

Combining Xenoestrogens at Levels below Individual No-Observed-Effect Concentrations Dramatically Enhances Steroid Hormone Action

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The low potency of many man-made estrogenic chemicals, so-called xenoestrogens, has been used to suggest that risks arising from exposure to individual chemicals are negligible. Another argument used to dismiss concerns of health effects is that endogenous steroidal estrogens are too potent for xenoestrogens to contribute significantly to estrogenic effects. Using a yeast reporter gene assay with the human estrogen receptor α , we tested these ideas experimentally by assessing the ability of a combination of 11 xenoestrogens to affect the actions of 17 β -estradiol. Significantly, each xenoestrogen was present at a level well below its no-observed-effect concentration (NOEC). To derive accurate descriptions of low effects, we recorded concentration–response relationships for each xenoestrogen and for 17 β -estradiol. We used these data to predict entire concentration–response curves of mixtures of xenoestrogens with 17 β -estradiol, assuming additive combination effects. Over a large range of concentrations, the experimentally observed responses decisively confirmed the model predictions. The combined additive effect of the 11 xenoestrogens led to a dramatic enhancement of the hormone's action, even when each single agent was present below its NOEC. Our results show that not even sub-NOEC levels of xenoestrogens can be considered to be without effect on potent steroidal estrogens when they act in concert with a large number of similarly acting chemicals. It remains to be seen to what degree these effects can be neutralized by environmental chemicals with antiestrogenic activity. Nevertheless, potential human and wildlife responses induced by additive combination effects of xenoestrogens deserve serious consideration. **Key words:** 17 β -estradiol, additivity, mixture effects, xenoestrogens, yeast estrogen screen (YES). *Environ Health Perspect* 110:917–921 (2002). [Online 12 August 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p917-921rajapakse/abstract.html>

The enormous discrepancies between the high concentrations of xenoestrogens often required to produce effects in laboratory assays and their low levels in human tissues and the environment have fueled the belief that synergisms between these chemicals need to be invoked to explain possible health risks to humans and wildlife. However, initial reports of strong synergisms between binary combinations of estrogenic pesticides (Arnold et al. 1996) could not be reproduced (Ashby et al. 1997; Ramamoorthy et al. 1997) and had to be withdrawn (McLachlan 1997). Furthermore, numerous tissues contain potent steroidal estrogens at biologically active levels. This has led to the view that exposure to xenoestrogens may not pose any harm because they are unable to impact the strong effects of steroidal estrogens (Safe 1995).

However, the perceived “weakness” (or otherwise) of a xenoestrogen alone does not necessarily signal absence of risks, when considering the effects of xenoestrogens in relation to potent steroidal estrogens. In a recent study of mixtures of estradiol and the weak xenoestrogens bisphenol A and *o,p'*-DDT (Rajapakse et al. 2001), we found that both the hormone and the xenoestrogens contributed in equal measure to the observed combination effects when combined at concentrations that produced similar responses. Furthermore, model calculations carried out by our group suggested that combinations of a large number of xenoestrogens might

modulate the effects of 17 β -estradiol, even when each individual xenoestrogen is present at concentrations that alone would not produce measurable effects (Kortenkamp and Altenburger 1999). In view of the implications for hazard assessment, we set out to test this idea experimentally.

Mixture experiments involving a large number of chemicals place very high demands on the reproducibility of biologic responses. To meet these requirements, we used the yeast estrogen screen (YES) (Routledge and Sumpter 1996), which has proven to yield highly reproducible results (Payne et al. 2000; Rajapakse et al. 2001).

