Problems for Risk Assessment of Endocrine-Active Estrogenic Compounds

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Estrogenic industrial compounds such as bisphenol A (BPA) and nonylphenol typically bind estrogen receptor (ER) α and ERβ and induce transactivation of estrogen-responsive genes/reporter genes, but their potencies are usually ≥ 1000-fold lower than observed for 17β-estradiol. Risk assessment of estrogenic compounds on the basis of their potencies in simple reporter gene or binding assays may be inappropriate. For example, selective ER modulators (SERMs) represent another class of synthetic estrogens being developed for treatment of hormone-dependent problems. SERMs differentially activate wild-type ERα and variant forms expressing activation function 1 (ER-AF1) and AF2 (ER-AF2) in human HepG2 hepatoma cells transfected with an estrogen-responsive complement C3 promoter-luciferase construct, and these *in vitro* differences reflect their unique *in vivo* biologies. The HepG2 cell assay has also been used in our laboratories to investigate the estrogenic activities of the following structurally diverse synthetic and phytoestrogens: 4′-hydroxytamoxifen; BPA; 2′,4′,6′-trichloro-4-biphenylol; 2′,3′,4′,5′-tetrachloro-4-biphenylol; *p-t*-octylphenol; *p*-nonylphenol; naringenin; kepone; resveratrol; and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane. The results show that synthetic and phytoestrogens are weakly estrogenic but induce distinct patterns of ER agonist/antagonist activities that are cell context- and promoter-dependent, suggesting that these compounds will induce tissue-specific *in vivo* ER agonist or antagonist activities. These results suggest that other receptors, such as the aryl hydrocarbon receptor, that also bind structurally diverse ligands may exhibit unique responses *in vivo* that are not predicted by standard *in vitro* bioassays. *Key words:* agonists, antagonists, estrogen receptor, estrogens, structure–activity. *Environ Health Perspect* 110(suppl 6):925–929 (2002).
http://ehpnet1.niehs.nih.gov/docs/2002/suppl-6/925-929safe/abstract.html

Hazard and risk assessment of toxic chemicals is usually focused on individual compounds and used to limit or regulate exposures. This approach has been particularly valuable for setting standards for occupational exposures and for emissions of various industrial compounds or their byproducts. It is more difficult to develop regulations for chemical mixtures because compound interactions may result in additive, synergistic, or antagonistic effects. The halogenated aromatic (HA) industrial chemicals and their byproducts are complex mixtures of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) that are regulated using toxic equivalents (TEQs), which integrate the additive contributions of individual compounds in the mixture (*1–5*). Risk assessment of HAs initially focused on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic member of this class of compounds; however, TCDD is a minor component of many samples containing PCDDs/PCDFs. Therefore, hazard and risk assessment of the most highly toxic 2,3,7,8-substituted PCDDs and PCDFs uses an approach (Equation 1) where the TEQs of a mixture is the summation of the concentrations of individual congeners times their toxic equivalency factor (TEF), which is their fractional potency compared with TCDD (TEF = 1.0). The biological plausibility of the TEQ approach is supported by the aryl hydrocarbon receptor (AhR)-mediated mechanism of action for the toxic 2,3,7,8-substituted PCDDs/PCDFs. TEFs/TEQs have been used extensively for regulating industrial emissions of PCDDs/PCDFs and for estimating body burdens (adipose tissue, blood, and milk) of these compounds in wildlife and human populations. However, there are also significant problems in applying the TEF/TEQ approach for estimating toxicity/genotoxicity and for predicting adverse health effects associated with dietary intakes of PCDDs/PCDFs. The diet contains PCB mixtures, and their concentrations are orders of magnitude higher than the TEQs for PCDDs/PCDFs. Many individual PCB congeners and mixtures exhibit AhR antagonist activities, and the TEQ approach will therefore overestimate toxicity (*6–9*). Moreover, recent studies have identified a number of phytochemicals, including indole-3-carbinol and related compounds, bioflavonoids, alkaloids, diverse phenolics, and carotenoids that are either weak AhR agonists or antagonists (*10–20*). The dietary intake and/or serum levels for some of these compounds are several orders of magnitude higher than observed for HA–TEQs. Potential interactions of phytochemical AhR agonists/antagonists and HA–TEQs have not been studied extensively; however, there are examples of inhibition of TCDD-induced responses by phytochemical AhR antagonists (*10–13,15,16*). These interactions have not been incorporated in a recent U.S. Environmental Protection Agency (U.S. EPA) evaluation of the potential adverse effects of current dietary intakes of HA-derived TEQs. An understanding of interactions of HAs with high levels of natural AhR agonists/antagonists in the diet is required for a science-based risk assessment.

