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Ten Years of Mixing Cocktails – a Review of Combination Effects of Endocrine Disrupting Chemicals

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AGD – anogenital distance

AhR - arylhydrocarbon receptor

AR – androgen receptor

E2 - 17β-estradiol

EE2 - 17α-ethinylestradiol

EGF – epidermal growth factor

ER – estrogen receptor

GD – gestational day

IGF – insulin-like growth factor

MAPK – mitogen-activated phosphokinases

NOAEL – no observed adverse effects level

NOEC – no observed effect concentration

NOEL – no observed effect level

NR – nipple retention

PCB – polychlorinated biphenyls

PCDD – polychlorinated dibenzodioxins

PCDF – polychlorinated dibenzofurans

PND – postnatal day

TCDD – 2.3.7.8 Tetrachloro dibenzodioxin

TEF/TEQ – TCDD equivalency factor – TCDD equivalents

YES – yeast estrogen screen

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Outline of section headers

Abstract

In the last ten years, good evidence has become available to show that the combined effects of endocrine disrupters belonging to the same category (e.g. estrogenic, anti-androgenic or thyroid-disrupting agents) can be predicted by using dose addition. This is true for a variety of endpoints representing a wide range of organisational levels and biological complexity. Combinations of endocrine disrupters 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 endocrine disrupters. Nevertheless, it is argued that the accumulated evidence seriously undermines continuation with the customary chemical-by-chemical approach to risk assessment for endocrine disrupters. Instead, the ground is prepared for seriously considering group-wise regulation of classes of endocrine disrupters. Great care should be taken to define such classes by using suitable similarity criteria. It is suggested that criteria should focus on common effects, rather than common mechanisms. The review ends by highlighting research needs, and lack of information about exposure scenarios is identified as a knowledge gap that seriously hampers progress with endocrine disrupter risk assessment. It is recommended that future research should focus on investigating the effects of combinations of endocrine disrupters from different categories with considerable emphasis on elucidating mechanisms. This strategy may lead to better defined criteria for grouping endocrine disrupters for regulatory purposes. Steps should be taken to come to dedicated mixtures exposure assessment for endocrine disrupters.

Introduction

The topic of combined exposures to endocrine disrupters has long been regarded as important, not least because of the continuing discovery of ever new chemicals with endocrine disrupting potential and the realisation that exposure is to a multitude of chemicals simultaneously, not to single agents. Yet, Yang's observation that over 95% of the resources in toxicological research are devoted to the study of single chemicals, with an almost complete neglect of mixture studies (Yang 1994), also applies to endocrine disrupter 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 endocrine disrupters 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 endocrine disrupters. In addition, mixture studies are perceived to be challenging, both conceptually and experimentally - concerns which have led the USEPA Science Advisory Board 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).

Despite these difficulties, perceived or real, a large number of papers on combination effects of endocrine disrupters have appeared during the last ten 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 evaluate the potential risks on an individual basis. Over twenty years ago, this risk assessment approach has found entry into the regulation of poly-halogenated 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 endocrine disrupters to call for similar cumulative risk assessment approaches? Where are knowledge gaps, and what are conceptual difficulties? A review of the earlier work on endocrine disrupter mixtures, leading up to 1997, has been published (Kortenkamp and Altenburger 1998). This paper concentrates on studies that appeared after 1997 in the peer-reviewed literature.

In studying endocrine disrupter mixtures, many researchers have followed what has been called a "whole mixture approach" (U.S.EPA 1986) where a combination of many chemicals is investigated as if it were a single agent, without assessing the individual effects of all the components. 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 endocrine disrupters is uncertainty about their potential to act together in an additive or synergistic manner (Daston et al. 2003). To address these concerns the review focuses on studies that have assessed endocrine disrupter 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, e.g. one chemical, without itself inducing 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, and 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 appreciated that it is not synonymous with additivity in the mathematical sense. In toxicology, mixture "additivity" describes the case where chemicals "act together" to produce effects without enhancing or diminishing each others 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 readers interested in more detail are referred to Berenbaum (1981, 1989) and Greco et al. (1995).

