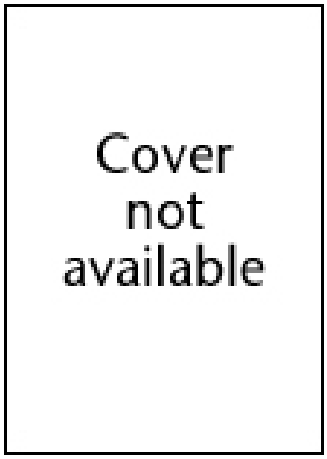

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ENDOCRINE DISRUPTION: A CRITICAL REVIEW OF ENVIRONMENTAL ESTROGENS FROM A MECHANISTIC PERSPECTIVE

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ENDOCRINE DISRUPTION: A CRITICAL REVIEW OF ENVIRONMENTAL ESTROGENS FROM A MECHANISTIC PERSPECTIVE

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With the aid of such systems as the E-screen assay (MCF-7 human breast cancer cells) and/or estrogen receptor (ER) binding assays, estrogenic activity has been identified in a wide variety of nonsteroidal substances, such as polychlorobiphenyls, alkylphenols, bisphenols, pesticides (e.g., DDT derivatives, methoxychlor, kepone), pharmaceutical agents (e.g., diethylstilbestrol [DES], tamoxifen, raloxifene), and phytoestrogens. With few exceptions (notably DES), most xenoestrogens show weak estrogenic and ER binding activity (e.g., 1/1000 to 1/1,000,000 that of the endogenous hormone, estradiol). A series of observations and episodes, some controversial, have led to the perception that endocrine disruption via hormonally active environmental substances is a hazard to wildlife and humans. As a result of Congressional legislation, the U.S. Environmental Protection Agency (EPA) formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to develop and implement a multitiered program for the screening and testing of potentially 87,000 chemicals and mixtures for hormonal (primarily estrogenic) activity and endocrine disruptive effects. This review explores the issue of xenoestrogens from a mechanistic perspective. Many nonsteroidal substances can interact with the ligand binding domain of the ER, albeit weakly, because they share characteristics common to both estradiol and DES, these being a ring structure (preferably unencumbered phenolic) along with a hydrophobic center. However, ER binding does not explain the nature of the biological response. As exemplified by a diversity of estrogenic and antiestrogenic effects exhibited by estradiol, tamoxifen, and raloxifene and by the antiestrogenic effect of DDT in the tiger salamander, different ER ligands evoke distinct response profiles that appear to be influenced by the target tissue and species. These distinct profiles are determined by the ligand itself and the diversity of ER isoforms (α or β), response elements, and individual coregulatory proteins that associate with ER. Endocrine disruption may also be influenced by the role of plasma binding on the delivery of xenoestrogens to cells, chirality of xenoestrogens, cross-talk between ER signaling and other signaling systems (e.g., aryl hydrocarbon receptor), and alternate mechanisms (e.g., antiandrogen effects). In view of the complexities pertaining to mechanisms of action of xenoestrogens and knowledge yet to be obtained in this area, it would appear that the screening and testing approach undertaken by EDSTAC is premature at this time.

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The possibility that environmental chemicals can adversely affect development, normal endocrine function, and general well-being of an organism via endocrine disruption has been recognized for some time. Endocrine disruption can occur through a variety of pathways. Xenobiotics may act on target tissues through hormone receptor or nonreceptor mechanisms, may influence hormone secretion or its clearance from the body, and may exert influence indirectly through modulation of pituitary–target organ feedback relationships. Within the area of endocrine disruption, the major focus has been on hormonally active chemicals or those that interact with hormone receptors (American Council on Science and Health, 1998; EDSTAC, 1998; Juberg, 2000; LeBlanc et al., 1997; National Research Council, 1999; Stancel et al., 1995). Although androgenic and thyroid hormonal effects are under investigation, most of the emphasis in endocrine disruption, and the focus of this review, has been on environmental chemicals that interact with the estrogen receptor.

Since the initial observation by Allen and Doisy (1923) of estrogen-induced vaginal epithelial cell cornification in the ovariectomized or immature female rodent, a wide variety of nonsteroidal chemicals have been shown to mimic this action, as well as other known estrogenic actions such as enlargement and/or growth of rodent uteri or avian oviducts (Cook et al., 1933; Cook & Dodds, 1933; Dodds & Lawson, 1936; Reid & Wilson, 1944; Bitman et al., 1968; Eroschenko, 1981; Lieberman, 1996; Ashby, 1998). Such compounds have been referred to as estrogen mimics, xenoestrogens, or environmental estrogens.

ENVIRONMENTAL ESTROGENS

In recent years, estrogenic activity of nonsteroidal substances has been evaluated by in vitro methods. Most recognized among these is the E-screen assay, which employs cultured estrogen sensitive MCF-7 human breast cancer cells. Under defined in vitro conditions, addition of an estrogenic substance to the cells stimulates their proliferation in a dose-dependent fashion. By comparing the dose-dependent cellular response of xenoestrogens with that of the naturally occurring estrogen, estradiol-17ß (estradiol), one can estimate the relative proliferative potency (RPP) of the former compared to estradiol, as well as relative proliferative effect (RPE), the magnitude of the maximal proliferative effect of the compound in question compared to that of estradiol. The latter can determine whether an estrogen mimic behaves as a full agonist (RPE of 100%) or partial agonist (RPE < 100%). While cell proliferation appears to be the most prevalent endpoint in E-screen assays, others

have also been employed to assess xenoestrogens in MCF-7 cells, such as estrogen-induced estrogen receptor processing (i.e., decline in receptors), induction of a marker specific protein (such as pS2, cathepsin, and progesterone receptors), or relative affinities of these chemicals to estrogen receptors (Soto et al., 1995). Although the MCF-7 cell line is primarily regarded as a system to identify mitogenic substances that interact with the estrogen receptor, proliferation of this cell line can also be promoted by a variety of other agents, namely, insulin and insulin-like growth factors (IGFs) I and II (De Leon et al., 1992), epidermal growth factor (EGF) (Hofland et al., 1995; Taylor et al., 1995), relaxin (Bigazzi et al., 1992), and lithium (Taylor et al., 1995). Thus, great care should be exercised when employing this system for identifying potential xenoestrogens.

The E-screen and other whole-cell in vitro assays (e.g., cells transfected with estrogen receptors and reporter genes) have detected estrogenic activity in a diversity of nonsteroidal compounds derived from a variety of sources, namely, industrial chemicals, pesticides, pharmaceutical agents, and substances of plant (phytoestrogen) and fungal (mycoestrogen) origin (Krishnan et al., 1993; Soto et al., 1995; Miksicek, 1995; Routledge et al., 1998). With the notable exception of synthetic estrogens such as diethylstilbestrol (DES) (which have estrogenic potency that approximate or exceed that of estradiol), most xenoestrogens are weak. Although the mycoestrogen zearalenone is about 1/100 as potent as estradiol (RPP = 1%), other xenoestrogens are much weaker, with RPPs ranging from about 0.1% to 0.0001% (1/100,000 or 1/1,000,000) or less (Krishnan et al., 1993; Soto et al., 1995; Miksicek, 1995; Routledge et al., 1998).