$$TEQ = \Sigma\ PCDD_i \times TEF_i + \Sigma\ PCDF_i \times TEF_i \quad [1]$$

The development and applications of the TEF/TEQ approach for TCDD and related HAs illustrate the utility and limitations of this method for hazard and risk assessment. There has been significant public, regulatory, and scientific concern regarding the potential adverse health effects of other endocrine-active chemicals, particularly those compounds that exhibit estrogenic/antiestrogenic, androgenic/antiandrogenic, and thyroid hormonelike activity (*21–25*). Research in this area has focused primarily on compounds that bind hormone receptors, and chemicals that interact with the ER have been a major concern. This resulted in a congressional mandate under the Food Quality Protection Act and Safe Drinking Water Amendments (*26,27*) for the U.S. EPA to develop screening programs for compounds with estrogenic and other endocrine activities. These assays were developed by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) and include a series of both *in vitro* and *in vivo* bioassays that can detect endocrine-active chemicals (*28,29*).

Development of Bioassays for Estrogenic Compounds

Figure 1 illustrates some industrial compounds that exhibit estrogenic activity. These include organochlorine pesticides and their breakdown products/metabolites, phenolics such as bisphenol A (BPA), hydroxy-PCBs,

This article is part of the monograph *Application of Technology to Chemical Mixture Research.*

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Financial support from the National Institutes of Health (ES09106 and ES04917) and the Texas Agricultural Experiment Station is gratefully acknowledged.

Received 18 December 2001; accepted 31 May 2002.

nonylphenols, and phthalates. Human exposure to these estrogenic compounds in the diet is relatively low and is accompanied by significantly higher levels of phytoestrogens such as flavonoids, other hydroxylated aromatics present in vegetables, fruits, nuts, and other products (*22*). The EDSTAC has outlined several *in vitro* and *in vivo* bioassays for estrogenic compounds (*28,29*), and these assays can provide data on relative estrogenic potencies for individual compounds or estrogen equivalents (EQs) for mixtures (*30,31*). For example, we have used multiple bioassays to show that the EQs in 200 mL of red wine (*30*) were at least 1,000 times higher than EQs for the average daily intake of known estrogenic pesticides in the diet. The use of individual bioassays and EQs is comparable to the TEF/TEQ method for hazard and risk assessment of TCDD and related HAs and is based on their common mechanism of action through initial binding to the ERα or ERβ. The applications and limitations of the TEF/TEQ approach for HAs have been discussed (*7–9*), and it is important to evaluate proposed applications of bioassays for quantitating synthetic and natural estrogens (e.g., EQs) and other classes of endocrine-active compounds.

Structure–Activity Relationships for Estrogenic Compounds: Selective Estrogen Receptor Modulators

