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Monograph

Ten Years of Mixing Cocktails: A Review of Combination Effects of Endocrine-Disrupting Chemicals

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Abstract

In the last 10 years, good evidence has become available to show that the combined effects of endocrine disruptors (EDs) belonging to the same category (e.g., estrogenic, antiandrogenic, or thyroid-disrupting agents) can be predicted by using dose addition. This is true for a variety of end points representing a wide range of organizational levels and biological complexity. Combinations of EDs are able to produce significant effect, even when each chemical is present at low doses that individually do not induce observable effects. However, comparatively little is known about mixtures composed of chemicals from different classes of EDs. Nevertheless, I argue that the accumulated evidence seriously undermines continuation with the customary chemical-by-chemical approach to risk assessment for EDs. Instead, we should seriously consider group-wise regulation of classes of EDs. Great care should be taken to define such classes by using suitable similarity criteria. Criteria should focus on common effects, rather than common mechanisms. In this review I also highlight research needs and identify the lack of information about exposure scenarios as a knowledge gap that seriously hampers progress with ED risk assessment. Future research should focus on investigating the effects of combinations of EDs from different categories, with considerable emphasis on elucidating mechanisms. This strategy may lead to better-defined criteria for grouping EDs for regulatory purposes. Also, steps should be taken to develop dedicated mixtures exposure assessment for EDs.

Keywords: combination effect, combined exposure, endocrine-disrupting chemical, mixture, review

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The topic of combined exposures to endocrine disruptors (EDs) has long been regarded as important, not least because of the continuing discovery of ever new chemicals with endocrine-disrupting potential and the realization that exposure is to a multitude of chemicals simultaneously, not to single agents. Yet, Yang's observation (Yang 1994) that > 95% of the resources in toxicologic research are devoted to the study of single chemicals, with an almost complete neglect of mixture studies, also applies to ED research. A contributing factor to this imbalance is no doubt the inaccessibility of theoretical concepts in mixture toxicology and the resulting uncertainty as to how to proceed experimentally. To complicate matters further, the early work on mixtures of EDs was motivated by a systematic search for synergisms. In 1996, a report claiming spectacular synergisms between binary mixtures of estrogenic pesticides was published (Arnold et al. 1996), but had to be withdrawn because the experimental results could not be reproduced by other laboratories (Ashby et al. 1997; Ramamoorthy et al. 1997). This episode has led many to question the overall importance of combination effects of EDs. In addition, mixture studies are perceived to be challenging, both conceptually and experimentally—concerns that have led the U.S. Environmental Protection Agency (EPA) Science Advisory Board (SAB) and Science Advisory Panel to recommend a delay in the screening and testing of mixtures for hormonal potential until the feasibility of such approaches could be assessed with the benefit of data on individual chemicals (SAB 1999).

In spite of these difficulties, perceived or real, many articles on combination effects of EDs have been published in the last 10 years, and it is timely to assess what progress has been made. A review of the evidence is also motivated by the fact that certain legislative and regulatory frameworks in some countries mandate consideration of groups of chemicals that act via the same mechanism, rather than evaluating the potential risks on an individual basis. Over 20 years ago, this risk assessment approach found entry into the regulation of polyhalogenated dioxins and furans, where the application of the toxic equivalency factor/toxic equivalences (TEF/TEQ) concept is now common practice (van den Berg et al. 1998). Is there sufficient evidence about combination effects of EDs to call for similar cumulative risk assessment approaches? Where are knowledge gaps, and what are conceptual difficulties? A review of the earlier work on ED mixtures, leading up to 1997, has been published (Kortenkamp and Altenburger 1998). In the present article, I concentrate on studies that appeared after 1997 in the peer-reviewed literature.

In studying ED mixtures, many researchers have followed what has been called a "whole mixture approach" (U.S. EPA 1986), in which a combination of many chemicals is investigated as if it were a single agent, but the individual effects of all the components are not assessed. This type of experiment is useful for studying complex mixtures, or on a case-by-case basis, but leads to difficulties in extrapolating from one mixture to the other because small variations in composition may lead to significant changes in its toxic effects. Furthermore, whole mixture approaches do not answer whether chemicals act in an additive, antagonistic, or synergistic fashion. However, one of the major difficulties in assessing EDs is uncertainty about their potential to act together in an additive or synergistic manner (Daston et al. 2003). To address these concerns, I focus on studies that have assessed ED mixtures in terms of additivity, antagonism, or synergy. Typically, such studies attempt to predict additive combination effects on the basis of information about the effects of all components in the mixture. Not all types of mixtures lend themselves to this approach; for example, one chemical, which by itself does not induce the effect of interest, can modify the responses provoked by a second agent. In these cases, the resulting combination effect is difficult to predict from knowledge about the effect profile of the individual components. Often, however, all mixture components themselves induce the effect of interest; in these cases, it may be possible to anticipate the resulting joint effect by making assumptions about expected additivity.

The use of the term "additivity" in mixture toxicology still causes much confusion, partly because it is not always synonymous with additivity in the mathematical sense. In toxicology, mixture "additivity" describes the case in which chemicals "act together" to produce effects without enhancing or diminishing each other's action. There are various models for dealing with this kind of additivity, and the choice of a "correct" one is of

great importance, because it is in relation to these additivity expectations that combination effects are evaluated in terms of synergisms (effects greater than additive) or antagonisms (effects falling short of additivity). Choosing an inappropriate additivity expectation as a point of reference may result in combination effects being erroneously determined as additive, synergistic, or antagonistic. Thus, before reviewing endocrine mixtures, a brief introduction into concepts for calculating mixture additivity is in order. An in-depth discussion of this topic is beyond the scope of this review, but additional information has been published by Berenbaum (1981, 1989) and Greco et al. (1995).