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 becomes obvious when the case of 10 agents is considered that each provoke, say, 15% of a certain response. The anticipation that the resulting joint effect should be 10 x 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 and furans (PCDD, PCDF). 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 equi-effective 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 PCDD and PCDF (Safe 1998; van den Berg et al. 1998). A great deal of the work on endocrine disrupter mixtures has utilised dose addition or concentration addition as the concept for calculating additivity expectations. Considering that the majority of mixture studies were based on endpoints relatively close to the steps following hormone-receptor binding and activation, the choice of dose addition appears to be well-founded.

Independent action (often 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 employed rarely for mixtures of endocrine disrupters.

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 above example with 10 compounds that each induce a 15% effect.

In the following, work with the three most frequently studied hormone receptors, the estrogen, androgen and thyroid receptors, will be considered. There is a rich literature concerning the Ah-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 endocrine disrupters will be considered.

Mixtures of estrogenic chemicals

Estrogenic chemicals have been the focus of most of the work on endocrine disrupters, and it is not surprising that this group of substances has been the topic of the majority of endocrine disrupter mixture studies. While the earlier efforts have mainly employed binary mixtures (reviewed in Kortenkamp and Altenburger 1998), work carried out since 1998 has made significant contributions to the analysis of multi-component mixtures containing three, often five and up to 12 estrogenic chemicals. “Estrogenicity” can be defined in various ways. At the functional, physiological level, the term denotes the ability of a chemical to evoke responses similar to 17β-estradiol (E2), such as cornification of the vaginal epithelium, and uterine cell proliferation. Of toxicological concern is the role of estrogens in breast and ovarian cancer, and 17β-estradiol and synthetic estrogens are recognised human carcinogens. Advances in our understanding of the mode of action of estrogens have led to further definitions which refer to specific steps at various molecular levels, and this suggests itself as a way to structure the evidence on estrogen mixtures: Thus, ”estrogenicity” can mean affinity to the estrogen receptor (ERα or 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 the time of writing, no post-1998 multi-component study with ER binding as the endpoint was available.

Estrogen receptor activation

(Payne et al. 2000) studied combinations of two, three and four estrogenic chemicals in the yeast estrogen screen (YES), an ERα-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; Silva et al. 2002) have extended this approach to the analysis of mixtures involving eight and twelve estrogenic agents, respectively. In both cases, the mixture responses seen using the YES agreed excellently with the effects predicted by using concentration addition. In an attempt to verify the assumption that concentration addition is an appropriate model for estrogen mixtures, the observed mixture effects were also compared with additivity predictions calculated using independent action. In the paper 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 ERα 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 E2, 17α-ethynyl estradiol (EE2) and diethylstilbestrol 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 which the authors attributed to saturation effects. In a second paper, the same group investigated ternary combinations of further estrogenic chemicals. While 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 E2, genistein and *o,p'*-DDT were antagonistic both in the low and the high concentration range (Charles et al. 2002b).

Activation of ERα was monitored by measuring expression of the *TFF1* gene (coding for the pS2 protein) to study the effects of combinations of estrogenic UV filter substances (Heneweer et al. 2005). Binary mixtures of 2-hydroxy-4-methoxy-benzophenone and its metabolite 2,4-dihydroxybenzophenone showed concentration additive effects, as did a combination of these two chemicals with octyl methoxycinnamate and 3-(4-methylbenzylidene) camphor. In a TEQ approach the authors expressed effect concentrations of the test chemicals in terms of 17β-estradiol 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 E2, EE2, estrone, genistein and α-zeralenol, with effects well in agreement with concentration addition.

Cell proliferation

The effects of *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE and β-HCH on the proliferation of estrogen dependent MCF7 cells (E-Screen assay) were found to be concentration additive at two different mixture ratios, but the observed resposes were equally well predicted by independent action (Payne et al. 2001). Suzuki et al. (2001) tested binary mixtures of natural and synthetic estrogenic chemicals including E2, 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 E2 and

bisphenol A, while the remaining eight binary combinations gave additive, antagonistic or weakly synergistic effects. However, the interpretation of these results is complicated by the fact that additivity expectations were calculated by multiplication of unscaled effect measures, a method inconsistent with independent action. Rajapakse et al. (2004) analysed mixtures containing E2, EE2, 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 E2, EE2, and genistein produced excellent agreement with predicted concentration additivity, and the same was observed for a four-component mixture with E2, EE2, genistein and bisphenol A. The presence of 4-nonylphenol and 4 *tert*-octylphenol appeared to be associated with the observed antagonisms. It is conceivable that differential activation of metabolising enzymes (e.g. cytochrome P450) or efflux pumps by mixture components has led to removal of other constituents, but this hypothesis awaits experimental confirmation.