Two ER subtypes (ERα and ERβ) bind structurally diverse endogenous steroids, phytoestrogens, and synthetic chemicals. Relative binding affinities of estrogenic compounds for ERα and ERβ are similar for most compounds. 17β-estradiol (E_2) and diethylstilbestrol bind ERs with high affinity, whereas most phytoestrogens and synthetic (industrial) compounds bind ERα and ERβ with relatively low affinity (*32–34*). *In vitro* binding affinities do not distinguish between ER agonists or antagonists nor do they predict tissue-specific estrogenic or antiestrogenic activity. For example, tamoxifen, a widely used drug for treatment and prevention of breast cancer, binds ERα and ERβ with moderate affinity and exhibits ER antagonist (antiestrogenic) activity in breast tumors, but also exhibits estrogenic activity in the uterus, bone, and vascular system. Tamoxifen and selective ER modulators (SERMs) with tissue-specific ER agonist/antagonists are currently being developed for treatment of hormone-dependent tumors, vascular disease, and osteoporosis, and as hormone replacement therapy for postmenopausal women (*35–39*). The structure-dependent properties of SERMs are due, in part, to ligand-induced conformational changes in the ER that affect the subsequent tissue-specific recruitment of other nuclear factors required for ligand-induced gene expression and physiologic responses. X-ray analysis of E_2 and SERMs bound to the ligand-binding domain of ERα and ERβ confirms that different structural classes of estrogenic compounds modulate ER conformation (*40–42*). We have confirmed this by showing that interaction profiles of polypeptides with ligand-bound ERα and ERβ are highly variable and dependent on ligand structure (*43–48*). Further confirmation of ligand structure-dependent activity of SERMs has been shown in studies with human HepG cells transfected with an E_2-responsive pC3-luc construct (containing the human complement C3 promoter linked to a luciferase reporter gene) and wild-type hERα or variant forms with a deletion of the activation factor 1 (AF1) domain (ERα-AF2) or critical mutations in the AF2 domain (ERα-AF1) (*49,50*). Table 1 illustrates the distinct pattern of induced luciferase activity by E_2 and SERMs tamoxifen/4′-hydroxytamoxifen, ICI 182,780, and raloxifene. These *in vitro* differences are consistent with the unique biologies of these compounds in estrogen-responsive tissues/organs (*50*).