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What Is Additivity?

It is often said that the effects of a combination of chemicals may be smaller or larger than the sum of the individual effects of all components. Without further justification, this is frequently taken to mean that the anticipated combination effect is accessible by calculating the simple arithmetic sum of the individual effects of all chemicals. The fallacy of this expectation has been discussed elsewhere (Berenbaum 1981; Kortenkamp and Altenburger 1998), but it becomes obvious when we consider, for example, 10 agents, each of which provokes 15% of a certain response. The anticipation that the resulting joint effect should be 10 × 15% = 150% turns out to be biologically impossible if the maximally inducible effect can only be 100%.

Thus, approaches are required that provide more reliable calculations of mixture effects, such that a reference point for the assessment of combination effects in terms of synergisms, additivity, and antagonism can be defined. For this purpose, two concepts are available: dose addition (often referred to as concentration addition) and independent action. These concepts are used depending on the presumed modes of action of the mixture components.

Dose addition is applied to mixtures of chemicals that exert their effects through similar modes of action. Examples include organophosphorus pesticides and polychlorinated dioxins (PCDDs) and polychlorinated furans (PCDFs). Because these chemicals interact with well-defined molecular targets, it is thought that one chemical can be replaced totally or in part by an equal fraction of an equieffective concentration of another without diminishing the overall combined effect (Loewe and Muischnek 1926). A widely used application of dose addition is the TEF/TEQ concept for the assessment of mixtures of PCDDs and PCDFs (Safe 1998; van den Berg et al. 1998). A great deal of the work on ED mixtures has utilized dose addition or concentration addition as the concept for calculating additivity expectations. Considering that the majority of mixture studies have been based on end points relatively close to the steps following hormone-receptor binding and activation, the choice of dose addition appears to be well-founded.

Independent action (also called “response addition”) is used for combinations of agents with diverse modes of action. By activating differing effector chains, every component of a mixture of dissimilarly acting chemicals is thought to provoke effects independent of all other agents that might also be present. The resulting combined effect can be calculated from the effects caused by the individual mixture components by adopting the statistical concept of independent events (Bliss 1939). Independent action (often confusingly also referred to as “response addition” or “effect multiplication”) has been used rarely for mixtures of EDs.

Both dose (or concentration) addition and independent action are able to account for saturation effects at higher effect doses and will not produce paradoxical predictions of supra-maximal combination effects such as in the example described above with 10 compounds, each inducing a 15% effect.

In this article, I will discuss work with the three most frequently studied hormone receptors: the estrogen receptor, the androgen receptor (AR), and the thyroid receptor. There is a rich literature concerning the aryl hydrocarbon receptor (AhR), which has been reviewed elsewhere (van den Berg et al. 1998) and will consequently not be dealt with here, but interactions between AhR agonists and other EDs will be considered.

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Mixtures of Estrogenic Chemicals

Estrogenic chemicals have been the focus of most of the work on EDs, and it is not surprising that this group of substances has been the topic of the majority of ED mixture studies. Although the earlier efforts mainly employed binary mixtures (reviewed by Kortenkamp and Altenburger 1998), work carried out since 1998 has made significant contributions to the analysis of multicomponent mixtures containing 3, often 5, and up to 12 estrogenic chemicals. “Estrogenicity” can be defined in various ways. At the functional, physiologic level, the term denotes the ability of a chemical to evoke responses similar to those of 17β-estradiol (E_2), such as cornification of the vaginal epithelium and uterine cell proliferation. Of toxicologic concern is the role of estrogens in breast and ovarian cancer, and E_2 and synthetic estrogens are recognized human carcinogens. Advances in our understanding of the mode of action of estrogens have led to further definitions that refer to specific steps at various molecular levels. These definitions provide a way to structure the evidence on estrogen mixtures; thus, ”estrogenicity” can mean affinity to the estrogen receptor (ER-α or ER-β) (although this does not distinguish agonists from antagonists), the ability to activate expression of estrogen-dependent genes, or stimulation of cell proliferation of ER-competent cells. At present, no post-1998 multicomponent study with ER binding as the end point is available.

ER activation

Payne et al. (2000) studied combinations of two, three, and four estrogenic chemicals in the yeast estrogen screen, an ER-α–based gene reporter system. Individual dose–response curves for *o,p′*-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol were recorded, and this information was used to successfully predict the joint effects of *o,p′*-DDT, genistein, 4-nonylphenol, and 4-*n*-octylphenol for mixtures with a fixed ratio. Rajapakse et al. (2002) and Silva et al. (2002) extended this approach to the analysis of mixtures involving 8 and 12 estrogenic agents, respectively. In both cases, the mixture responses observed using the yeast estrogen screen agreed excellently with the effects predicted using concentration addition. In an attempt to verify the assumption that concentration addition is an appropriate model for estrogen mixtures, the authors also compared the observed mixture effects with additivity predictions calculated using independent action. In the study by Payne et al. (2000), both concepts produced very similar predictions. However, Silva et al. (2002) and Rajapakse et al. (2002) found that independent action underestimated the observed mixture effects by a large margin.