Before we could proceed with the studies presented here, we had to investigate whether weak xenoestrogens, in the absence of the potent hormone 17 β -estradiol, would act together when combined at levels below no-observed-effect concentrations (NOECs). Furthermore, information about the validity of a variety of concepts for the prediction and assessment of xenoestrogen mixture effects was required. In a recent study involving eight weak xenoestrogens and the YES assay, we addressed these points (Silva et al. 2002). We were able to show that significant mixture effects occurred when the chemicals were mixed at levels equal to 50% of their individual NOEC. The effects of combinations of estrogenic chemicals were predicted on the basis of concentration–response relationships of individual mixture components (“fixed

mixture ratio design”) (Altenburger et al. 2000; Backhaus et al. 2000). We found that the additivity predictions calculated by using the concept of concentration addition (CA) (Loewe and Muischnek 1926) proved to agree excellently with experimental observations. In contrast, the competing concept of independent action (Bliss 1939) led to considerable underestimations of measured effects (Silva et al. 2002). Thus, CA can be used with confidence for the prediction and assessment of the joint effects of xenoestrogens in the YES assay. When dealing with agents that exhibit sigmoidal concentration–response curves, as is the case with estrogenic chemicals, it is not possible to calculate expected additive effects by forming the arithmetic sum of individual responses (effect summation; ES). Although the pitfalls of this approach have been discussed extensively (Berenbaum 1985; Kortenkamp and Altenburger 1998; Payne et al. 2000), its shortcomings are not widely acknowledged. For this reason, we have included ES here for purposes of comparison.

The clarification of the above issues provided a firm basis for the studies presented here. We created a pool of 11 weak xenoestrogens with a mixture ratio in proportion to the EC₀₁ (concentration producing 1% of the maximally inducible effect in the YES assay) of all individual components. This ensured that no single xenoestrogen contributed disproportionately to the overall mixture effect. The 11 xenoestrogens were in turn mixed with 17 β -estradiol at defined mixture ratios, ranging from 1:25,000 to 1:100,000 (17 β -estradiol:pool of xenoestrogens). Predictions were calculated by using CA and then tested experimentally.

We determined concentrations of test agents that failed to produce measurable effects relative to untreated yeast by establishing NOECs and by using regression-based approaches that estimate low effects by interpolation on the basis of entire dose–response curves (benchmark concentrations) (Moore

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and Caux 1997; Van der Hoeven 1997). We have approximated “insignificant effects” as EC_{01} values.

Finally, it was necessary to restrict the selection of test agents to those that produced a maximal response similar to 17 β -estradiol. The reason for this constraint lies in the mathematical features of the CA concept: CA estimates concentrations of mixtures (and single agents) associated with predetermined effect levels. With estrogenic chemicals that yield only submaximal effects at saturating concentrations, this would have led to mixture effect prediction curves not exceeding the lowest maximal effect of any single mixture component. In many cases this might have complicated the assessment of agreement between experimental observation and predicted mixture effects. To avoid such ambiguities, we used only xenoestrogens with maximal effects of at least 60% of that seen with 17 β -estradiol in mixture studies.

Materials and Methods

Chemicals. We purchased 17 β -estradiol ($\geq 98\%$ pure) and genistein (98% pure) from Sigma Chemical Company Ltd. (Dorset, UK); resorcinol monobenzoate (99% pure), phenyl salicylate (99% pure), benzyl-4-hydroxyphenol (99% pure), and 2,4-dihydroxybenzophenone (99% pure) from Aldrich Chemicals (Dorset, UK); bisphenol A (4,4'-isopropylidene diphenol; 97% pure) from Acros Organics (Geel, Belgium); and 4'-chlorobiphenyl-4-ol ($> 95\%$ pure), 2,3,4-trichlorobiphenyl (99.9% pure), 2',5'-dichlorobiphenyl-4-ol ($> 95\%$ pure), 2,3,4,5-tetrachlorobiphenyl ($> 97\%$ pure), and 2',3',4',5'-tetrachlorobiphenyl-4-ol (95% pure) from Ultra Scientific (North Kingston, RI, USA). We used the agents as supplied; we prepared 1 mM stock solutions in HPLC-analyzed absolute ethanol (Mallinckrodt Baker, Deventer, Holland). Stock solutions of the mixtures were also

made at 1 mM by combining appropriate volumes of stock solutions. Stocks and subsequent dilutions were kept in critically cleaned glass containers and stored at -20°C . All other chemicals used were research grade from Sigma Chemical Company, unless otherwise stated. We selected many of the compounds that were used by Miller et al. (2001).

