease in gene expression.
+ The actions of estradiol plus HPTE were additive.
– HPTE antagonized the actions of estradiol.
* Indicates activity unique to HPTE.
[1]Indicates cases where HPTE also displayed antiandrogenic activity.
[2]From [60]

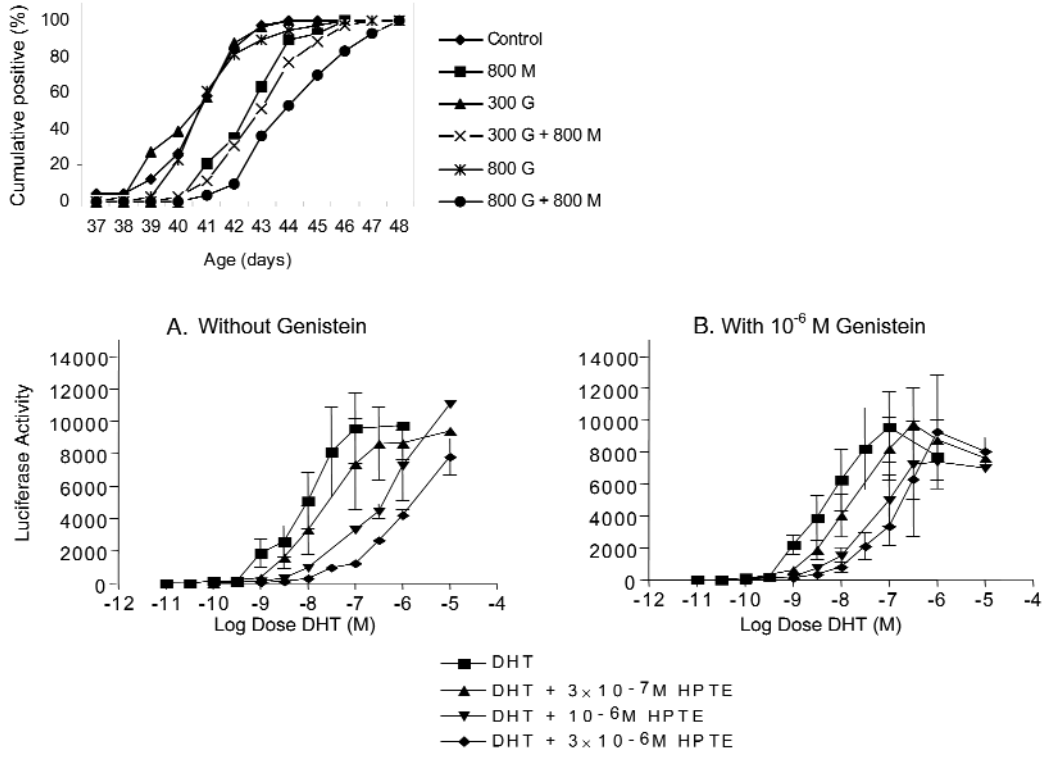

Fig. 3 *Antiandrogenic interaction of the binary mixture of genistein and methoxychlor.* Upper panel: Time course of cumulative percentage positive for preputial separation (PPS), a developmental landmark of androgenic action, in male rats treated with the xenochemicals genistein and methoxychlor. The age at which PPS occurred was delayed by methoxychlor. Genistein by itself did not have an effect, but the presence of 800-ppm genistein enhanced the effect of methoxychlor. Lower panel: Androgen receptor- (AR-) dependent transcriptional activation assay using expression plasmid-transfected HepG2 cells. The methoxychlor metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) antagonized the AR-activating effect of dihydrotestosterone (DHT) by shifting the dose–response curves to the right (A). The presence of genistein had no effect on the AR antagonism of HPTE (B). The *p*Kb value for HPTE was 6.42 ± 0.17 in the absence of genistein and 6.64 ± 0.17 in the presence of genistein [from 61].

methoxychlor antiandrogenicity by genistein is not predicted based on in vitro androgen receptor transactivation assays (Fig. 3).

In a parallel set of experiments, we examined interactions of genistein and methoxychlor on mammary gland development in juvenile rats. Inguinal mammary glands were obtained from both female and male pups. In male rats, methoxychlor caused elongation of the glandular ducts, while genistein enhanced ductile branching. The two compounds together promoted development of alveolar-lobular structure, an effect not observed with either compound alone.

Together, these studies highlight the complexity of steroid hormone receptor-mediated responses and the difficulties for predicting endocrine activities of chemical mixtures based on the actions of these chemicals individually in short-term in vitro and in vivo bioassays.

SUMMARY

The TEF/TEQ approach for risk assessment was developed for chemicals such as halogenated aromatics that induce their effects through ligand-activated receptors. For persistent halogenated aromatic AhR

agonists, this approach has some limited utility. However, the structure-dependent interactions of SRMs have been extensively investigated, and the results suggest that a TEF/TEQ approach for these compounds is not appropriate due to their unique tissue-specific agonist and antagonist activities. Ligands that bind AhR and other nuclear receptors also induce tissue-, species-, and age-dependent responses. Therefore development of mechanisms-based hazard risk assessment of receptor agonists/antagonists must account for these multiple variables.

ACKNOWLEDGMENTS

S. Safe acknowledges financial support from the National Institutes of Health (ES09106 and ES04917).

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