Examinations of the effects of ternary mixtures of estrogenic chemicals in an ER-α gene reporter system based on MCF7 cells were carried out by Charles et al. (2002a). All mixtures were examined in a factorial design involving 64 treatment groups and response surfaces constructed. Combinations of E_2, 17 α-ethinylestradiol (EE_2), and diethylstilbestrol (DES) showed concentration additive effects when all components were present at levels that fell within the linear range of their individual dose–response curves. At higher concentrations, however, the combined effect of the three estrogens fell short of expected additivity, a phenomenon that Charles et al. (2002a) attributed to saturation effects. In a second paper, Charles et al. (2002b) investigated ternary combinations of additional estrogenic chemicals. Although combinations of benzo[*a*]pyrene, 1,2-benzanthracene, and chrysene and of methoxychlor, *o,p′*-DDT, and dieldrin showed concentration additivity over a wide range of mixture ratios, the joint effects of E_2, genistein, and *o,p′*-DDT were antagonistic, both in the low and the high concentration ranges.

Heneweer et al. (2005) monitored the activation of ER-α by measuring expression of the *TFF1* gene (trefoil factor 1; coding for the pS2 protein) to study the effects of combinations of estrogenic ultraviolet filter substances. Binary mixtures of 2-hydroxy-4-methoxybenzo-phenone and its metabolite 2,4-dihydroxy-benzophenone showed concentration additive effects, as did a combination of these two chemicals with octyl methoxycinnamate and 3-(4-methylbenzylidene) camphor. In a TEQ approach, Heneweer et al. (2005) expressed effect concentrations of the test chemicals in terms of E_2 equivalents. Le Page et al. (2006) developed a reporter gene assay based on glial cells (U251-MG) transfected with three zebrafish ER subtypes and the brain aromatase promoter linked to luciferase. This system was used to study a mixture of E_2, EE_2, estrone, genistein, and α-zeralenol, with effects well in agreement with concentration addition.

Cell proliferation

Payne et al. (2001) found the effects of *o,p′*-DDT, *p,p′*-DDT, *p,p′*-DDE, and β-hexachlorocyclohexane on the proliferation of estrogen-dependent MCF7 cells (E-SCREEN assay) to be concentration additive at two different mixture ratios, but the observed resposes were equally well predicted by independent action. Suzuki et al. (2001) tested binary mixtures of natural and synthetic estrogenic chemicals, including E_2, estrone, bisphenol A, butyl benzylphthalate, endosulfan, methoxychlor, and pentachlorophenol, for proliferative effects in MCF7 cells. Using an effect-multiplication method to construct contour plots, the authors observed

apparent synergisms with E_2 and bisphenol A, whereas the remaining eight binary combinations gave additive, antagonistic, or weakly synergistic effects. However, the interpretation of these results is complicated by the fact that Suzuki et al. (2001) calculated additivity expectations by multiplication of unscaled effect measures, a method inconsistent with independent action. Rajapakse et al. (2004) analyzed mixtures containing E_2, EE_2, genistein, bisphenol A, 4-nonylphenol, and 4-*tert*-octylphenol in the E-SCREEN assay. A small deviation from concentration additivity was observed. Interestingly, the omission of genistein produced an even more pronounced antagonism. However, a three-component mixture composed of E_2, EE_2, and genistein produced excellent agreement with predicted concentration additivity, and the same was observed for a four-component mixture containing E_2, EE_2, genistein, and bisphenol A. The presence of 4-nonylphenol and 4-*tert*-octylphenol appeared to be associated with the observed antagonisms (Rajapakse et al. 2004). It is conceivable that differential activation of metabolizing enzymes (e.g., cytochrome P450) or efflux pumps by mixture components can lead to removal of other constituents, but this hypothesis awaits experimental confirmation.

Vitellogenin induction in fish

In fish, the induction of vitellogenin is controlled by ER-α, and this response can be used to monitor exposure to estrogenic chemicals in juvenile or male fish. Thorpe et al. (2001) were the first to exploit this end point to study the effects of binary mixtures of estrogenic chemicals on juvenile rainbow trout (*Oncorhynchus mykiss*). Over a large range of response levels, a binary mixture of E_2 and 4-nonylphenol followed the effects predicted by concentration addition, but a mixture of E_2 and the pesticide methoxychlor was less than additive. A binary mixture of E_2 and EE_2 also produced concentration additive effects (Thorpe et al. 2003). In the largest investigation of this kind so far, Brian et al. (2005) recorded concentration–response relationships for E_2, EE_2, bisphenol A, 4-nonylphenol, and 4-*tert*-octylphenol for vitellogenin induction in fathead minnows (*Pimephales promelas*) and used this information to predict the responses to a mixture of all five chemicals. This study was truly predictive because the combination effect predictions had to rely on single chemical effect data recorded more than a year before commencement of the mixture experiment. The observed effects agreed excellently with the concentration addition expectation (Brian et al. 2005).

Xie et al. (2005) used the juvenile trout vitellogenin assay to evaluate mixtures of the pesticides 2,4-dichlorophenoxyacetic acid (2,4-D), triclopyr, diquat dibromide, and plyphosate with two alkyl-phenol ethoxylate–containing surfactants [R11 (Wilbur-Ellis Co., San Francisco, CA, USA) and Target Prospreader Activator (TPA; Target Specialty Products Fresno, CA, USA)]. Of all pesticides, only 2,4-D and triclopyr caused enhancements in vitellogenin levels when given individually, and R11 and TPA were also effective. Using a TEQ approach, the additivity expectations were derived by addition of E_2 equivalents. Binary combinations of 2,4-D with R11 or with TPA produced essentially concentration-additive mixture effects (Xie et al. 2005). However, responses in excess of expected concentration additivity were seen with triclopyr and TPA.