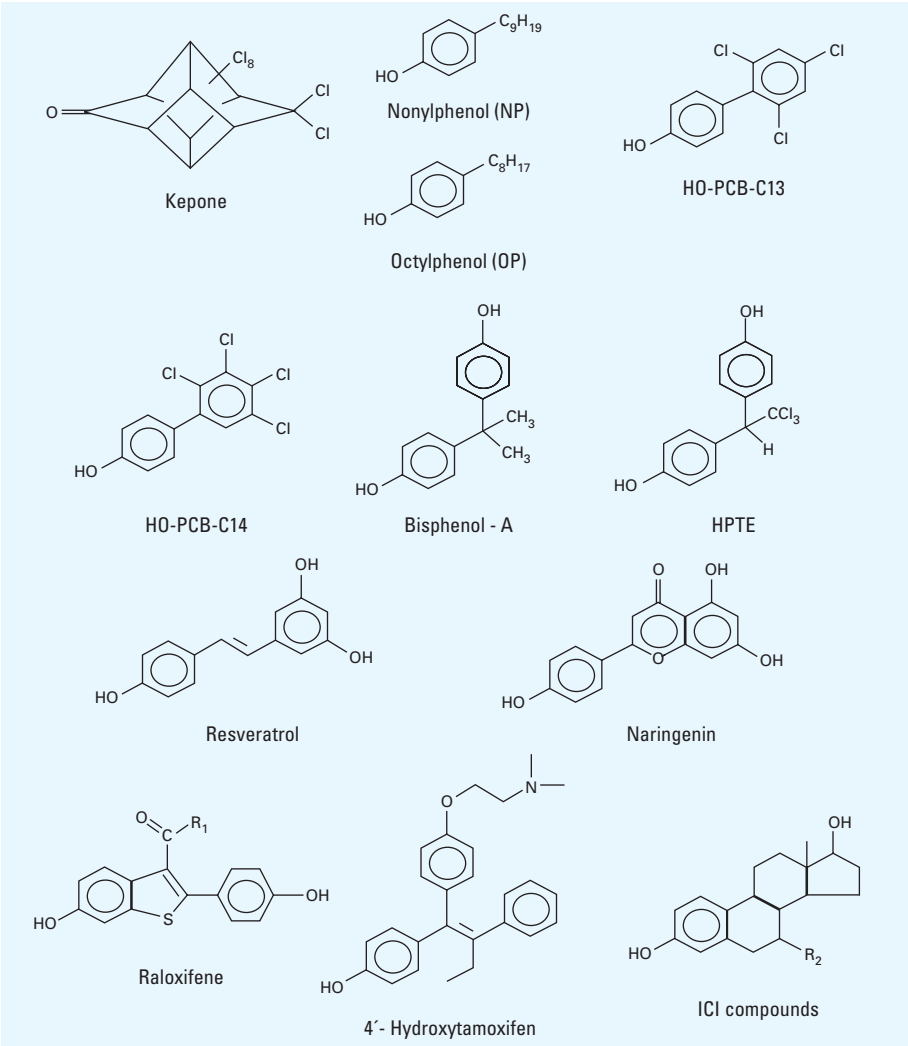

Figure 1. Structures of synthetic and naturally occurring estrogenic compounds.

Activation of Wild-Type and Variant hERα by Synthetic and Natural Estrogenic Compounds

Most studies on synthetic/industrial estrogenic compounds and phytoestrogens indicate that these compounds are weakly active

Table 1. Activation of pC3-luc in HepG2 cells treated with SERMs (*55*).

SERM	hERα	hERα-AF1	hERα-AF2[a]
E_2 (10^{-9} M)	+++	+++	0
Tamoxifen	+[a]	+	0
Raloxifene	0	+	
ICI 164,384	0	0	0

+, significant ($p < 0.05$) induction (<40% of E_2; +++, represents maximal (100%) induction by E_2. [a]hERα-AF2 does not activate pC3-luc in HepG2 cells with E_2 or SERMs.

in ER binding, reporter gene, or cell proliferation assays (*32–34*). Both natural and synthetic estrogenic compounds are characterized by the structural diversity in their background ring structures and substituents (*51,52*). E_2 and estrone contain tetracyclic ring structures with one fully aromatic ring, and changes in this backbone structure or aromaticity result in loss of hormonal activity. In contrast, synthetic/natural estrogenic compounds include substituted benzenes, stilbenes, biphenyls, diphenylmethanes, diphenylethanes, flavones, isoflavones, flavonols, and polycyclic aromatic compounds. Figure 1 illustrates structurally diverse synthetic/natural estrogenic compounds used in our studies to investigate the effects of ligand structure on activation of estrogen-responsive constructs containing complement C3 (pC3-luc) or estrogen response element (ERE) ($pERE_3$) promoter inserts and cotransfected with hERα, hERα-AF1, and hERα-AF2 expression plasmids (*53,54*). These studies have adapted the HepG2 cell assay that distinguishes between the biological activities of SERMs and include additional cell lines (U2 and MDA-MB-231 cells) and two promoters (pC3-luc and $pERE_3$) (*53,54*). In addition, we investigated partial hERα antagonist activities of these weakly estrogenic compounds. Results of initial studies in HepG2 cells using pC3-luc showed that the phenolic compounds (mono- and dihydroxy) gave similar but not identical patterns of induced gene expression clearly different from those observed for the phytoestrogens naringenin and resveratrol and the chlorinated hydrocarbon kepone (Table 2) (*53*). Cell context was also an important determinant for some responses induced by the phenolics and the phytoestrogens. For example, resveratrol induced reporter gene activity in U2 human osteogenic sarcoma cells transfected with pC3-luc and hERα-AF1 and naringenin was inactive, whereas these induction activities were reversed in U2 cells. In HepG2 cells cotreated with E2 plus synthetic/natural estrogens, only BPA (hERα, hERα-AF1, and hERα-AF2) and naringenin (hERα) exhibited partial antiestrogenic activity with one or more forms of wild-type or variant hERα, and these inhibitory effects have also been observed *in vivo* in rodent (uterus) studies (*55,56*). Ongoing studies using $pERE_3$, wild-type and variant forms of hERα in HepG2, U2, and ER-negative MDA-MB-231 breast cancer cells show that activation of luciferase activity by natural/synthetic estrogens depends on ligand structure, cell context and form of hERα expressed in these ER-negative cell lines (Table 3) (*54*). Moreover, using $pERE_3$, most of the test compounds exhibit antiestrogenic activity in one or more of these assays and results of both estrogenic and antiestrogenic assays also differentiate between individual phenolic compounds.