Vitellogenin induction in fish

In fish, the induction of vitellogenin is controlled by ERα, 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 endpoint 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 E2 and tert-nonylphenol followed the effects predicted by concentration addition, but a mixture of E2 and the pesticide methoxychlor was less than additive. A binary mixture of E2 and EE2 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 E2, EE2, 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.

Xie et al. (2005) have used the juvenile trout vitellogenin assay to evaluate mixtures of the pesticides 2,4-dichlorophenoxyacetic acid (2,4D), triclopyr, diquat dibromide and plyphosate with two alkyl-pheno ethoxylate-containing surfactants (R11 and Target Prospreader Activator, TPA). Of all pesticides, only 2,4D and triclopyr caused enhancements in vitellogenin levels, when given individually, and R11 and TPA were also effective. In a TEQ approach, the additivity expectations were derived by addition of estradiol equivalents. Binary combinations of 2,4D with R11, or with TPA produced essentially concentration additive mixture effects. 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 E2, ethynyl estradiol and diethylstilbestrol using uterine proliferation in immature CD-1 mice as the endpoint. 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) have presented a study involving eight estrogenic chemicals using the uterotrophic assay with 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.

Mixtures of anti-androgens

Androgens are key regulators of male sexual differentiation during the *in utero* and early postnatal 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 endpoints. 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 Jr et al. 2001). Thus, anti-androgens can be defined narrowly as androgen receptor (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 Jr et al. 2001).

By applying the isobole method which is another application of dose addition (Berenbaum 1981; Loewe and Muischnek 1926) it was found that procymidone and vinclozolin, both AR antagonists, additively inhibited testosterone binding to the AR (Nellemann et al. 2003). Administration of a 1:1 mixture of both fungizides to castrated, testosterone-treated male rats led to dose additive alterations in reproductive organs weights, androgen levels and androgen receptor-dependent gene expression. Birkhoj et al. (2004) have 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 a combination of these three chemicals with simazin and tribenuron-methyl was given 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 Jr et al. 2001). A combination of procymidone and di-butyl 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-organised 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 (DEHP) and di-(2-ethylhexyl)adipate (DEHA), but the effects of the mixture were not different from those of the single chemicals.

Mixtures of thyroid-hormone-disrupting chemicals

Compared with estrogens and anti-androgens, thyroid-disrupting chemicals are the least well studied endocrine disrupters. 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 (T4). Most of the studies of thyroid disrupting effects have analysed the effects of mixtures without recording responses induced by individual mixture components, and 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 analysed effects on thyroid histopathology. Desaulniers et al. (2003) used the TCDD equivalents method and found that the effects of 16 polychlorinated biphenyls, dioxins and furans on circulating thyroxin levels could be predicted well.

Crofton et al. (2005) have presented an in-depth study of a mixture of 18 polyhalogenated hydrocarbons (2 PCDDs, 4 PCDFs and 12 co-planar and non-coplanar PCBs) to investigate the hypothesis that their joint effect on reducing T4 levels is dose-additive. Young female rats were treated for four days with individual mixture components and dose-response relationships with altered T4 levels as the

endpoint 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.

Summary of studies with similarly acting EDC

Taken together, there is good evidence that endocrine disrupting chemicals produce combination effects in a dose additive manner. This applies to a wide range of endpoints reflecting various hierarchical levels of hormone action in a variety of organisms. Where deviations from expected additivity occurred (Charles et al. 2002ab; 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 endocrine disrupters with similar effects.

The reported deviations are nevertheless interesting from a conceptual view point. Toxicokinetic interactions such as differential activations of metabolising 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 by

(Rajapakse et al. 2004). Similar considerations may apply to the mixture of thyroid disrupting chemicals analysed by Crofton et al. (2005) where many diverse mechanisms are at play leading to reductions in circulating thyroxin levels.