Uterotrophic assays

Charles et al. (2002a) were the first to confirm the additive effect of combinations of E_2, EE_2, and DES using uterine proliferation in immature CD-1 mice as the end point. Response surfaces constructed for permutations of each chemical at three dose levels demonstrated that the combined effects of all agents were additive. Tinwell and Ashby (2004) presented a study involving eight estrogenic chemicals using the uterotrophic assay in immature rats, but their aim was not to investigate agreement with additivity expectations. The combined effect of all chemicals was always larger than the responses observed with individual components.

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Mixtures of Antiandrogens

Androgens are key regulators of male sexual differentiation during *in utero* and early post-natal development. Chemicals that counteract androgen action at some stage in this period can lead to malformations of the reproductive tract. Changes in the anogenital distance, retained nipples, and alterations in the weight of sexual organs and accessory glands are frequently studied end points. These effects can arise through antagonism of androgens at the steroid receptor level and/or via suppression of testosterone synthesis in Leydig cells (Fisher 2004; Gray et al. 2001). Thus, anti-androgens can be defined narrowly as AR antagonists, but a broader definition in terms of counteracting the effects of androgens in a functional sense (which would include inhibition of uptake of testosterone precursors, and of testosterone synthesis steps) has also been proposed (Gray et al. 2001).

By applying the isobole method, which is another application of dose addition (Berenbaum 1981; Loewe and Muischnek 1926), Nellemann et al. (2003) found that procymidone and vinclozolin, both AR antagonists, additively inhibited testosterone binding to the AR. Administration of a 1:1 mixture of both fungizides to castrated, testosterone-treated male rats led to dose-additive alterations in reproductive organ weights, androgen levels, and AR-dependent gene expression. Birkhoj et al. (2004) extended the use of the isobole method to three-component mixtures of the pesticides deltamethrin, methiocarb, and prochloraz. An equimolar mixture of the three pesticides additively suppressed AR activation *in vitro*. When the authors gave a combination of these three chemicals with simazin and tribenuronmethyl to castrated testosterone-treated rats, weight changes of the adrenal gland and the levator ani, as well as alterations in gene expression of AR-associated genes were observed. The combination of all five chemicals showed effects that were not found for the individual pesticides, but whether these responses were additive could not be assessed.

A mixture of the AR antagonists procymidone and vinclozolin was evaluated in the Hershberger assay, where they acted additively in reducing ventral prostate and levator ani weights (Gray et al. 2001). A combination of procymidone and dibutyl phthalate, an inhibitor of androgen synthesis, significantly enhanced the occurrence of hypospadias in male offspring when given to pregnant rats during gestational days 14–18. Wolf et al. (2004) observed that vinclozolin and testosterone proprionate, two chemicals with opposing effects on male sexual differentiation, antagonized one another during sexual development of the male rat. A mixture of butyl benzyl phthalate, an inhibitor of testosterone synthesis, and linuron, an AR antagonist, decreased testosterone production and caused alterations of androgen-organized tissues in a dose-additive fashion (Hotchkiss et al. 2004). Jarfelt et al. (2005) studied changes in anogenital distance and retained nipples of male offspring of female rats treated with di-(2-ethylhexyl) phthalate and di-(2-ethylhexyl)adipate, but the effects of the mixture were not different from those of each chemical alone.

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Mixtures of Thyroid-Disrupting Chemicals

Compared with estrogens and antiandrogens, thyroid-disrupting chemicals are the least well studied EDs. It is therefore not surprising that few mixture studies exist using this kind of agents.

Thyroid-disrupting chemicals can alter structure and function of the thyroid gland, as well as the homeostasis of thyroid hormones, by interfering with associated regulatory enzymes. Changes in the circulating levels of thyroid hormones are often the consequence. A wide variety of chemicals are able to affect thyroid hormone levels in differing ways. PCDDs, PCDFs, and PCBs are thought to suppress circulating thyroid hormone levels by up-regulating hepatic enzymes that glucuronidate thyroxin (T_4). Most of the studies of thyroid-disrupting effects have analyzed the effects of mixtures without recording responses induced by individual mixture components; this complicates assessment of combination effects in terms of additivity, synergism, or antagonism. Wade et al. (2002) exposed rats to a combination of organochlorines and two heavy metals and analyzed effects on thyroid histopathology. Using the TCDD (2,3,7,8-tetrachloro-*p*-dibenzodioxin) equivalents method, Desaulniers et al. (2003) found that the effects of 16 PCBs, PCDDs, and PCDFs on circulating T_4 levels could be predicted well.

Crofton et al. (2005) presented an in-depth study of a mixture of 18 poly-halogenated hydrocarbons (2 PCDDs, 4 PCDFs, and 12 coplanar and noncoplanar PCBs) to investigate the hypothesis that their joint effect on reducing T_4 levels is dose additive. Young female rats were treated for 4 days with individual mixture components, and dose–response relationships with altered T_4 levels as the end point were recorded. This information was used to predict the dose-additive response to a mixture of all 18 chemicals. The mixture ratio was chosen to be proportional to the levels of the chemicals reported in breast milk, fish, and other human food sources. The dose-additivity model yielded anticipated effect doses that were higher by a factor of 2–3 than the observed responses. This deviation was statistically significant, and the joint effect of all polyhalogenated pollutants in this model can therefore be classed as synergistic. Nevertheless, the extent of underestimation of observed effects was small.