A recent screening revealed estrogen receptor (ER) binding activity in 91 of 153 nonsteroidal compounds (Blair et al., 2000). In general, these compounds corresponded to those exhibiting estrogenic activity in vivo or in cell culture. Among these were synthetic estrogens (e.g., DES), antiestrogens (e.g., tamoxifen), alkylphenols, diphenyl derivatives (e.g., bisphenols and diphenyl ethane derivatives), congeners of dichlorodiphenyltrichloroethane (DDT) and polychlorobiphenyls (PCBs), methoxychlor derivatives, Kepone, parabens (alkyl hydroxy benzoate derivatives), phthalates, benzophenones (diphenylketones), oxyphenols, and assorted products with multiple benzene and/or phenol rings (e.g., phenolphthalein, phenol red, aurin, and nordihydroguariaretic acid). A few of these had a high affinity for ER. For example, DES and 4-OH-tamoxifen exhibited binding affinities for ER that were two- to fourfold that of estradiol (i.e., the relative binding affinities [RBA] of diethylstilbestrol [DES] and 4-OH-tamoxifen were 175% and 400%, respectively, compared to an RBA for estradiol of 100%). For most xenoestrogens,

Table 1 Nonsteroidal substances with estrogen activity by E-screen or related assay or by estrogen receptor binding activity

Industrial/commercial agents	Pesticides	Pharmaceutical agents	Plant/fungal agents
Selected PCBs	*o,p′*-DDT	DES	Zearalenone
Alkylphenols	*p,p′*-DDT	Tamoxifen[a]	Coumestrol[c]
Bisphenol-A	*o,p′*-DDE	Raloxifene[a]	Genistein[c]
Phenol red	*p,p′*-DDE	Benzyloxyphenol[b]	Daidzein[c]
Benzyl butyl phthalate	Dieldrin		Biochanin A[c]
Alkylhydroxybenzoates (parabens)	Toxaphene		Resveratrol
	Methoxychlor		Foods:
	Kepone		Soybean
	Endosulfan		Cabbage

Note. A representative sampling, not a complete list (Krishnan et al., 1993; Soto et al., 1995; Miksicek, 1995; Routledge et al., 1998; Blair et al., 2000).

[a]SERM (selective estrogen receptor modulator), an estrogen receptor ligand that exhibits either estrogenic or antiestrogenic activity, depending upon the target cell.

[b]Therapeutic depigmentation.

[c]Flavonoids.

however, the RBAs ranged from 0.1% to 0.0001% (from 1/1000 to 1/1,000,000 that of estradiol, as described earlier). In several cases, ER binding was detectable but too low to quantitate (Blair et al., 2000). In another report, several other xenoestrogens have been noted as having relatively high RBAs, namely, the mycoestrogens, zearalenol (10–100%) and zearalenone (about 3%), the phytoestrogens, coumestrol (5–94%) and genistein (up to 5%), and the PCB 3,4,3′,4′-tetrachlorobiphenyl (5%). The remaining xenoestrogens examined generally had RBAs under 0.1% (Hyder et al., 1999b). Table 1 shows a representative sampling of the various types of nonsteroidal substances that exhibit estrogenic activity.

REPORTS OF ENDOCRINE DISRUPTION IN HUMANS AND WILDLIFE

A series of observations led to the perception that xenoestrogens are hazardous to exposed wildlife and humans. A list of selected observations is shown in Table 2, and these have been discussed elsewhere (Stone, 1994; Colborn et al., 1996; Juberg, 2000; Safe, 2000). Most of the field observations noted have been confirmed in the laboratory where large doses of environmental estrogens have produced reproductive abnormalities in exposed animals (Colborn et al., 1996; Safe, 2000). Since most xenoestrogens have low potency relative to estradiol, the last two observations listed in Table 2, those pertaining to "synergy" and the "inverted U effect," are of particular interest since they suggest that xenoestrogens could produce endocrine disruptive effects at environmental

Table 2 Observations relevant to the concept of endocrine disruption due to environmental estrogens

Diethylstibestrol (DES) during pregnancy associated with vaginal cancer in offspring (Herbst et al., 1971).
Kepone (chlordecone) exposure associated with decreased sperm count (Guzelian, 1982).
Clover disease (impaired fertility) in grazing livestock attributable to phytoestrogens (Hughes, 1988).
Demasculinized alligators in Lake Apopka, Florida associated with *p,p′*-DDE contamination (Guillette et al., 1995).
Reproductive abnormalities in aquatic birds of the Great Lakes associated with organochlorine contamination (Fry, 1995).
Hermaphroditic fish in effluents of treatment plants (effluent induction of hepatic vitellogenin) (Sumpter & Jobling, 1995).
Association between breast cancer and tissue levels of the DDT residue, DDE (Wolff & Toniolo, 1995).
Meta-analysis of 61 published studies suggesting a 50% decline in sperm production from 1940 to 1990—attributed to exposure in utero to a "sea of estrogens" in the environment (Carlsen et al., 1992; Sharpe & Skakkebaek, 1993).
Report of "synergy of action" of xenoestrogen—binary mixtures of xenoestrogens produced an effect that exceeded the sum of two individual actions (by 10- to 1600-fold) (Arnold et al., 1996).
Report of "inverted-U" or dose-dependent biphasic effect—exposure to low levels of estrogen or xenoestrogen in utero sensitizes the prostate to androgen later in life, whereas higher levels of these compounds produce an antiandrogenic effect (vom Saal et al., 1997; Nagel et al., 1997).

levels. Furthermore, the "inverted U effect" suggests that developing organisms are particularly sensitive to hormonally active environmental substances.

The issue of endocrine disruption, however, has not been without controversy and detractors. Although persistent in the environment and body, xenoestrogens are usually very weak relative to estradiol. It has been suggested that, except for extreme circumstances (e.g., toxic spills, etc.), environmental levels of these pollutants are unlikely to have an adverse effect. Under usual circumstances, body levels of xenoestrogens would be small relative to endogenous estrogens, and thus would have little impact. It has also been suggested that any effect of environmental estrogens would be balanced by environmental agents that exhibit estrogen antagonism, such as dioxins and related compounds (Stone, 1994; Thomas, 1996; Juberg, 2000; Safe, 1995, 2000).