The recombinant yeast estrogen screen. We followed a protocol developed by Routledge and Sumpter (1996) exactly as described by Rajapakse et al. (2001). We obtained single-agent data from at least three experiments run in duplicate, and mixture samples were run in duplicate on at least two separate occasions. Nominal concentrations were used.

Dosimetry. We constructed scatter plots of corrected absorbance values (effect) versus log concentration and analyzed them using the best-fit approach (Scholze et al. 2000). We selected the best fit from a number of nonlinear regression models for final data analysis. In these studies, we used the asymmetric (or three-parameter) Hill function:

$$\text{Effect} = \text{Min} + \frac{(\text{Max} - \text{Min})}{1 + \left(\frac{c}{EC_{50}}\right)^{-p}}, \quad [1]$$

where Min and Max are the minimal and maximal observed effects, respectively; c is the concentration of test agent; EC_{50} is the concentration of test agent yielding half-maximal effects; and p is the slope parameter. The 95% confidence intervals (CIs) of the best estimate of mean effects were also calculated. Nonlinear curve fitting was carried out using SigmaPlot (version 5.0; SPSS Inc., Chicago, IL, USA).

Determinations of low effect concentrations. We used Dunnett's t -test (Dunnett 1955) to estimate NOECs. EC_{01} values were determined by interpolation using the best-fit regression model for each tested chemical.

Calculation of predicted mixture effects. After comprehensive concentration-response analysis of the single agents, the responses of mixtures of 17 β -estradiol with the xenoestrogen pool (i.e., all 11 chemicals combined in proportion to their EC_{01} values; Table 1) were predicted assuming additive combination effects. Despite the numerous reports highlighting the shortcomings of ES, it is still being applied incorrectly. For this reason, the expected effects for three mixture ratios were calculated using not only the appropriate concept of CA but also ES, in the hope of demonstrating experimentally its failure under these conditions. A detailed description of the concepts and their mathematical derivation was published by Rajapakse et al. (2001).

Assessing mixture predictions. The validity of the predicted mixture effects was evaluated experimentally using 1:100,000, 1:50,000, and 1:25,000 (17 β -estradiol:xenoestrogen pool) mixtures. Master stock solutions (1 mM) were made and serially diluted to cover the range of concentrations modeled in the predictions.

The impact of xenoestrogens in mixtures with 17 β -estradiol becomes discernible when mixture effects are plotted in terms of the concentration of steroid hormone in the mixture, along with the dose-response curve of the hormone alone. A shift of the mixture dose-response curve to the left of the 17 β -estradiol curve represents the impact of the xenoestrogens. The modulation is considered significant when the 95% CIs for the mixture and 17 β -estradiol regressions do not overlap.

Results

Concentration-response analysis of individual mixture components. All tested agents induced activation of the human estrogen receptor α (hER α) in a concentration-dependent manner.

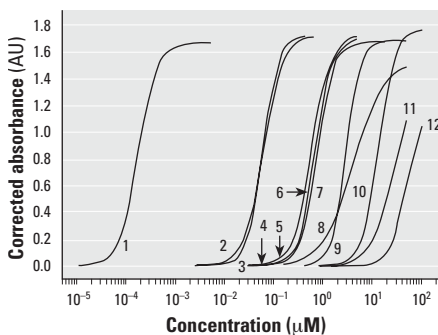


Figure 1. Concentration-response curves for the 11 xenoestrogens and 17 β -estradiol in the YES assay, showing the best-fit regression models (asymmetric Hill function). AU, absorbance units. Test agents: 1) 17 β -estradiol; 2) 2',3',4',5'-tetrachlorobiphenyl-4-ol; 3) 2',5'-dichlorobiphenyl-4-ol; 4) 4'-chlorobiphenyl-4-ol; 5) genistein; 6) 2,4-dihydroxybenzophenone; 7) benzyl-4-hydroxyphenol; 8) 2,3,4,5-tetrachlorobiphenyl; 9) bisphenol A; 10) resorcinol monobenzoate; 11) 2,3,4-trichlorobiphenyl; 12) phenyl salicylate.