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Summary of Studies with Similarly Acting EDs

Taken together, there is good evidence that EDs produce combination effects in a dose-additive manner. This applies to a wide range of end points reflecting various hierarchical levels of hormone action in a variety of organisms. Where deviations from expected additivity occurred (Charles et al. 2002a, 2002b; Crofton et al. 2005; Rajapakse et al. 2004; Thorpe et al. 2001), the differences between anticipated and observed effects were small. Thus, it is safe to say that for regulatory purposes, the concept of dose addition is sufficiently accurate for predicting combination effects of groups of EDs with similar effects.

The reported deviations are nevertheless interesting from a conceptual view point. Toxicokinetic interactions such as differential activations of metabolizing enzymes in the mixtures may have played a role, and this requires further experimental study. For example, some estrogenic organochlorines may induce specific subsets of cytochrome P450 enzymes involved in steroid metabolism, thus leading to increased removal of steroidal estrogens from the mixture, with a certain loss of activity. This may explain the slightly lower-than-expected combination effects observed in the E-SCREEN assay by Rajapakse et al. (2004). Similar considerations may apply to the mixture of thyroid-disrupting chemicals analyzed by Crofton et al. (2005), where many diverse mechanisms are at play leading to reductions in circulating T_4 levels.

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Combination Effects between Different Classes of EDs

Comparatively little work has been performed with mixtures of different classes of EDs, such as estrogenic agents combined with anti-estrogenic chemicals, or EDs combined with other toxicants. In terms of design and data assessment, this type of study differs from those discussed so far, because not all components present in the mixture may induce the effect chosen for analysis. In these cases, a “modulatory” influence of toxicants on the effects of other chemicals is studied. It is important to realize that the magnitude of such effect modulations cannot be predicted by adopting additivity concepts such as concentration addition or independent action.

Perhaps the best-known example of effect modulation is the inhibitory effect of AhR agonists, such as PCDDs and co-planar PCBs, on the action of estrogenic chemicals. Although not estrogenic themselves, AhR agonists have been reported to suppress some E_2-induced responses, not by antagonizing hormone binding to the ER but by down-regulation of ER expression, induction of steroid-metabolizing enzyme systems such as CYP 1A1 and 1A2, and inhibiting various growth factors and cell cycle regulators (Chen et al. 2001, Reen et al. 2002, Safe 1998). There is a rich literature about the molecular biology underlying the interactions between dioxins and estrogens (Sone and Yonemoto 2002).

Somewhat misleadingly, the action of AhR agonists has been called antiestrogenic, when it is perhaps more appropriate to view them as disruptors of estrogen signaling. The dioxin TCDD has been reported to inhibit the estrogen-induced proliferation of uterine tissue in immature mice (Gallo et al. 1986) and to lead to diminuitions of ER levels in the liver and the uterus. Modulations of ER levels by TCDD were also described in rats (Astroff and Safe 1988; Romkes and Safe 1988; Romkes et al. 1987). Although down-regulation of ER expression by AhR agonists in cell models is not controversial, difficulties with reproducing the effects in rodents have led to questions about the relevance of antiestrogenic effects of AhR *in vivo*. White et al. (1995) examined the impact of TCDD on the keratinization of the vaginal epithelium and uterine proliferation in Sprague-Dawley rats induced by E_2, but they failed to observe any inhibitory effects of TCDD. Uterine ER and progesterone receptor levels were also not affected, although toxicity typical of TCDD (reductions in thymus weight, induction of hepatic CYP 1A1) occurred. Similarly, Desaulniers et al. (2003) did not observe an influence of a mixture of 16 AhR agonists (various PCDDs, PCDFs, and PCBs) on uterine growth stimulated by EE_2 in prepubertal female Sprague-Dawley rats. Although the reasons for these contradictory findings remain to be fully elucidated, Desaulniers et al. (2003) pointed to reports by Petroff et al. (2001) and Sarkar et al. (2000) of enhancements of TCDD-induced AhR expression and CYP 1A1 induction in the presence of E_2. This could explain the lack of antiestrogencity of AhR agonists in their study. White et al. (1995) even questioned the validity of ascribing a specific antiestrogenic property to TCDD in the rat. They pointed out that inhibitory actions of TCDD on E_2-induced effects reported by Safe and colleagues (Astroff and Safe 1988; Romkes and Safe 1988; Romkes et al. 1987) occurred at TCDD doses similar to the median lethal dose (LD_{50}) for the Sprague-Dawley and Long-Evan strains. Because TCDD induces a well-known wasting syndrome, it is conceivable that the antiestrogenicity of TCDD is in fact the result of such systemic toxicity, rather than due to specific effects opposing the action of the hormone. Thus, more work is required to evaluate whether disruption of estrogen signaling by AhR agonists occurs at realistic doses, and whether doses shown to interfere with estrogen-mediated biochemical effects, such as changes in gene expression, also lead to suppression of estrogen action with more apical end points, such as cell proliferation.