Key observations suggesting endocrine disruptive effects in humans have lacked consistency or have been criticized on technical grounds. For example, several reports have been unable to confirm an association between breast cancer risk and tissue levels of organochlorines (Zheng et al., 2000; Juberg, 2000; Safe, 1995, 2000). With regard to the observation of declining sperm count over the last 50 years, concern has been raised about potential sources of bias, regional variations in sperm count, statistical treatment of the data, and failure to observe

such effects in single center studies (Heinze, 1998; Orejuela et al., 1998; Swan et al., 1997, 1998; Juberg, 2000; Safe, 2000). Several laboratories have been unable to confirm the original observation of synergy produced by binary mixtures of various xenoestrogens using a variety of in vivo and in vitro assay systems to test the original chemical mixtures as well as other combinations in a wide range of doses (Arcaro et al., 1998; Ramamoorthy et al., 1997a, 1997b, 1997c; McLachlan et al., 1997; Ashby et al., 1997). In fact, the original observation (Arnold et al., 1996) was formally withdrawn after the laboratory that initially reported this effect was unable to confirm the initial observation (McLachlan, 1997). Finally, attempts to repeat the "inverted U-effect" have provided inconsistent results. While several laboratories have reported failure to demonstrate heightened sensitivity of offspring to in utero exposure to a variety of xenoestrogens (Ashby et al., 1999; Cagen et al., 1999), a recent report from another laboratory completely confirmed this effect (Gupta, 2000).

EDSTAC

Controversy notwithstanding, the issue of the adverse effects of endocrine disruption to wildlife and humans has received considerable publicity, as indicated by articles appearing in the popular media (Stone, 1994) and a book entitled *Our Stolen Future* (Colborn et al., 1996). The latter supported the position that environmental chemicals, via endocrine disruption, pose a serious threat to humans and wildlife. Ultimately, the issue of endocrine disruptors became the topic of Congressional hearings in 1996, leading to its inclusion in the Food Quality Protection Act and Amendments to the Safe Water Drinking Act. This legislation mandated that the U.S. Environmental Protection Agency (EPA) develop and implement a screening program to determine whether environmental chemicals had hormonal activity. Although most attention was given to estrogen effects, androgen and thyroid hormone activity were also considered.

Accordingly, the U.S. EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), comprised of representatives from academia, industry, and public health advocacy groups with the task of developing and implementing a screening program that was to be in operation by 1999 (progress report by August 2000). In excess of 87,000 chemicals (e.g., constituents of pesticides, industrial chemicals, food additives, cosmetics, naturally occurring substances), as well as representatives from distinct classes of mixtures (e.g., contaminants in human breast milk, mixtures common to hazardous waste sites, and pesticide–fertilizer mixtures), were to be considered for testing.

EDSTAC recommended a "tiered approach" to accomplish its objective. Following *Initial Sorting* and *Priority Setting*, determination of substances to be deferred or examined further, *Tier 1 Screening (T1S)* determined whether a substance or mixture exhibited hormone agonist activity using a variety of recommended in vitro (e.g., estrogen receptor binding/reporter gene) and in vivo (e.g., rodent uterotrophic) assays. T1S assays were to be of maximal sensitivity, and to consider all possible endpoints and species variations. In anticipation of the large number of substances to be considered in T1S, a "high-throughput prescreening" (HTPS) approach was recommended where preliminary hormonal and biological information were to be obtained in assays using automated/robotic high-speed systems in preference to manual methodologies. *Tier 2 Testing (T2T)* was developed to test for endocrine-mediated adverse effects from those substances identified as having this potential from T1S or from earlier priority setting. The assays recommended for T2T (e.g., two-generation mammalian reproductive toxicity study, avian reproduction test, amphibian development and reproduction test) were incorporated to include the most sensitive developmental life stage and to identify and quantitate a specific hazard in a variety of species. The conclusion as to the endocrine disruptive effects of a particular substance would be based on the weight of the evidence from T1S and T2T (EDSTAC, 1998).

CONSIDERATION OF THE PHENOMENON OF ENDOCRINE DISRUPTION FROM THE MECHANISTIC PERSPECTIVE

Ligand–Estrogen Receptor Interactions

Among the intriguing issues pertaining to endocrine disruptors is the apparent "promiscuity" of the estrogen receptor (i.e., its affinity for such a diverse array of compounds). To address this issue, one must first consider the estrogen receptor signaling pathway and general aspects of the estrogen–ER interaction. The ER, a protein located within the nucleus of the cell, is a member of the nuclear hormone receptor superfamily of transcription factors (e.g., steroids, thyroid hormones, retinoids, and vitamin D derivatives) that share common structural characteristics and modes of action. In general, the interaction between ER and its ligand, estradiol, forming a ligand–receptor complex, results in "activation" of the receptor (which involves release from restraint by heat-shock protein), dimerization of ligand:ER complexes, and interaction of the dimer with specific genomic sequences or "response elements" (REs) within the regulatory region of the target gene. In the course of interacting with the RE, the ligand–ER complex recruits other proteins, transcriptional adaptors, or coregulatory proteins, which participate in the process of

transcription. The interaction of the ligand–ER complex with the RE can have stimulatory or inhibitory regulatory influences (Paige et al., 1999; Gustafsson, 2000).

The ER is a protein containing about 500 amino acids, with its ligand binding domain (LBD) comprised of about 250 amino acid residues on the C-terminus of the molecule. On the basis of x-ray crystallographic examination of ER and other members of this receptor family, the LBD of ER is predicted to have an elongated globular shape comprised of 12 linked α-helices arranged in an "antiparallel triple sandwich" topology containing the binding pocket in a hydrophobic core (Anstead et al., 1997; Brzozowski et al., 1997). Much insight regarding the ligand–ER interaction has been gained by comparing the chemical structures of estradiol with the potent nonsteroidal synthetic estrogen, DES. As shown in Figure 1, estradiol contains a 17-carbon steroid nucleus comprised of 3 hexane rings (A, B, C) and a pentane ring, (D), a methyl group (carbon 18) located between rings C and D, and 2 polar hydroxyls at either end of the steroid nucleus. One hydroxyl group is located off the A ring at carbon 3 (forming a phenolic A-ring), and the other occurs off the D ring at carbon 17 (17ß-OH). Both hydroxyls interact with the receptor via hydrogen bonding, with the 3-OH initiating binding and interacting with an amino acid in helix 3 of the LBD and the 17-OH interacting with one in helix 11. While the polar interaction with the binding pocket is relatively weak, most of the binding energy derives from the hydrophobic interactions between rings B, C, D, and about 20 amino acid residues along the binding pocket (Anstead et al., 1997). Although structurally quite distinct from estradiol, DES shares some common characteristics with the latter, primarily two lateral hydroxyl groups linked by a hydrophobic center, in this case the diethylstilbene. As a result of similarities in steric–electrostatic characteristics (e.g., polar–hydrophobic relationships and distances and three-dimensional characteristics of estradiol and DES) and flexibilities in the LBD of ER, both compounds can interact with the ER and evoke an estrogenic response. As a matter of fact, unlike estradiol, which requires a distinct orientation of the 3-OH and 17β-OH to insert itself into the binding pocket of ER, DES, by virtue of its symmetry, can insert itself into the LBD with either one of the hydroxyls interacting with the 3-OH initiating site and the other hydroxyl interacting with the other hydroxyl binding site (Duax et al., 1985; Wiese et al., 1995; Anstead et al., 1997).