Table 1. Summary of asymmetric Hill parameters^a for test agents in the YES.

Compounds ^b	Fraction in XE mix ^c	NOEC (μM)	EC_{01} (μM) ^d	X_{50} (μM)	p -Value	Max
1. 17 β -Estradiol	—	1.15×10^{-5}	2.3×10^{-5}	1.8×10^{-4}	2.273	1.657
2. 2',3',4',5'-Tetrachlorobiphenyl-4-ol	0.0004	0.005	0.008	0.061	2.202	1.711
3. 2',5'-Dichlorobiphenyl-4-ol	0.0009	0.011	0.011	0.060	2.699	1.713
4. 4'-Chlorobiphenyl-4-ol	0.0054	0.054	0.074	0.599	2.161	1.665
5. Genistein	0.0074	0.038	0.086	0.595	2.337	1.672
6. 2,4-Dihydroxybenzophenone	0.0144	0.073	0.124	0.702	2.617	1.713
7. Benzyl-4-hydroxyphenol	0.0102	0.116	0.130	0.787	2.519	1.703
8. 2,3,4,5-Tetrachlorobiphenyl	0.0159	0.295	0.177	4.921	1.324	1.493
9. Bisphenol A	0.0554	0.422	0.632	2.879	2.983	1.676
10. Resorcinol monobenzoate	0.1950	0.990	2.236	13.49	2.546	1.765
11. 2,3,4-Trichlorobiphenyl	0.1592	1.501	2.420	30.13	1.755	1.522
12. Phenyl salicylate	0.5360	9.486	8.824	53.66	2.355	1.232
13. Sum (excluding 1)	1.000	13	14.7	—	—	—

Abbreviations: Max, maximum; XE, xenoestrogen.

^aDefined in “Materials and Methods”; absorbance units for Max. ^bNumbers correspond to those in Figure 1. ^cProportion of each component in “xenoestrogen pool” before combining with estradiol at ratios described in the text. ^dConcentration producing effect 0.017 absorbance units (i.e., 1% of maximal response in YES).

There was relatively little variation in the responses produced by the test chemicals. The regression lines of all the mixture components were of similar shapes and slopes, with the exception of bisphenol A, 2,3,4-trichlorobiphenyl, and phenyl salicylate, which produced somewhat shallower curves (Figure 1). However, there were considerable variations in potency. For example, the median effect concentration of 17 β -estradiol was approximately 350 times lower than that of the most potent tested xenoestrogen, 2',3',4',5'-tetrachlorobiphenyl-4-ol, and over 300,000 times lower than that of phenyl salicylate, the weakest of all tested agents. The concentration–response data for 2,3,4-trichlorobiphenyl and phenyl salicylate did not show the leveling off normally expected to occur at higher concentrations because, due to concerns of limited aqueous solubility, we did not test concentrations above 100 μ M.

Parameters of the regression models and the mixture ratio of the pool of the 11 xenoestrogens are summarized in Table 1.

Estimation of low effect concentrations. We have estimated low effect concentrations of all tested chemicals in two ways: *a*) NOECs were determined using Dunnett's test, and *b*) concentrations producing 1% of the maximum induced effect in the YES assay (i.e., 1% of 1.765 absorbance units, given by saturating

concentrations of resorcinol monobenzoate) were calculated.

As shown in Table 1, the low effect concentrations estimated using the two methods agreed well, although the NOECs of most chemicals were slightly lower than their corresponding EC₀₁.