Epidermal growth factor (EGF) and insulin-like growth factor (IGF) are able to enhance estrogen signaling by inducing ER phosphorylation and other signaling events (Aronica and Katzenellenbogen 1993; Ignar-Trowbridge et al. 1996). These observations prompted Charles et al. (2002a) to study the impact of EGF and IGF on E_2-induced activation of ER in an MCF7 cell–based reporter gene system. Several combinations of all three agents were investigated and response surfaces recorded. Although EGF and IGF, on their own, did not promote gene transcription in this model, there were enhancements of the effects of E_2, mostly caused by EGF. These results indicate that the presence of growth factors may sensitize ER-competent cells to the action of the hormone, with significant consequences in terms of lowered effect thresholds. It remains to be seen whether similar effects also occur with estrogen-like environmental pollutants. Without a doubt, the potential for greater-than-additive interactions through interference with interacting signaling pathways deserves further attention and should be investigated systematically.

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Low-Dose Mixture Effects

In the context of discussions about EDs, various often-conflicting definitions of the term “low dose” have been used. “Low dose” is variously taken to mean “doses lower than used normally in toxicity testing”; “doses that approach, or are equal to, those encountered by humans”; or “doses associated with low effects” (National Toxicology Program 2001; vom Saal and Hughes 2005). Not all of these definitions have proven useful in guiding experimental work aimed at investigating whether mixtures of EDs provoke effects at low doses. Many of the chemicals suspected of being EDs have not yet been subjected to toxicity testing and consequently, “doses lower than used normally in toxicity testing” are difficult to define. Similarly, the resolving power of most *in vivo* and many *in vitro* assays is insufficient to demonstrate effects of combinations of agents at doses approaching human exposure levels. For these reasons, low-dose levels in mixture studies were selected by adhering to the last of the above definitions [i.e., “low dose” in the sense of doses that produce low effects, usually around or below no observed effect levels (NOELs)]. Although such doses may be relatively large compared to human exposure levels, the relevant experimental studies provided valuable insights into the potential of EDs to act together at low doses.

The concept of dose addition implies that every effective agent in the mixture, at any dose, contributes more or less to the overall combination effect. Crucially, this also holds true when the individual doses are without effect. Thus, combination effects should also result from agents present at or even below effect thresholds, provided sufficiently large numbers of components sum up to a sufficiently high total effect dose. It may be helpful to illustrate these implications of dose addition by adopting a thought experiment first presented by Berenbaum (1981). Let us consider a large number of chemicals that by chance all exhibit the same sigmoidal dose–response curve. At small doses, the effect produced by one single component is too small to be distinguishable from untreated controls. However, the response expected from combining 10 chemicals at this same low dose is equivalent to the effect of a 10-fold higher dose, because all components are assumed to exhibit the same dose–response relationship. The procedure can be repeated with infinitesimally small doses below effect thresholds; as long as there are sufficiently high numbers of chemicals present simultaneously, combination effects should result.

The good agreement of ED mixture effects with dose addition makes it an attractive proposition to review whether these theory expectations are in line with experimental observation. Silva et al. (2002) assessed

combinations of eight xenoestrogens in the yeast estrogen screen at concentrations of 50% of their no observed effect concentrations (NOECs) and observed responses of up to 40% of a maximal estrogenic effect. Using the same assay, Rajapakse et al. (2002) set out to investigate whether low levels of weak xenoestrogens would be able to modulate the effects of E_2. A combination of 11 xenoestrogens, all present at levels around their individual NOECs, led to a doubling of the effects of E_2. Tinwell and Ashby (2004) combined 8 estrogenic chemicals at doses that gave no significant uterotrophic responses when tested on their own. When administered together, quite strong uterotrophic effects were observed. The mixture experiments with 5 estrogenic chemicals in fathead minnows (*Pimephales promelas*) presented by Brian et al. (2005) also demonstrated combination effects at concentrations that individually did not induce vitellogenin synthesis. The 18 thyroid-disrupting chemicals chosen by Crofton et al. (2005) to analyze changes in T_4 levels were all present at doses equivalent to, or even below, their individual NOELs.

These examples clearly demonstrate that combinations of EDs with similar effects are able to act together at doses that when used alone do not lead to observable effects. The experimental evidence is in line with the assumptions of dose addition. Combination effects may result from cumulative exposure to EDs if they are present in sufficiently large numbers at levels equivalent to fractions of their individual NOELs. However, whether mixture effects will indeed occur is difficult to anticipate without comprehensive information about the levels and the identity of EDs in the environment and in human tissues. This is where one of the major challenges for mixture assessment currently lies: Our information about the occurrence of EDs in humans and the environment is patchy. Considerable efforts in mixture exposure assessment need to be made to fill these gaps; exposure assessment, and not hazard assessment, currently represents the “bottleneck” for making further progress in this important area.

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Implications for Regulatory Strategies

It is evident that the traditional chemical-by-chemical approach to risk assessment is inadequate when dealing with EDs (and chemicals with other toxic profiles). The biological reality of combination effects from exposure to multiple agents at low doses highlights the potential for underestimating risks when mixture effects are not taken into account. This underlines the need to modify current risk assessment practice if humans and the environment are to be protected adequately from multiple exposures to EDs. As a first step in the direction of implementing better risk assessment, the idea of grouping EDs according to suitable similarity criteria suggests itself, as is already common practice with the group-wise assessment of AhR agonists such as PCDDs, PCDFs, and PCBs in the TEF/TEQ approach. For example, in a recent opinion paper, the European Scientific Committee on Toxicology, Ecotoxicology and the Environment (Commission of the European Communities 2004) pointed out that

> For compounds with an identical mode of action, such as oestrogenic hormones and xenoestrogens that act through an oestrogen receptor, the performance of individual risk assessments is problematic. For example, the effects of natural and synthetic oestrogens may be additive, especially since these chemicals co-occur in the aquatic environment.