Although incomplete, our general understanding of why other xenoestrogens bind ER derives from common chemical characteristics similar to those of estradiol and DES. As shown in Table 3 and Figure 1, environmental estrogens are either chlorinated or nonchlorinated and most, *but not all*, are aromatic. The aromatic compounds can contain one, two,

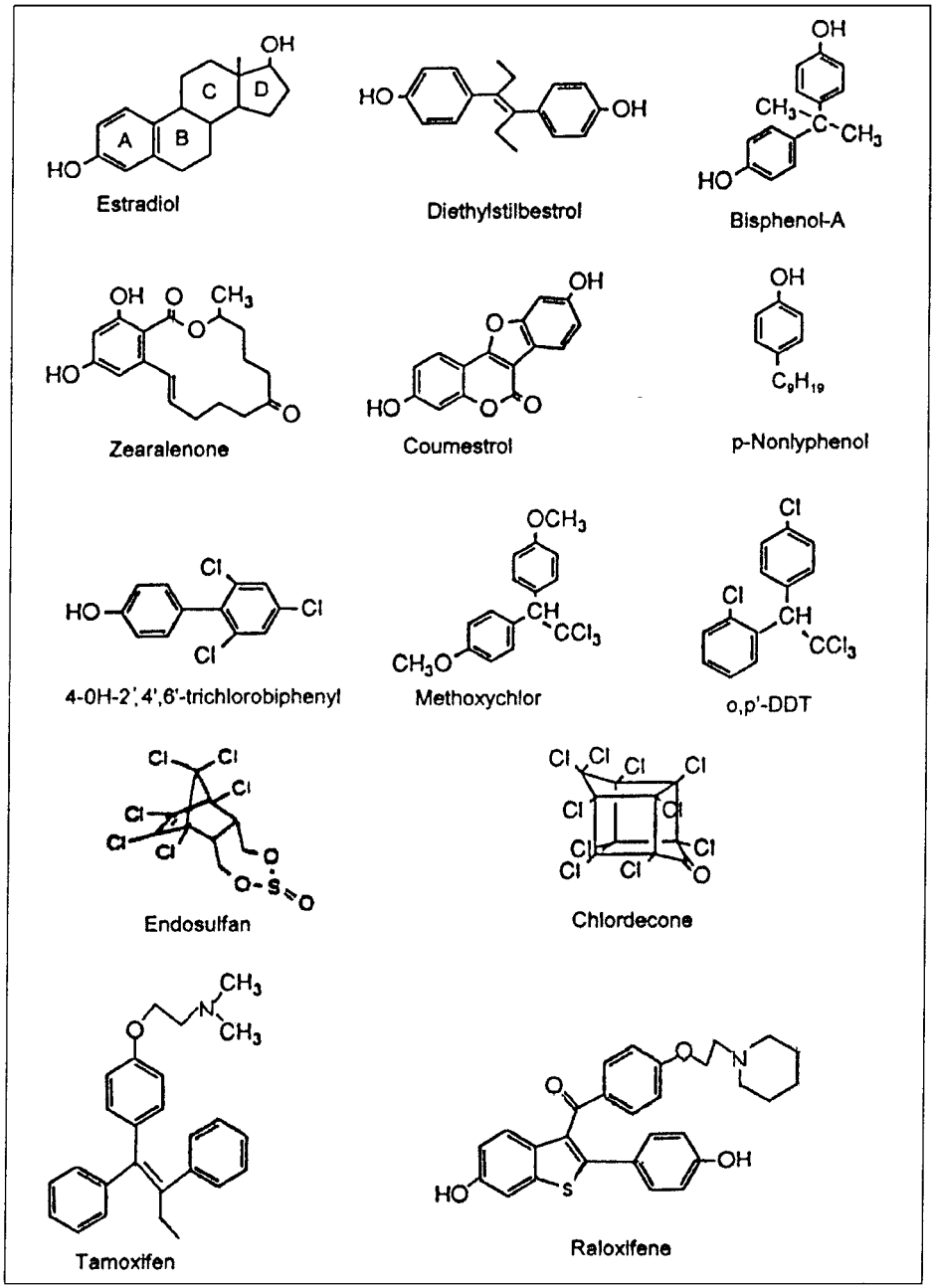

Figure 1 Chemical structures of estradiol and representative xenoestrogens, nonsteroidal compounds that bind to the estrogen receptor and exhibit estrogenic biological activity.

Table 3 Chemical diversity of nonsteroidal estrogen receptor ligands

Chlorinated
Aromatic
Phenolic: selected OH-PCBs,
Nonphenolic: selected PCBs, *o*′,*p*′-DDT, methoxychlor[a]
Nonaromatic
Organochlorine insecticides (endosulfan, Kepone, dieldrin, toxaphene)
Nonchlorinated
Aromatic
Phenolic:
Poly: DES, phenol red, bisphenol-A, phytoestrogens (flavonoids), raloxifene,[b] equol, enterolactone[c]
Mono: Zearalenone,[d] alkylphenols (*p*-nonaphenol)
Nonphenolic: Tamoxifen,[b] benzyl butyl phthalate

[a]Polychlorinated compound containing two *O*-methylated phenyl groups that can be metabolically transformed to a compound containing one or two phenols.

[b]A selective estrogen receptor modulator (SERM), an estrogen receptor ligand that can exhibit either estrogenic or antiestrogenic activity, depending upon the target cell.

[c]An isolate of human urine produced by microbial metabolism in intestine.

[d]A fungal product.

or more phenyl rings, and these may either be nonphenolic or contain one or more phenolic rings. The most common chemical motif present in xenoestrogens, and an apparent requisite of ER binding, is a ring structure. This includes the nonaromatic organochlorine pesticides (such as kepone, dieldrin, and toxaphene) (Katzenellenbogen, 1995; Soto et al., 1995; Wolff & Toniolo, 1995, Blair et al., 2000).