The pattern of pC3-luc activation by BPA and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) in HepG2 cells was similar. However, results obtained using $pERE_3$ in HepG2, U2, and MDA-MBA-231 cells clearly distinguish between the two 4,4′-dihydroxydiphenylmethane analogs that differ only in their methylene bridge substituents. HPTE and BPA exhibit similar ER agonist and antagonist activities in HepG2 cells. However, their activities are significantly different in MDA-MB-231 and U2 cells. BPA is consistently a stronger agonist in MDA-MB-231 and U2 cells transfected with hERα or hERα-AF1, whereas both compounds exhibit similar activity in U2 cells transfected with hERα-AF2. Thus, BPA and HPTE differentially activate variant/wild-type hERα, and recent studies in our laboratories also show that BPA but not HPTE activates hERβ in HepG2 cells. HPTE acts as an hERβ antagonist and an androgen receptor antagonist (*57,58*).

Differences in ligand-dependent activation of ERα and ERβ are related to ligand-induced conformational changes in the receptors and their subsequent recruitment of coactivators and other nuclear factors required for transactivation. We have used peptide libraries to identify specific peptides that exhibit ligand-dependent interactions with ERα, ERβ, and

Table 2. Ligand-structure-dependent activation of pC3-luc in HepG2 and U2 cells.

	hERα		hERα-AF1		hERα-AF2	
Natural/synthetic SERM	HepG2	U2	HepG2	U2	HepG2	U2
E_2 (10^{-9} M)	+++	+++	+++	+++	+++	+++
Phenolics (10^{-5} M)	++	++	++	++	Variable	Variable
Kepone (10^{-5} M)	0	0	+	0	0	0
Naringenin (10^{-5} M)	0	0	++	0	0	0
Resveratrol ($10^{-5/4}$ M)	0	0	0	+	0	0

++, significant ($p < 0.05$) induction (>40% of E_2); +, significant ($p < 0.05$) induction (<40% of E_2); +++, represents maximal (100%) induction by E_2.

Table 3. Comparative ER agonist/antagonist activities of BPA and HPTE in different cell lines transfected with $pERE_3$ and wild-type or variant forms of hERα (*53*).

		BPA		HPTE	
Cell line	ERα/variant	Agonist[a]	Antagonist[b]	Agonist[a]	Antagonist[b]
HepG2	hERα	+++	+	+++	0
	hERα-AF1	+	++	+	++
	hERα-AF2	+++	0	+++	+
MDA-MB-231	hERα	++	0	+	0
	hERα-AF1	+	+	0	0
	hERα-AF2	++	0	0	0
U2	hERα	++	0	+	0
	hERα-AF1	+++	0	0	0
	hERα-AF2	+++	++	+++	0

[a]+, significant ($p < 0.05$) induction (<40% of E_2); ++, significant ($p < 0.05$) induction (40–80% of E_2); +++, significant ($p < 0.05$) induction (>80% of E_2). [b]+, significant ($p < 0.05$) inhibition (<40%); +++, significant ($p < 0.05$) inhibition (>40%).