Criteria for defining similar modes of action

The challenge lies in defining what “identical modes of action” could mean for EDs, and how this could be translated into workable criteria for grouping EDs according to similar modes of action. The issue is linked to the general problem of defining “similar action” for purposes of mixture assessments, but unfortunately there are currently no unambiguous criteria for what should constitute similar action (Mileson et al. 1998). Often, the induction of the same phenomenological effect is deemed sufficient for accepting similar action. At the other extreme of the spectrum of opinions, an identical molecular mechanism involving the same active intermediate is required to fulfill the similarity assumption. A middle position is occupied by the view that interactions with the same site, tissue, or target organ should qualify for similarity.

One suggestion would be to group EDs according to the steroid receptors with which they interact. Thus, all estrogens, for example, could be regulated together. However, in taking this approach, the criteria chosen for grouping should be considered carefully. Too narrow a focus on molecular mechanisms might lead to problems and prove unworkable. The issue can be illustrated using anti-androgens as an example. With a narrow focus on identical modes of action, all AR antagonists could be considered, but this would leave out agents that are able to disrupt male sexual development by interfering with steroid synthesis, such as certain phthalates. Thus, application of a phenomenological similarity criterion (all agents that disrupt male sexual development by inducing changes in anogenital distance) would serve the group of anti-androgens better.

A similar case can be made for estrogenic or estrogen-like chemicals. Grouping these chemicals according to their ability to activate the ER-α would leave out ER-β agonists, although there is considerable overlap. However, the molecular mode of action is different in both cases. Furthermore, the phenomenon of ligand-independent activation of steroid receptors (e.g., by phosphorylations via mitogen-activated phosphokinase cascades and activation of receptor tyrosine kinases) has become well-established (Picard 2003), and steroid hormones themselves are able to induce these signaling events. If a similarity criterion for estrogens is defined in a strict molecular way, for example, solely in terms of binding to the E_2-binding pocket with subsequent activation of the helix 12 “mousetrap” mechanism, then a wealth of additional mechanisms of ER activation would be left disregarded, although these processes may well contribute to joint effects in living organisms. In this context, the question of sensitization to the effects of xenoestrogens by growth factors is particularly relevant. Thus, for estrogens, it would be more appropriate to adopt a phenomenological similarity criterion and to use the classical definition of estrogens (induction of proliferation of tissues of the female reproductive tract) for purposes of grouping in terms of similar action.

In the case of thyroid-disrupting chemicals, many different mechanisms are at play that all may lead to reductions in thyroid hormone levels. These include, but are not limited to, inhibition of uptake of iodide into the thyroid gland, disruption of thyroid hormone synthesis by inhibition of thyroid peroxidase, and alterations of the levels of circulating thyroid hormones by increased activity of uridine diphosphoglucuronosyl transferases. These enzymes are inducible by nuclear receptors such as PXR and CAR, which in turn respond to a wide variety of chemicals with differing structural features. Thus, it would be impossible to define thyroid-disrupting chemicals in terms of strict molecular similarity, and only a phenomenological approach has any prospect of producing workable grouping criteria.

A particular problem arises with chemicals that have been shown to interfere with signaling from several steroid receptors. An example is the ubiquitous environmental pollutant *p,p′*-DDE, which is a weak ER agonist and also an AR antagonist. It appears that many AR antagonists turn out to be ER agonists, and vice versa (Kojima et al. 2004).

The TEF approach for EDs?

The usefulness of the TEF/TEQ approach for hazard and risk assessment of ED mixtures has been reviewed by Safe (1998). The TEF approach is an application of the concept of dose addition. Given the good agreement of ED mixture effects with dose addition and considering that the TEF approach is relatively straightforward to use, it would appear uniquely suited for the joint assessment of specific groups of EDs. For many PCDD/PCDF mixtures, calculated TEQs agree well with experimentally determined TEQs (Desaulniers et al. 2003; Hamm et al. 2003; Safe 1998; van den Berg et al. 1998).

However, as Safe (1998) pointed out, the main problem in adopting the TEF approach for ED mixtures lies in the biological reality of interactions between different response pathways and signaling webs activated by diverse agents. The available evidence in the literature (Charles et al. 2002a) demonstrates that such interactions may lead to enhancements or suppressions of effects not captured by the additivity assumption of the TEF concept. This may become particularly relevant when nonlinear toxicokinetic factors are at work that alter TEFs derived from *in vivo* studies at higher doses. Interactions of this kind may also compromise the usefulness of TEFs derived from *in vitro* assays when comparisons to the *in vivo* situation are made.

The TEF concept relies on a standard or reference compound that is used to define TEFs for individual chemicals in the same class of compounds. In the case of PCDDs and PCDFs, this is TCDD; for estrogenic chemicals, the endogenous hormone E_2 suggests itself as a reference for defining TEFs. However, for lack of an endogenous agent, it is not straightforward to define a standard anti-androgen, although in principle this problem can be overcome by agreeing on an arbitrary choice of a particular chemical.

More difficult to deal with are violations of another assumption implicit in the use of the TEF approach: the requirement that the dose–response curves for all congeners of a group of chemicals should be parallel. If this assumption is not fulfilled, the TEF will vary depending on the effect levels chosen for deriving their numerical values. Parallel dose–response curves have often been observed with EDs but are by no means the general rule, and this militates against the general applicability of the TEF approach for ED mixtures.