Most active xenoestrogens are phenolic, unhindered in the *ortho* position and usually *para* to a bulky hydrophobic structure as in the case of alkyl phenols, bisphenols, hydroxylated PCBs, or phytoestrogens (Figure 1; Table 3). One phenol is presumed to simulate the action of the 3-OH group of estradiol that initiates ER binding (hydrogen binding within helix 3). ER binding or biological activity (in cell culture) may still be demonstrable in some aromatic compounds lacking an oxygen or an hydroxyl group (e.g., *o*,*p*′-DDT, select PCBs) or when the hydroxyl group is obstructed by *O*-methylation (e.g., methoxychlor) (Figure 1). However, such activity is significantly enhanced when appropriate hydroxyl groups are formed by hydroxylation (e.g., hydroxylated PCBs, 4-hydroxytamoxifen) or are generated by demethylation [e.g., formation of 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) from methoxychlor]. While the less active nonphenolic homologue may have some ER binding activity, the more active phenolic derivatives may also be generated from their less active precursors in vivo by metabolic bioactivation (Katzenellenbogen, 1995; Soto et al., 1995; Wolff & Toniolo, 1995, Blair et al., 2000). The estrogenic activity observed in certain nonaromatic organochlorine compounds has been attributed to the fact that these

compounds contain unique structures that are functionally equivalent to phenols. For example, the cyclic sulfite present in endosulfan is polar and can function in hydrogen bonding, as can the ketone present in chlordecone (Kepone), which is converted via hydration to a gem diol (i.e., two hydroxyls emanating from a single carbon) (Guzelian, 1982; Katzenellenbogen, 1995) (Figure 1).

The modulation of hydrophobic activity can also impact estrogenicity of these xenoestrogens. For example, hydrophobicity and estrogenicity can be enhanced by chlorination (Katzenellenbogen, 1995), lengthening of alkyl side chains (up to nine carbons) in the case of alkylphenols (Tabira et al., 1999), and aliphatic branching at the bridging carbon in the case of bisphenol derivatives (Perez et al., 1998). Binding data suggest that chemicals containing two-ring structures separated by two carbons (e.g., estradiol, DES, diphenyl ethanes) have greater binding affinity to ER than two-ring structures separated by a single carbon (e.g., bisphenol A derivatives, benzophenones) or chemicals with a single ring (e.g., alkylphenols, phthalates, and parabens). This reflects the importance of the critical spatial relationship between hydrophobic and hydrogen-bonding sites as it pertains to the ligand–ER interaction (Blair et al., 2000).

Diversity of Biologic Effects of ER Ligands and the Complexity of the ER Signaling Pathway

At the present time, an examination of relative ER binding affinity among xenoestrogens is being used to develop models of the ligand ER interaction via the quantitative structure–activity relationship (QSAR) approach. These data are to be implemented into comparative molecular field analysis (COMFA) QSAR and related methods based on steric and electrostatic properties of molecules, ultimately for the purpose of predicting estrogenic endocrine disruptors in accord with the objectives of EDSTAC (Blair et al., 2000). Although the common chemical motifs among steroid and nonsteroid substances may explain their affinity for the ER, they do not necessarily explain the biological responses evoked by target tissues. It appears that the situation is far more complicated than can be explained by merely a ligand–receptor interaction, since there appears to be considerable diversity in the biological response of the organism to an ER ligand.

An example of this diversity is evident from a comparison of biologic effects by estradiol and two nonsteroidal therapeutic agents, tamoxifen, and raloxifene. Tamoxifen, a triphenylethylene derivative, and raloxifene, a benzothiophene derivative (Figure 1), are classified as selective estrogen-receptor modulators (SERMs) because they as well as

Table 4 Diversity of biological effects of ER ligands

	Target			
ER ligand	Mammary	Uterus	Blood cholesterol (LDL)	Bone
Estradiol	Mitogenic	Mitogenic	Lowering	Antiosteoporotic
Tamoxifen	Antimitogenic	Mitogenic	Lowering	Antiosteoporotic
Raloxifene	Antimitogenic	Antimitogenic	Lowering	Antiosteoporotic

Note. See text for references.

other compounds (e.g., nafoxidine and clomophine) produce either an antiestrogenic or estrogenic effect that appears to be determined by the tissue itself. This is illustrated in the following comparison of biological effects between estradiol, tamoxifen, and raloxifene (Table 4). Among the more well-known and clinically significant actions of estradiol are stimulation of epithelial cell mitogenesis in mammary gland and uterus, a lowering of blood LDL cholesterol, and inhibition of bone resorption. These four actions pertain to the appropriateness of estradiol replacement for postmenopausal symptoms. While estradiol replacement might lower the risk of postmenopausal cardiovascular disease and osteoporosis, the possibility of increased risk of breast and endometrial cancer with such treatment is an important consideration (Nabulsi et al., 1993; Black et al., 1994; Beral et al., 1999; Sullivan & Fowlkes, 1996; Guzzo, 2000).

On the other hand, tamoxifen and raloxifene exhibit biologic effect profiles that are distinctly different from estradiol as well as from one another (Table 4). Tamoxifen antagonizes estrogen action in breast and is an estrogen agonist in uterus, blood cholesterol, and bone (Assikis & Jordan, 1997; Bentram & Jordan, 1999), while raloxifene is an antiestrogen in breast *and* uterus and mimics estradiol action for the latter two endpoints (Black et al., 1994; Draper et al., 1996; Bentrem & Jordan, 1999). Although tamoxifen, by virtue of its action on the breast, has been promoted as a therapy for breast cancer, concern has been raised about its potential as a risk factor for endometrial cancer (Assikis & Jordan, 1997; Bentram & Jordan, 1999). Further diversity is seen at the molecular level. Estradiol and tamoxifen in vivo produce distinct differences in the kinetics and magnitude of induction of select mRNAs of immature rat uterus, such as c-jun, jun-b, c-myc, and c-fos (Hyder et al., 1999b).

The diversity of ER ligand effects is also evident from a recently published developmental study in the tiger salamander, *Ambystoma tigrinum* (Clark et al., 1998). Immersion of larval female animals in a sublethal solution of technical-grade DDT (containing 80% *p,p′*-DDT and 20% *o,p′*-DDT) antagonized the proliferative effect of estradiol on

Müllerian epithelial cells. In other words, DDT, a substance that usually exhibits estrogenic activity in mammalian assay systems, is antiestrogenic in this species. This latter observation may be of particular significance for the endocrine disruptor issue if, as has been suggested, animal species serve as the sentinels for the evaluation of potential humans hazards of hormonally active substances among other types of environmental pollutants (van der Schalie et al., 1999).