Combination effects between different classes of endocrine disrupters

Comparatively little work has been carried out with mixtures of different classes of endocrine disrupters, such as estrogenic agents combined with anti-estrogenic chemicals, or endocrine disrupters combined with other toxicants. In terms of design and data assessment, these studies differ 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 realise 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 polychlorinated dioxins and co-planar polychlorinated biphenyls, on the action of estrogenic chemicals. Themselves not estrogenic, AhR agonists are reported to suppress some E2-induced responses not by antagonising hormone binding to the ER, but by down-regulation of ER expression, induction of steroid-metabolising enzyme systems such as CYP 1A1 and 1A2, and by 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 which is not the topic of this review. Interested readers may wish to refer to Sone and Yonemoto (2002).

Somewhat misleadingly, the action of AhR agonists has been called "anti-estrogenic", when it is perhaps more appropriate to view them as disrupters of estrogen signalling. The dioxin TCDD was 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). While 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 "anti-estrogenic" effects of AhR *in vivo*. White et al. (1995) examined the impact of TCDD on the keratinisation of the vaginal epithelium and uterine proliferation in Sprague-Dawley rats induced by E2, but 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 polychlorinated dioxins, furans and bipenyls) on uterine growth stimulated by EE2 in pre-pubertal 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 E2. This could explain the lack of "anti-estrogencity" of AhR agonists in their hands. White et al. (1995) even questioned the validity of ascribing a specific "anti-estrogenic" property to TCDD in the rat. They pointed out that inhibitory actions of TCDD on E2-induced effects

reported by Safe and associates occurred at TCDD doses similar to the LD50 for the Sprague-Dawley and Long-Evan strains. Since TCDD induces a well-known wasting syndrome, it is conceivable that the "anti-estrogenicity" 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 signalling 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 endpoints such as cell proliferation.

Epidermal growth factor (EGF) and insulin-like growth factor (IGF) are able to enhance estrogen signalling by inducing ER phosphorylation and other signalling 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 E2-induced activation of ER in a 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 E2, mostly due to EGF. These results indicate that the presence of growth factors may sensitise 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 signalling pathways deserves further attention and should be investigated systematically.

Low dose mixture effects

In the context of discussions about endocrine disrupters, 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” (NTP 2001; vom Saal and Hughes 2005). Not all of these definitions have proven useful in guiding experimental work aimed at investigating whether mixtures of endocrine disrupters provoke effects at low doses. Many of the chemicals suspected of being endocrine disrupters 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. Although such doses may be relatively large compared to human exposure levels, the relevant experimental studies provided valuable insights into the potential of endocrine disrupters 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 a combining, say, 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 endocrine disrupter 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 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 E2. A combination of eleven xenoestrogens, all present at levels around their individual NOECs, led to a doubling of the effects of E2. Tinwell and Ashby (2004) combined eight 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 five 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 analyse changes in T4 levels were all present at doses equivalent to their individual NOELs, or even below.

These examples clearly demonstrate that combinations of endocrine disrupters with similar effects are able to act together at doses that on their own 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 endocrine disrupters 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 endocrine disrupters 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 endocrine disrupters 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.

Implications for regulatory strategies

It is evident that the traditional chemical-by-chemical approach to risk assessment is inadequate when dealing with endocrine disrupters (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 endocrine disrupters. As a first step in the direction of implementing better risk assessment, the idea of grouping endocrine disrupters according to suitable similarity criteria suggests itself, as is already common practice

with the group-wise assessment of AhR agonists such as PCDD, PCDF and PCB in the TEF/TEQ approach. For example, in a recent opinion paper, the European Scientific Committee on Toxicology, Ecotoxicology and the Environment (SCTEE 2004) pointed out that "*for compounds with identical mode of action, such as oestrogenic hormones and xenoestrogens (…) the performance of individual risk assessments is problematic. …The effects may be additive, especially since these chemicals co-occur in the aquatic environment"*.

Criteria for defining "similar modes of action"

However, the challenge lies in defining what "identical modes of action" could mean for endocrine disrupters, and how this could be translated into workable criteria for grouping endocrine disrupters 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 fulfil 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 endocrine disrupters according to the steroid receptors they interact with. Thus, all estrogens, androgens, anti-androgens etc. 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 into problems and prove unworkable. The issue can be illustrated by taking 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, etc…") 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 ERα would leave out ERβ agonists, although there is considerable overlap. But undoubtedly, 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 MAPK cascades and activation of receptor tyrosine kinases, has become well-established (Picard 2003), and steroid hormones themselves are able to induce these signalling events. If a similarity criterion for "estrogens" is defined in a strict molecular way, e.g. solely in terms of binding to the estradiol 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 real living organisms. In this context, the question of sensitisation to the effects of xenoestrogens by growth factors is particularly relevant. Thus, for "estrogens" too it would be more appropriate to adopt a phenomenological similarity criterion, and to utilise 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 signalling 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 toxic equivalency factor approach for endocrine disrupters?