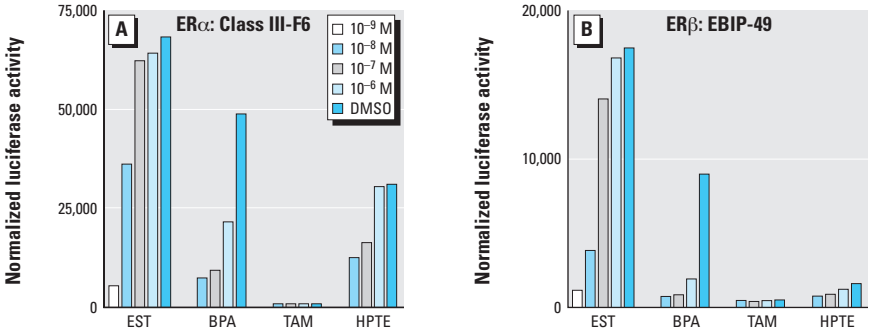

Figure 2. Evaluation of selected xenoestrogens with ERα- and ERβ-selective peptides in HepG2 cells. Mammalian two-hybrids were performed to characterize the interaction of estradiol (EST), BPA, hydroxytamoxifen (TAM), and HPTE with ERα and ERβ. Each ER expression construct includes the VP16 activation domain sequence fused 5′ to the complete coding sequence for human ERα (*A*) and ERβ (*B*). Human HepG2 hepatoma cells were transiently transfected with the pVP16-ERα or pVP16-ERβ expression vector together with a peptide-Gal4DBD fusion construct, a 5XGAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid (a constitutively active transfection and toxicity control). Cells were treated with dimethyl sulfoxide (vehicle control), or the indicated chemical for 24 hr. Luciferase values were normalized to the β-galactosidase activity. Results are presented as mean values for three separate experiments.

other nuclear receptors (*43–48*). Figure 2 summarizes results of mammalian two-hybrid assays using pVP16ERα or pVP16hERβ and ER-interacting peptides fused to the DNA binding domain of the yeast GAL4 protein. HepG2 cells are transfected with the interacting proteins and the 5XGAL4-Luc reporter and treated with different concentrations of E_2, tamoxifen, HPTE, or BPA. Previous studies have demonstrated that interaction of peptide EBIP-49 with hERβ is ligand dependent (*47*), and E_2 induces reporter gene activity in HepG2 cells transfected with EBIP-49, pVP16hERβ, and 5XGAL4-Luc. BPA also induces activity at the highest concentration (10^{-6} M), whereas HPTE and tamoxifen are inactive (Figure 2). A similar experiment using pVP16hERα and the class III-F6 peptide (*46*) shows that tamoxifen is inactive, whereas E_2, HPTE, and BPA induce reporter gene activity with different dose–response curves. These preliminary data from the mammalian two-hybrid assays complement results showing that HPTE and BPA differentially activate pC3-luc (Tables 2, 3) and suggest that synthetic/natural estrogens (e.g., Figure 1) exhibit SERM-like estrogenic/antiestrogenic activity.

Summary

The TEF/TEQ approach for risk assessment was developed for chemicals such as HAs that induce their effects through ligand-activated receptors. For persistent HA AhR agonists, this approach has some limited utility. However, the use of TEFs/TEQs for estimating the toxicity of low-level dietary exposures is confounded by concurrent exposures to high levels of phytochemicals that are also AhR agonists/antagonists. For example, daily TEQ intakes of TCDD and related compounds are 50–200 pg and serum values < 5 ppt (lipid weight), or approximately 0.1 pM. In contrast, total daily intakes of flavonoids can be as high as 1 g, and serum levels of flavonoids such as quercitin and genistein can be in the nanomolar to low micromolar range, where these compounds act as AhR antagonists (*16*). Structure-dependent interactions of SERMs with hERα and hERβ have been extensively investigated, and the results suggest that a TEF/TEQ approach for these compounds is not appropriate because of their unique tissue-specific ER agonist/antagonist activities. Results of this study demonstrate that synthetic/natural estrogenic chemicals also exhibit SERM-like activity, and *in vitro* binding or reporter gene bioassays would not necessarily predict their estrogenic/antiestrogenic activity for any given response *in vivo*. Thus, although the xenoestrogens resveratrol and naringenin (Figure 1) exhibit weak binding affinity for ERα, these data would not predict interactions with ERβ or other ligand-activated receptors. In addition there is also evidence that naringenin, BPA, and resveratrol exhibit both ERα agonist and antagonist activities in cell culture and/or *in vivo* assays (*55,56,59–62*). Resveratrol also interacts with the AhR (*11,15*). Ligands that bind other nuclear receptors and the AhR also induce tissue-, species-, and age-dependent responses, and therefore development of mechanism-based hazard/risk assessment of receptor agonists/antagonists must account for these multiple variables.

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