Recent findings pertaining to the complexity of the ER signaling pathway in the target cell appear to add insight regarding the diversity of effects exhibited above by ER ligands. A second isoform of the ER, derived from a distinct gene, was isolated from rat prostate in 1996, leading to the renaming of the original protein as ERα and the new isoform as ERβ. Although the DNA binding domains of ERα and ERβ are very homologous (95%), the ligand binding domain (LBD) in the C-terminus exhibits partial (50–60%) homology. As suggested by the partial homology of the LBD, steroidal estrogens, as well as some xenoestrogens, may exhibit similar affinities and structure–function relationships with ERα and ERβ. On the other hand, several xenoestrogens (e.g., coumestrol, genistein, bisphenol-A, and methoxychlor) preferentially bind to ERβ (Chang & Prins, 1999; Kuiper et al., 1997, 1998).

ERα and ERβ also exhibit different developmental patterns and functionalities, as demonstrated by localization studies (Fisher et al., 1997) and studies in the knockout mouse (Ogawa et al., 1998). Furthermore, ERα and ERβ appear to exhibit distinct tissue distributions. For example, ERα mRNA expression predominates in the rat epididymis, testis, pituitary, kidney, and adrenal, and that of ERβ predominates in prostate, bladder, and lung, whereas rat ovary and uterus express both messages (Kuiper et al., 1997). The two ER isoforms also localize to different structures within the same organ. In the ovary, for example, ERα localizes to the theca and interstitium, whereas ERβ is found in the granulosa cells (Sar & Welsch, 1999; Shughrue et al., 1998). A similar situation exists in the testes, where ERα is found in the Leydig cells and ERβ is detected in the Sertoli cells, spermatogonia, spermatocytes, and spermatids (Fisher et al., 1997; Saunders et al., 1998; van Pelt et al., 1999). Finally, the presence of α and β isoforms of ER in the same cell suggests that the binding of activated ERs to the genomic response element could involve heterodimerization as well as homodimerization of α and β isoforms of ER. This appears to be the case (Chang & Prins, 1999).

In addition to ER heterogeneity, numerous estrogen response (or enhancer) elements (EREs) have been identified. The original was a consensus ERE, a 13-mer pallindromic sequence (GGTCAnnnTGACC) located in the 5′-flanking region of the vitellogenin gene (of *Xenopus*

and chicken) that bound the ER homeodimer. Other EREs have also been identified (e.g., ovalbumin, calbindin, prolactin, LHβ, creatine kinase, uteroglobin, MCF-7 pS2, oxytocin, progesterone receptor, c-fos, c-jun, lactoferrin, cathepsin D, and choline acetyl transferase) (Stancel et al., 1995; Hyder et al., 1999a). These other EREs usually contain a pallindromic half-site that is identical to the consensus ERE and a second half-site containing one to three nucleotide modifications in the pallindromic sequence. EREs are regarded as estrogen receptor receptors (ERRs), and individual ERRs exhibit distinct ER affinities. As a reflection of this heterogeneity, different estrogen-induced biological responses including specific genes (e.g., organ weight, glucose oxidation, creatinine kinase, VEGF, and c-fos) exhibit distinct dose-response and time-course profiles (Stancel et al., 1995; Hyder et al., 1999a, 1999b).

In addition to the conventional EREs, there are other means by which ERs interact with the genome to evoke transcription. ERs can also complex with other transcription factors (e.g., Sp1, Fos, and Jun) and bind to the genomic response elements (e.g., Sp 1 and AP1) in an indirect fashion associated with these other proteins instead of the direct binding to conventional EREs (Paech et al., 1997; Safe, 1999; Gustafsson, 2000; Saville et al., 2000). The coregulatory proteins that bind to ER–genomic complexes and influence transcription in a stimulatory (coactivators) and inhibitory (corepressors) mode are also heterogeneous. Among the coactivator proteins found to associate with ER are SRC (steroid receptor coactivator)-1, RIP (receptor interacting protein) 140, and CPB (CAMP response element binding protein) and ERAP (estrogen receptor associating protein) 140 (Cavailles et al., 1995; Nguyen et al., 1999; Shibata et al., 1997), whereas one such corepressor is NCoR (nuclear receptor corepressor) (Chien et al., 1999).

Finally, recent observations indicate that the conformation of ER is altered by the ligand–ER interaction. Each ligand (even closely related ones) produces distinct receptor conformational alterations. In addition, conformation changes occurring in ERα and ERβ are distinct for a given ligand. Conformation of ER determines the site where the coregulatory protein binds to it. Hence, conformation of ER determines the nature of the ligand-induced biological response (Norris et al., 1999; Paige et al., 1999; Wijayaratne et al., 1999). Furthermore, orientation of the ligand itself within the binding pocket influences the conformation of the LBD and, in so doing, may determine whether or not a coregulatory protein binds to the receptor (Brzozowski et al., 1997; Shiau et al., 1998). On the basis of the foregoing heterogeneity of ER isoforms, response elements, coregulatory adaptor and repressor proteins, and the influence of ligand on ER structure and affinity for coregulatory proteins, it would appear that biological responses to a naturally occurring estrogen or

Table 5 Effect of ER isoforms and/or DNA enhancer (response) elements of the nature of the biologic response to an ER ligand

Ligand	Receptor	DNA enhancer element	Response
With transfected He La cells (Paech et al., 1997)			
Estradiol	ERα	ERE	Agonist
Raloxifene	ERα	ERE	Antagonist for estradiol
Estradiol	ERβ	ERE	Agonist
Raloxifene	ERβ	ERE	Antagonist for estradiol
Estradiol	ERα	AP1	Agonist
Raloxifene	ERα	AP1	Agonist (weak), antagonist for estradiol
Estradiol	ERβ	AP1	Antagonist for raloxifene
Raloxifene	ERβ	AP1	Agonist
With transmitted human HepG2 cells (Gaido et al., 1999)			
Estradiol	ERα	ERE	Agonist
HPTE	ERα	ERE	Agonist
Estradiol	ERβ	ERE	Agonist
HPTE	ERβ	ERE	Antagonist for estradiol

xenoestrogen are a function of the ligand itself and the tissue context of the ER signaling pathway.

The influence of ER signaling pathway constituents is illustrated by a recent report by Paech et al. (1997). Using HeLa cells transfected with ERα or ERβ, as well as distinct DNA response (or enhancer) elements and a reporter gene, these investigators showed that for a given ligand (e.g., estradiol and raloxifene) the type of response evoked (agonist or antagonist) was a function of the receptor isoform and enhancer element. These data are summarized in Table 5. In the presence of one type of ERE (consensus ERE), estradiol exhibited the expected agonist response and raloxifene exhibited the antiestrogen response regardless of ER isoform. When the consensus ERE was replaced by another DNA response element (e.g., AP1) and the ERα isoform was present, estradiol behaved as an agonist, and raloxifene exhibited weak agonist activity alone as well as antagonist activity in the presence of estradiol, similar but not identical to that seen with consensus ERE. In the presence of ERβ and AP1, the situation was *reversed* from that seen with ERα or ERβ and consensus ERE. Raloxifene exhibited agonist activity, whereas estradiol behaved as the antagonist of raloxifene action. In other words, the presence of another enhancer element (AP1 instead of consensus ERE) completely reversed the ER response profile to individual ligands.