Suggestions for a temporary solution

Safe's (1998) main argument against the generalized use of the TEF approach for ED mixtures (i.e., the richness and variety of activation of interacting signaling webs with their potential of nonadditive joint effects) carries a lot of force. There can be no doubt that future research should further characterize these interactions and their potential to modulate the action of hormone-like agents. On the other hand, the overwhelming evidence showing that groups of estrogenic, antiandrogenic, and thyroid-disrupting chemicals act together in an additive fashion cannot be ignored. The progress that has been made in the last 10 years in assessing ED mixtures severely compromises the credibility of continued use of the chemical-by-chemical approach to risk assessment. It is likely that future research into ED signaling cross-talk will uncover better criteria for dealing with these chemicals in a more holistic way, but until then, lack of this knowledge should not prevent regulators from making the best use of available empirical evidence.

Therefore, I suggest that EDs be temporarily grouped and that these groups be subjected to common hazard and risk assessment. Great care should be taken not to apply inappropriately restrictive criteria in carrying out these classifications. EDs should be arranged according to their ability to provoke similar effects, rather than according to similar mechanisms of action. Given that the expectation of parallel dose–response curves is unrealistic, use of the TEF concept should be avoided. Instead, dose addition should be preferred for calculating quantitative additivity expectations, if necessary, even in the absence of empirical data about mixture effects.

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Research Recommendations

As I have demonstrated in this article, comparatively little information exists concerning the ways in which EDs belonging to different classes may interact to produce combined effects. As a result, research efforts should be focused to pursue this area of study. For example, antiandrogens, including AR antagonists and inhibitors of steroid synthesis, should be combined with estrogenic agents that also possess antiandrogenic properties to allow the study of possible impacts on disruption of male sexual development *in vivo*. PCDDs, PCDFs, and PCBs are well-known disruptors of male sexual development, but very little is known about how they act together with antiandrogens and estrogens, and it is urgent to fill this gap.

Another knowledge gap that needs to be bridged concerns possible interactions between growth factors such as EGF and IGF with natural steroid hormones and xenoestrogens. A better understanding of the joint effects of these agents is required to evaluate environmental factors that are important in the formation of breast cancer. There is already evidence demonstrating a potential for synergism between steroid hormones and growth factors (Charles et al. 2002a), but studies building on this knowledge are required to evaluate potential interactions between growth factors and xenoestrogens. This area of investigation could be enhanced further by analyzing the role of signaling cross talk between natural dietary components that activate retinoid receptors and ER pathways.

Of particular interest are combinations of chemicals in which not all components produce the effect to be analyzed, but where some may significantly modulate the effects of others, without themselves being effective. By their very nature, the impact of such effect modulators will not be predictable quantitatively by employing the usual additivity expectations in mixture toxicology. However, it is necessary to explore whether the direction of such effect modulations can be anticipated in qualitative terms by analyzing interactions at the level of metabolism and transport. The approach taken could use existing databases for the visualization of complex gene and signaling networks as a mining and analytical tool for hypothesis generation (Ekins et al. 2006). Signaling nodes and interaction points identified in this way could then be targeted experimentally by gene expression profiling and proteomics techniques. This will also allow assessments of the usefulness of such data mining and experimental approaches for the grouping of EDs into classes with similar effect profiles.

Exposure assessment has revealed itself as another major limiting factor that is currently hampering progress with ED mixtures. Information exists about the concentrations of many individual chemicals in human tissues and in the environment, but data concerning the levels of multiple chemicals in the same samples are scarce. Put simply, we need to know whether women in agricultural areas in, say, Spain, who exhibit elevated levels of certain pesticides in their tissues also show high levels of phthalates from copious use of personal care products and cosmetics. To date, this information is not available, and it will require dedicated, targeted mixture exposure assessment strategies to fill this gap.

Perhaps the greatest challenge will be to develop ways in which concepts for mixture effect assessment can be used productively in epidemiology. Epidemiology has traditionally focused on defining the impact of single chemicals on disease outcomes, and only very few examples exist where the role of combinations of chemicals could be evaluated. As an example relevant to endocrine disruption, the shortcomings of traditional single-chemical epidemiology on elucidating causes for breast cancer have been previously discussed (Kortenkamp 2006). In order to develop viable approaches to solving this problem, the concerted efforts of mixture toxicology experts, exposure assessors, and epidemiologists will be required.

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Conclusion

In the last 10 years, considerable progress has been made in assessing the effects of multi-component mixtures of EDs. Work has focused on mixtures with components belonging to certain classes of EDs, such as estrogenic, antiandrogenic, and thyroid-disrupting chemicals; these studies have demonstrated the usefulness of the concept of dose addition in anticipating combination effects. Good evidence is available to show that joint effects occur even when all mixture components are present at levels below doses that cause observable effects. In view of this evidence, the traditional chemical-by-chemical approach to risk assessment is hard to justify, and the ground is prepared to seriously consider group-wise regulation of EDs. In spite of serious shortcomings in our understanding of signaling cross talk between categories of EDs, we should group these chemicals according to their ability to induce similar effects (as opposed to similar mechanisms) until better mechanistic information in forthcoming. This modus operandi is only viable with a concurrent, targeted research program aimed at improving our understanding of ED mixtures. Future research should particularly focus on combinations of EDs that belong to different categories.

Footnotes

This article is part of the monograph "Endocrine Disruptors—Exposure Assessment, Novel End Points, and Low-Dose and Mixture Effects."

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