Prediction and assessment of mixture effects. To avoid one xenoestrogen contributing disproportionately to the overall combination effect, we made a pool of xenoestrogens by mixing the agents in proportion to their EC₀₁ values. In turn, we combined this “pool” with 17 β -estradiol at mixture ratios of 1:100,000, 1:50,000, and 1:25,000 (17 β -estradiol:xenoestrogen pool). On the basis of the relative prevalence of each chemical in the mixture and of their concentration–response relationships, we calculated mixture effect predictions using CA and ES. The predictions were computed assuming additive combination effects and then tested experimentally.

There was good agreement between observed combination effects and those predicted by the CA concept (Figure 2). Thus, the combined effect of all the xenoestrogens and 17 β -estradiol can be called additive. In line with expectation, the observed mixture concentration–response curves shifted to lower concentrations, as the relative amount

of 17 β -estradiol increased (median effect concentrations were 6.2, 4.5, and 3.2 μ M for the 1:100,000, 1:50,000, and 1:25,000 17 β -estradiol:xenoestrogen pool, respectively).

In contrast, the predictions calculated using ES consistently and systematically underestimated the experimentally observed combination effects, independent of effect level. Furthermore, ES was conspicuously unable to model the leveling off of responses usually seen at high concentrations. Using ES as the assessment model, we would have concluded, erroneously, that the xenoestrogen–estradiol mixtures acted synergistically, because the observed combination effects exceeded those predicted by ES.

Impact of xenoestrogens on the effects of 17 β -estradiol. Due to the high potency of 17 β -estradiol, it is conceivable that the observed effects of the xenoestrogen–estradiol mixtures were almost entirely due to the action of the steroid hormone. Whether or not this is the case is not immediately obvious from the plots of mixture effects against the total concentration of all mixture components shown in Figure 2. To delineate the effect of the hormone from the effects contributed by the xenoestrogen pool, we normalized the total mixture concentrations for 17 β -estradiol levels and plotted the observed responses against the 17 β -estradiol content of

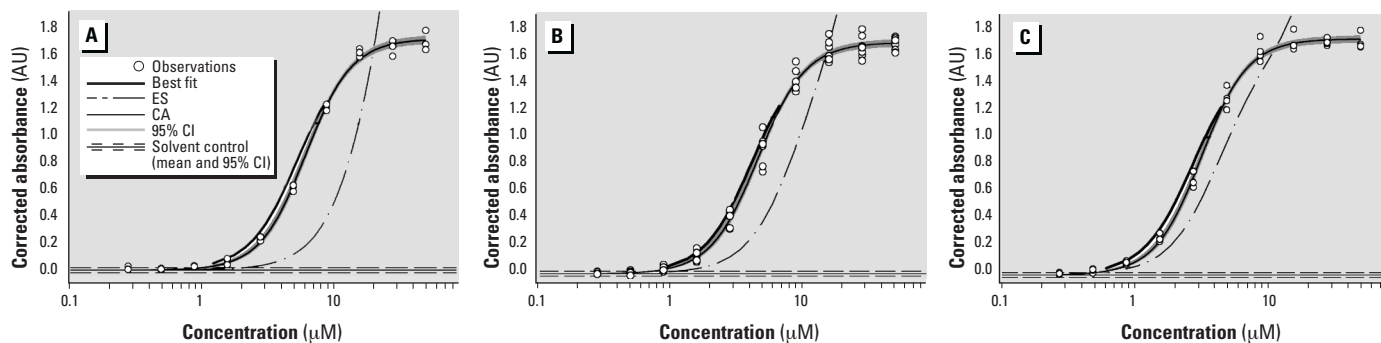


Figure 2. Predicted and observed mixture effects of mixtures of 17 β -estradiol and 11 xenoestrogens. AU: absorbance units. The xenoestrogens were pooled in proportion to their EC₀₁ values before being combined with 17 β -estradiol at mixture ratios of (A) 1:100,000; (B) 1:50,000 and (C) 1:25,000 17 β -estradiol:xenoestrogen pool.