A similar situation was demonstrated by Gaido et al. (1999). In this study, the influence of ER isoform was examined on the biological response profile of estradiol and HPTE, the demethylated metabolite of methoxychlor. Using human HepG2 cells transfected with ERE,

a reporter gene, and either ERα or ERβ isoforms, these investigators showed that with transfected ERα both estradiol and HPTE exhibited agonist activity, with estradiol being about one order of magnitude more potent than HPTE. When ERα was replaced by ERβ, estradiol retained its agonist activity, but now HPTE behaved like an estrogen antagonist, inhibiting the action of estradiol (Table 5).

Plasma Binding and Uptake of Xenoestrogens by Tissues

Although the employment of the E-screen or similar in vitro assays has been justified on the basis of costs and ethical concerns, some shortcomings of this approach relative to in vivo assays are noteworthy. Intact cell in vitro assays are only relevant in terms of the interaction of a substance in question with the target cell. While local metabolic transformations (i.e., bioactivation) may occur under these conditions, such conditions fail to address such considerations relevant to the in vivo (i.e., whole animal) situation, namely, biological half-life of the substance, most metabolic conversions, interactions with endogenous hormones, or the delivery of hormones to the target tissue from the extracellular fluid.

The delivery of estradiol to the target cell is influenced by the presence of plasma proteins that bind the steroid noncovalently and reversibly. While the nature and control of estradiol binding in plasma have been under examination for some time, the role of plasma proteins on the uptake of xenoestrogens has been examined only in a limited fashion. This is further complicated by the fact that an apparent species specificity exists with regard to estradiol binding in plasma. Estradiol is bound primarily in rodents by α-fetoprotein and in humans primarily by sex hormone binding globulin (Nagel et al., 1998). Similar species variations could exist for xenoestrogens. An assay has been developed using the uptake of [^{3}H] estradiol by cultured MCF-7 cells to quantitate the effect of plasma proteins on the uptake of hormone by target cells. By comparing the tissue uptakes of [^{3}H] estradiol by these cells (and, hence, the apparent K_d values) in the presence or absence of 100% adult male human serum, one can estimate the relative ratio of unbound to bound estradiol in plasma. This method indicates that about 3.5% of serum estradiol was unbound in human serum, consistent with estimates determined by other methods (Nagel et al., 1998).

A modification of this method has been developed to determine the influence of serum on the uptake of xenoestrogens by the target cell, the relative binding affinity–serum modified access (RBA-SMA) assay. The RBA-SMA assay is based upon a comparison of the ability of varying concentrations of the xenoestrogen with that of unlabeled estradiol to displace [^{3}H]estradiol from estrogen receptors of cultured MCF-7 cells

in the presence and absence of 100% human serum. The SMA of a xenoestrogen is calculated from the ratio of the RBA (relative to estradiol) in serum to that in serum free medium. From this assay one can determine whether a xenoestrogen behaves in serum like estradiol (SMA = 1), whether a xenoestrogen is less tightly bound by serum proteins than estradiol (SMA > 1), or whether a xenoestrogen is more tightly bound by serum protein than estradiol (SMA < 1). An SMA >1 would suggest that the xenoestrogen has greater access to the target cell, and hence greater potential biopotency in vivo than that predicted by the standard (i.e., serum-free) E-screen assay, whereas an SMA <1 would suggest that a xenoestrogen is more tightly bound by serum than estradiol and has reduced access to the target cell (hence, less potential biopotency in vivo) than that predicted by the E-screen (Nagel et al., 1998).

While the data are limited, the published observations are very provocative. These assays indicate that four phytoestrogens (daidzein, coumestrol, equol, and genistein) have elevated SMAs (ranging from 3.5 to 12), whereas another of this class, biochanin A, has reduced access to the cell (SMA = 0.4). The results were variable for four synthetic estrogens. DES and moxestrol are less restrained than estradiol (SMAs of about 6), whereas denestrol and hexestrol are more tightly bound by plasma (SMAs about 0.1). Variability was also the case for the SERMs, with raloxifene having an SMA of 1 and tamoxifen an SMA of 0.1 (Nagel et al., 1998). Another report revealed that bisphenol A has an SMA of about 1.7, whereas those of octylphenol and nonylphenol were 0.04 and 0.15, respectively (Nagel et al., 1997).

Chirality

The fact that enantiomers (or mirror images) of chiral (or asymmetric) molecules are stereochemically distinct from one another, and hence may differ biologically, has been recognized in the field of pharmacology for some time. However, the significance of chirality to environmental toxicology has been largely overlooked until recently, although many potential endocrine disruptors (e.g., PCBs, phthalates, and DDT derivatives) are chiral. A recent report addressed the fact that soil microbes may exhibit selectivity in their ability to degrade one or the other of a pair of enantiomers. Thus the existence of a particular enantiomer can be influenced by environmental conditions. Field studies and laboratory studies have demonstrated that such "enantioselectivity" and hence potential toxicity of a substance in the soil can be influenced by the degree of forestation (e.g., forest vs. pasture), condition of the soil (e.g., enrichment of soil with organic nutrients), or temperature (warming) (Lewis et al., 1999).

The importance of chirality to estrogen receptor binding, and hence its potential significance to endocrine disruptors, has also been demonstrated. For example, the *S*-enantiomer of the stilbestrol estrogen, indenestrol A (IA-*S*), is more estrogenic than its *R*-enantiomer (IA-*R*), and IA-*S* exhibits about a 15-fold greater affinity for the mouse ER than IA-*R* (Kohno et al., 1996). *cis*-Diethyltetrahydrochrysene (THC), a molecule containing four hexane rings, with the outer two being phenolic containing *p*-hydroxyl at either end, is an experimental ER ligand. A racemate of THC (a 1:1 mixture of both *R*,*R*-THC and *S*,*S*-THC) preferentially binds to ERβ with fourfold greater affinity than ERα. In the presence of ERα, racemic THC is agonistic, and with ERβ it exhibits antagonistic activity. This complex profile of receptor and biologic specificity of this substance appears to be explained entirely on the basis of chirality. The antagonistic activity is due to *R*,*R*-THC interaction with ERβ. *R*,*R*-THC exhibits agonist activity in the presence of ERα. *S*,*S*-THC is agonistic with both ERα and ERβ, with much lower affinity (about 1/20) for ERβ than *R*,*R*-THC (Sun et al., 1999).