The usefulness of the toxic equivalency factor / toxic equivalents (TEF/TEQ) approach for hazard and risk assessment of endocrine disrupter mixtures has been reviewed by (Safe 1998). The TEF approach is an application of the concept of dose addition. Given the good agreement of endocrine disrupter 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 endocrine disrupters. 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) has pointed out, the main problem in adopting the TEF approach for endocrine disrupter mixtures lies in the biological reality of interactions between different response pathways and signalling webs activated by diverse agents. As shown previously, the available evidence in the literature 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 non-linear 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 which is used to define TEFs for individual chemicals of the same class of compounds. In the case of PCDDs and PCDFs this is 2,3,7,8 TCDD, and for estrogenic chemicals, the endogenous hormone E2 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, and that is the requirement that the dose-response curves for all congeners of a group of chemicals should be parallel. If this assumption is not fulfilled, TEF will vary depending on the effect levels chosen for deriving their

numerical values. Parallel dose-response curves have often been observed with endocrine disrupters, but are by no means the general rule, and this militates against the general applicability of the TEF approach for endocrine disrupter mixtures.

Suggestions for a temporary solution

Safe's main argument against the generalised use of the TEF approach for endocrine disrupter mixtures, i.e. the richness and variety of activation of interacting signalling webs with their potential of non-additive joint effects, carries a lot of force. There can be no doubt that future research should further characterise 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, anti-androgenic 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 endocrine disrupter mixtures severely compromises the credibility of continued use of the chemical-by-chemical approach to risk assessment. It is likely that future research into endocrine disrupter signalling 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 best use of available empirical evidence.

On balance therefore, it is suggested to temporarily group endocrine disrupters and to subject these groups to common hazard and risk assessment. Great care should be taken not to apply inappropriately restrictive criteria in carrying out these classifications. Endocrine disrupters 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.

Research recommendations

As this review has demonstrated, comparatively little information exists about the ways in which endocrine disrupters 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, anti-androgens, including AR antagonists and inhibitors of steroid synthesis, should be combined with estrogenic agents that also possess anti-androgenic properties to allow the study of possible impacts on disruption of male sexual development *in vivo*. PCDDs, PCDFs and PCBs are well-known disrupters of male sexual development, but very little is known how they act together with anti-androgens 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 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 analysing the role of signalling cross talk between natural dietary components that activate retinoid receptors and ER pathways.

Of particular interest are combinations of chemicals where not all components produce the effect to be analysed, 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 analysing interactions at the level of metabolism and transport. The approach taken could be to utilise existing databases for the visualisation of complex gene and signalling networks as a mining and analytical tool for hypothesis generation (Ekins et al. 2006). Signalling 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 endocrine disrupters into classes with similar effect profiles.

Exposure assessment has revealed itself as another major limiting factor that is currently hampering progress with endocrine disrupter mixtures. Information exists about the levels of many individual chemicals in human tissues and the environment, but data about the levels of multiple chemicals in one and the same sample 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 chemicals epidemiology on elucidating causes for breast cancer have been discussed by Kortenkamp (2006). It will require the concerted efforts of mixture toxicology experts, exposure assessors and epidemiologists to develop viable approaches to solving this problem.

Conclusion

In the last ten years, considerable progress has been made with assessing the effects of multi-component mixtures of endocrine disrupters. Work has focused on mixtures composed of components belonging to certain classes of endocrine disrupters, such as estrogenic, anti-androgenic and thyroid-disrupting chemicals, and 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 endocrine disrupters. Despite serious shortcomings in our understanding of signalling cross talk between categories of endocrine disrupters, it is suggested to 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

programme aimed at improving our understanding of endocrine disrupter mixtures. Future research should particularly focus on combinations of endocrine disrupters that belong to different categories.

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