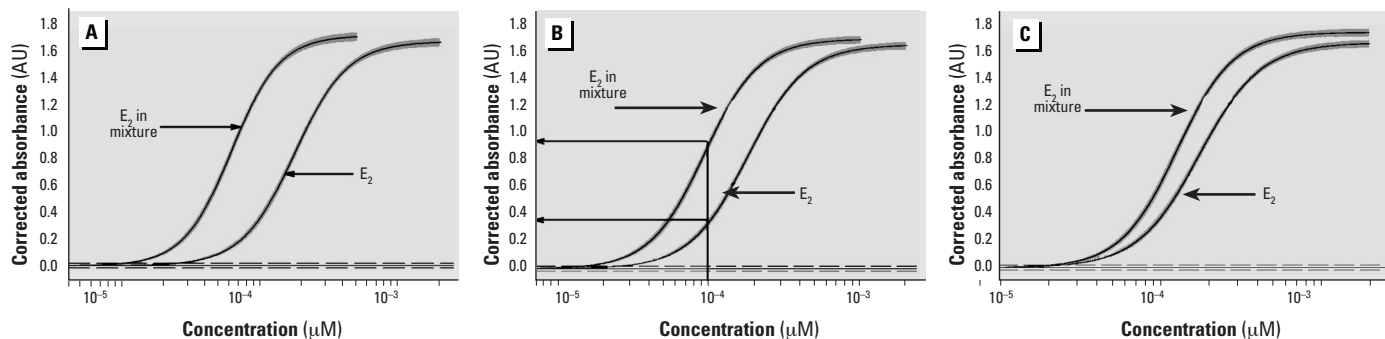


Figure 3. The impact of xenoestrogens on the effects of 17 β -estradiol (E₂) in three mixtures: (A) 1:100,000, (B) 1:50,000, and (C) 1:25,000 E₂:xenoestrogen pool. AU, absorbance units. Best fits, with 95% confidence belts, to the experimentally observed mixture data are plotted in terms of the E₂ concentrations, alongside the concentration–response curve for E₂ alone. The arrows in (B) indicate the concentration and the corresponding effects highlighted in Figure 4.

the mixtures. The resulting best-fit regression curves were then compared to that of the hormone on its own (Figure 3). If the xenoestrogens contributed significantly to the total combination effect, increases in response, resulting in displacements of the mixture curves toward lower concentrations, would be expected. The extent of this leftward shift relative to the 17 β -estradiol curve should be more pronounced, the higher the xenoestrogen content of the mixtures.

These expectations were borne out by our experimental observations. With all three mixtures, the contribution of the xenoestrogens revealed itself as a shift of the mixture concentration–response curves to the left of the 17 β -estradiol curve. This shift was most notable in the 1:100,000 mixture, in which the proportion of xenoestrogens was highest. Even with the 1:25,000 mixture, the xenoestrogens significantly modulated the action of the steroid hormone, judged by the lack of overlap of the respective 95% CIs of the best-fitted regression models.

Modulation of the effects of 17 β -estradiol by xenoestrogens at concentrations below individual NOECs. We became interested in comparing the joint effects of xenoestrogen–estradiol mixtures with the responses expected to occur after administration of each component at concentrations well below the individual NOEC. As shown by the data compiled in Table 1, the total mixture concentrations resulting from combining all 11 xenoestrogens and 17 β -estradiol at their NOECs or EC₀₁ levels are 13 and 14.7 μ M, respectively. Figure 4 shows the effect of 5 μ M of the 1:50,000 mixture. This concentration was chosen because it lies on the linear portion of the mixture concentration–response curve and is the lowest tested concentration where the dose–response curves of the mixture and of 17 β -estradiol are parallel. Additionally, this concentration is well below the sum of the NOECs and EC₀₁ values of the xenoestrogens in the mixture. We can therefore safely assume that none of the xenoestrogens would have produced effects detectable with the YES assay when applied singly at these levels.