Antiestrogenic Activity of Aryl Hydrocarbon Ligands

PCBs can exhibit estrogenic activity or estrogen antagonistic activity, depending upon distinct chemical characteristics that exist in this family of compounds. The chemical characteristics associated with estrogenicity of PCBs are (1) a nonhalogenated phenyl ring containing an OH ($p > m > o$); (2) optimal polyhalogenation of the remaining phenyl ring; and (3) noncoplanarity of the phenyl rings (Wolff & Toniolo, 1995). Optimal halogenation is exemplified by the fact that the most potent estrogenic PCB was 4-hydroxy-2′,4′,6′-trichlorobiphenyl (Figure 1), as measured by ER binding activity (Korach et al., 1988) and E-screen (Soto et al., 1995). Noncoplanarity of this compound is achieved by the presence of *ortho* chlorines (at positions 2 and 6) on one of the rings. Modification of PCBs by polyhalogenation (particularly on both phenyls) and coplanarity impart upon PCBs an affinity for the aryl hydrocarbon receptor (AhR) and assumption of biologic actions characteristic of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), among these the induction of cytochrome P450 (CYP1A family) isozymes and the inhibition of a variety of estrogen-inducible actions (Wolff & Toniolo, 1995). This antiestrogenicity, however, is different from those associated with competitive ligand binding to the ER (as in the case of SERMs). Antiestrogenicity associated with dioxinlike activity involves cross-talk between the AhR signaling pathway and the ER signaling pathway. Transactivation initiated by dioxin-like compounds involves the formation of a ligand–heterodimeric complex (AhR plus another protein, AhR nuclear

locator, ARNT). This heterodimeric complex then binds to a genomic site, the dioxin response element (DRE). Inhibitory DREs have been located within the promoter region of certain estrogen-responsive genes (e.g., cathepsin D, p52, c-fos). Under these conditions, the association between a ligand–AhR–ARNT heterodimeric complex and DRE disrupts the interaction of the estrogen–ER complex with its ERE, thus inhibiting estrogenic action (Safe et al., 1998; Safe, 1999).

Antiandrogenic Activity of Certain Xenoestrogens

Other studies suggest an alternate mechanism of action for some xenoestrogens, an antiandrogenic effect. In the developing male mammal estrogen exposure and antagonism of endogenous androgen action in utero produce similar effects in offspring (Sharpe, 1995). Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4,-dione], a commercially available fungicide, appears to be such an androgen antagonist. Exposure of pregnant rats to this substance produces male offspring with feminine characteristics, such as reduced anogenital distance, nipples, and cleft phallus with hypospadias. Two hydrolytic products of vinclozolin, 2-([(3,5-dichlorophenyl)-carbamoyl]oxy)-2-methyl-3-butenoic acid (M1) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), which are found in body fluids of exposed animals, were strong competitive inhibitors for radioactive ligand binding to the androgen receptor (AR), while vincolozolin was a relatively weak inhibitor of AR binding (K_i for M1 and M2 was 92 and 9.7 μM, respectively, whereas that of vinclozolin was >700 μM). None of these compounds inhibited the activity of 5α-reductase, another antiandrogenic mechanism. The observations suggest that the demasculinizing effects of vinclozolin exposure are attributable to the competitive AR antagonism of one or both of the hydrolytic products of vinclozolin, M2 and/or M1 (Kelce et al., 1995a).

A follow-up study revealed similar antiandrogenic activity in estradiol and some xenoestrogens, namely, DES, and certain DDT congeners, namely, *p,p′*-DDT, *p,p′*-DDE, and *o,p′*-DDT. Although these substances exhibited little or no 5α-reductase inhibition, they exhibited significant competitive antagonism to AR androgen binding. In fact, the DDT congeners exhibited greater AR binding activity than ER binding activity (Kelce et al., 1995b). Danzo (1997) also reported that *p,p′*-DDE and *p,p′*-DDT exhibited much more binding to AR than to ER, although estradiol did not exhibit AR binding activity. The suggested antiandrogenic activity of *p,p′*-DDE (the most active AR receptor antagonist) was verified in vivo. Feeding of pregnant rats with this substance produced male offspring with decreased anogenital distances and nipples (Kelce et al., 1995b). Feeding of this substance to adult males for 4 days produced

significant decreases in seminal vesicular and prostatic weights without differences in serum testosterone (Kelce et al., 1995b). It will be recalled that the feminization of alligators in Lake Apopka, FL, discussed earlier was attributed to the presumed estrogenic activity of *p,p′*-DDE (Table 2).

CONCLUDING COMMENTS

Two advisory bodies, the American Council on Science and Health (1999) and the National Research Council (1999), have examined the available literature concerning endocrine disruptors, and both have arrived at essentially the same general conclusion. They acknowledge that environmental substances exhibit hormonal activity and under extreme exposures, such as toxic spills and laboratory experiments, can evoke endocrine disruptive effects. However, at present, they indicate that the epidemiologic data do not support a consistent link between environmental pollutants and endocrine-disruptive effects in humans. The National Research Council noted that much is to be learned about the reproductive and developmental effects of hormonally active agents and recommended that more research be conducted.

The current analysis explored the issue of endocrine disruption of estrogenic substances from a mechanistic perspective. Many substances can interact with the estrogen receptor, albeit weakly, because of characteristics they share with two potent estrogenic substances, estradiol and diethylstilbestrol. Primarily, these features are a ring structure (preferably aromatic and an unencumbered hydroxyl group) along with a hydrophobic center capable of interacting with the core of the binding domain of the estrogen receptor. However, this affinity for the receptor does not explain the nature of the biological response. As exemplified by a diversity of systemic biological effects exhibited by estradiol, tamoxifen, and raloxifine, and by the antiestrogenic effect of DDT in the tiger salamander, different ER ligands give different profiles of biological response that appear to be influenced by tissue and species. These distinct profiles are dictated by such factors as the ligand itself, the ER isoform, the genomic site of ER binding, and assembly of coregulatory proteins associated with ER. Among the other factors that influence the nature of endocrine disruption are the role of plasma binding on the delivery of environmental agents to the tissue, chirality of these agents, cross-talk between signaling systems (e.g., between AhR and ER), and alternate mechanisms (e.g., antiandrogen effects). No doubt this is not a complete list of factors, since other aspects of ER were not addressed, such as the involvement of specific activation domains (AF-1 and AF-2) of the receptor in xenoestrogen action (Chang & Prins, 1999; Gustafsson, 2000).

As illustrated by the estrogen signaling pathway, hormone–tissue interactions are extremely complex, and much more about these complexities is to be learned. In view of the uncertainities inherent in the mechanisms associated with endocrine disruptive effects, it would appear that the aggressive approach to screen and test environmental chemicals developed and implemented by EDSTAC is inappropriate at this time. It would seem that a more productive use of resources would be to obtain greater insights about the mechanisms of hormonal and potentially endocrine-disruptive effects of these chemicals.

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