Figure 4 also shows the responses expected to result from the levels of 17 β -estradiol present in the mixture. Crucially, combination with 11 weak xenoestrogens led to dramatic enhancements of mixture responses, although each of the 11 xenoestrogens was present at levels well below those that induce measurable responses. There was more than a doubling of effects relative to those of 17 β -estradiol.

It becomes immediately obvious from Figure 4 that calculation of combination effects by computing the arithmetic sum of individual responses (ES) led to dramatic underestimation of observed joint effects. In

contrast, CA yielded a prediction that agreed well with experimental observation.

Discussion

Considering the feasibility of multicomponent mixture studies involving more than 10 chemicals, there were initial concerns that multiplication of errors would undermine the predictability of joint effects. However, our study shows that it is possible to predict accurately the joint effects of multicomponent mixtures of xenoestrogens. Together with earlier observations from our laboratory (Silva et al. 2002), the data presented here confirm the usefulness of CA in anticipating combination effects produced in the YES assay. With the 1:50,000 mixture, there was considerable overlap between the predicted concentration–response curve and the 95% CI of the regression model (Figure 2). Mainly because of the very tight CIs of the regression models for observed mixture effects, the predicted curves for the 1:100,000 and the 1:25,000 mixtures failed to meet the overlap criterion, but they were close enough to the observed values to justify the conclusion of additive joint effects. To a large degree, the small differences between prediction and observation were due to the low biologic variability of the YES assay. It remains to be seen whether results of similar quality can be achieved with bioassays that show greater variability.

Perhaps the most striking finding of our study is the demonstration that large numbers of weak xenoestrogens are able to modulate significantly the effects of the potent steroidal estrogen 17 β -estradiol. This modulation occurred even when each individual xenoestrogen was present at levels that did not induce measurable effects, well below the individual NOEC and EC₀₁ values. Although it may at first appear paradoxical, this phenomenon can be explained in terms of the premises of the CA concept. The concept assumes that all components of a mixture act in a similar way. From a pharmacologic point of view, they can be thought of as behaving as dilutions of one another. Thus, in interacting with the estrogen receptor, each component will add to the overall joint effect in proportion to its individual potency and its concentration. It follows that infinitesimally low concentrations of an agent will contribute to the mixture effect, even though, upon administration on its own, no response (zero effect) will be produced. If the number of agents is sufficiently large, their concentrations will combine to produce measurable responses. Provided these responses are sufficiently large, effect modulations of potent steroid estrogens will occur, as observed here. Simple addition of effects, as in the ES method, is unable to describe the behavior of mixtures of xenoestrogens and 17 β -estradiol (Figure 4) and will

underestimate biologic responses (Berenbaum 1985; Kortenkamp and Altenburger 1999; Silva et al. 2002).

It was our intention to model a scenario in which all components of a mixture interact with the same receptor, the hER α , in the genetic context of the yeast cell reporter gene construct. Our results show clearly that under such conditions, not even concentrations below NOECs can be considered to be without effect on steroidal estrogens, provided exposure is to sufficiently large numbers of receptor agonists. Due to the characteristics of the YES assay, however, the influence of other signal transduction pathways and effector chains responsible for estrogen-related effects (and their inhibition) could not be captured. It is well recognized that the effects of steroid hormone receptor agonists/antagonists not only are determined by ligand–receptor binding but also depend on interactions with specific effector molecules (Katzenellenbogen et al. 1996). One class of such effectors, accessory proteins called coregulators, are able to modulate the transcriptional activity of steroid hormone receptors, even in the absence of classical ligands (reviewed by Robyr et al. 2000; Rosenfeld and Glass 2001). It remains to be seen how the convergence of ligand-dependent and -independent mechanisms in different cellular contexts might impact the joint effects of xenoestrogens and related compounds. Similar considerations apply to the possible antagonizing effects of chemicals such as coplanar polychlorinated biphenyls, dioxins, and furans.

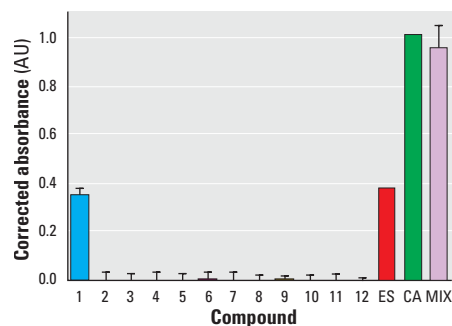


Figure 4. The effects produced by each mixture component at the concentrations present in 5 μ M of the 1:50,000 mixture. Also shown are the predicted mixture effects calculated by using ES and CA and the observed mixture effect (MIX). Test agents (individual concentration): 1) 17 β -estradiol (100 pM); 2) 2',3',4',5'-tetrachlorobiphenyl-4-ol (1.9 nM); 3) 2',5'-dichlorobiphenyl-4-ol (4.6 nM); 4) 4'-chlorobiphenyl-4-ol (26.9 nM); 5) genistein (37.1 nM); 6) 2,4-dihydroxybenzophenone (71.9 nM); 7) benzyl-4-hydroxyphenyl (71.9 nM); 8) 2,3,4,5-tetrachlorobiphenyl (79.5 nM); 9) bisphenol A (276.4 nM); 10) resorcinol monobenzoate (974.7 nM); 11) 2,3,4-trichlorobiphenyl (795.9 nM); 12) phenyl salicylate (2.68 μ M). Error bars indicate the upper 95% confidence limit of responses. In view of the good agreement between CA prediction and experimental observation (MIX) the combined effect of all agents may be called (concentration) additive.

These agents may oppose the effects of estrogenic chemicals by down-regulating ER α expression, up-regulating 17 β -estradiol-metabolizing enzymes, and altering the expression of estrogen-inducible genes (Gillesby and Zacharewski 1998). It will be revealing to investigate whether such effects occur at environmentally relevant concentrations.

Taken together, our findings from this and previous studies allow several interconnected conclusions to be drawn. First, the biologic effects of xenoestrogens cannot be dismissed as insignificant solely on the basis of their low potency compared with steroidal estrogens. Considered in isolation, the contribution of individual xenoestrogens at the concentrations found in wildlife and human tissues will always be small. However, such reasoning cannot be used to support claims of negligible health risks from weak xenoestrogens, because the number of xenoestrogens present in wildlife and humans is unknown but likely to be very large. Whether there are risks associated with xenoestrogens depends also on the influence of chemicals that antagonize estrogenic effects. It remains to be seen whether these chemicals, at environmentally relevant concentrations, are able to neutralize the effects of xenoestrogens.

Second, the fascination with synergistic effects that has gripped the endocrine disruptor field in the wake of the paper by Arnold et al. (1996) is misguided. It has led to a disregard of the significance of seemingly unspectacular additive combination effects. Our results show clearly that additivity is important and deserves serious attention in the assessment of chemicals with estrogenic activity.

Third, by not taking combination effects into account, significant underestimations of the effects associated with exposure to xenoestrogens are likely. In our experimental model, we have demonstrated in principle that every xenoestrogen, however weak, may add incrementally to the total estrogenic effect, even at very low concentrations, and even in the presence of potent endogenous steroidal estrogens. Although the limitations

of our assay system are obvious (it is unable to model antiestrogenic effects and transcriptional activation in different cellular contexts), it is safe to conclude that the discourse about endocrine disruptors cannot be limited to single agents and their effects. There have been reports of low-dose effects of estrogenic chemicals (Howdeshell et al. 1999; vom Saal et al. 1997). In most cases, however, the outcome of single-agent hazard and risk assessment exercises is the conclusion that individual estrogenic chemicals pose no hazard because they are present at very low, apparently ineffective levels in humans and wildlife. A more balanced approach to risk assessment is required. It is imperative to take stock of the total estrogenic burden of humans and wildlife and assess systematically the possible influence of environmentally relevant agents that may have the ability to neutralize the effects of estrogens.

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