Mixtures of Four Organochlorines Enhance Human Breast Cancer Cell Proliferation

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In view of the large differences between the concentrations of estrogenic chemicals needed to elicit effects in *in vitro* assays and their levels in human tissues, it is hard to explain possible health risks in terms of exposure to individual compounds. Human populations, however, are exposed to mixtures of estrogenic and estrogen-like agents and it is necessary to consider the impact of combined effects. We assessed the combined effects of 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (*o,p´*-DDT), 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p´*-DDE), β-hexachlorocyclohexane (β-HCH), and 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (*p,p´*-DDT) on the induction of cell proliferation in MCF-7 cells. All four compounds are persistent organochlorines that can be found in human tissues. We performed extensive concentration–response analyses with the single agents to predict the effects of two mixtures of all four compounds with different mixture ratios. We calculated the predictions by using the pharmacologically well-founded models of concentration addition and independent action and then tested them experimentally. *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT acted together to produce proliferative effects in MCF-7 cells. The combined effect of the four agents could be predicted on the basis of data about single agent concentration–response relationships. Regression analysis demonstrated that there were combination effects even when each mixture component was present at levels at or below its individual no-observed-effect-concentration. We assessed combination effects in two ways: First, evaluations in relation to the proliferative responses induced by single mixture components revealed that the combination effects were stronger than the effects of the most potent constituent. Thus, according to this method of evaluation, the combined effects may be termed synergistic. Second, comparisons with the expected effects, as predicted by concentration addition and independent action, showed excellent agreement between prediction and observation. With this approach, the combined effect of all four compounds can be termed additive. *Key words*: breast cancer, E-Screen assay, estrogenic agents, MCF-7 cells, mixture effects, organochlorine compounds. *Environ Health Perspect* 109:391–397 (2001). [Online 27 March 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p391-397payne/abstract.html

There is a continuing, worldwide rise in breast cancer and other endocrine-related cancers such as neoplasms of the testis and prostate (*1*). In breast cancer, cumulative exposure of breast tissue to the endogenous hormone 17β-estradiol [1,3,5(10)-estratriene-3, 17β-diol] is thought to be one of the main risk factors, and several epidemiological studies have shown that risk is indeed strongly linked to elevated serum levels of the free, bioavailable hormone (*2–4*). However, the rise in breast cancer is not explained entirely by internal exposure to endogenous hormones or genetic predisposition. It has been suggested that environmental chemicals that can mimic endogenous estrogens may play a role in the development of the disease (*1*), but this idea remains controversial.

Attempts to link cancer risks to environmental chemicals are complicated by the fact that their estrogenic potency is low compared to that of 17β-estradiol. The disturbing trends in the incidence of breast and testicular cancer may therefore be difficult to explain simply in terms of exposure to individual organochlorine compounds.

Several recent case–control studies of a possible association between breast cancer and serum levels of single chemicals such as 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (*o,p´*-DDT), 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p´*-DDE), or polychlorinated biphenyls (PCBs) have yielded negative or inconclusive results (*5–7*).

At any given time, human populations are exposed to a multitude of agents with estrogenic or estrogen-like activity—so-called xenoestrogens or exoestrogens (*8*). Through their lipophilicity and persistence, many of these agents accumulate in adipose tissue and are present in blood serum. They are likely to act not only with each other but also with endogenous estrogens. It is therefore crucial to assess the effects of mixtures of xenoestrogens, a challenge regarded by many government agencies and expert decision-making bodies all over the world as a major research priority (*9,10*).

Unfortunately, the assessment of combination effects of xenoestrogens is hampered by disputes and controversies. A report (*11*) describing synergism between estrogenic pesticides in a yeast reporter system could not be reproduced (*12,13*) and was eventually withdrawn (*14*). The ensuing debate has cast doubt on the relevance of mixture effects of xenoestrogens. Conclusive evidence of the ability of estrogenic chemicals to produce combination effects when present as mixtures is still missing. Recently, Shekhar et al. (*15*) have contributed an analysis of the combined effects of *o,p´*-DDT and 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (*p,p´*-DDT) in MCF-7 human breast cancer cells. They suggested that the presence of *p,p´*-DDT led to enhanced responses in combination with estradiol or *o,p´*-DDT. However, Davidson and Yager (*16*) pointed out that a lack of adequate dose–response analyses in Shekhar and colleagues' paper complicated the interpretation of their data and emphasized that accurate assessments of combination effects in terms of synergism, additivity, and antagonism require extensive dose–response analyses.

These discussions have motivated us to explore the combined effects of *o,p´*-DDT, *p,p´*-DDE, β-hexachlorocyclohexane (β-HCH), and *p,p´*-DDT, ubiquitous environmental pollutants that can be found in the adipose tissue of human subjects. All four compounds can induce cell proliferation in estrogen-dependent breast cancer cells (*17,18*), but exert their effects in differing and often poorly understood ways. Two of the chemicals, *o,p´*-DDT and *p,p´*-DDT, are estrogen receptor agonists (*17,19*), whereas β-HCH and *p,p´*-DDE stimulate cell division independent of estrogen receptor-mediated pathways (*18,20*). The first aim of our studies was to assess whether the four chosen agents can act together in causing cell proliferation in MCF-7 cells.

The combined effects of mixtures of agents are commonly assessed in terms of synergism, additivity, or antagonism. Such evaluations critically rely on quantitative estimations of what the expected effect of a mixture should be. If the observed responses are stronger or weaker than expected, the combined effect can be called synergistic or antagonistic, respectively. If expectations are met, the mixture effect is considered additive (*21*).

Combination effects are also frequently evaluated in relation to the effects of the most potent individual mixture components. Thus, synergism is thought to exist when the mixture effect exceeds those of the most effective single component (*22,23*). For our analysis we have used both approaches to evaluating combination effects.

Two main ways of modeling quantitatively the expected effects of mixtures can be distinguished. Some methods use information about underlying mechanisms of action to describe expected mixture effects (*24,25*). Alternatively, quantitative estimations of expected mixture effects can be derived from data about concentration–response relations of the individual mixture constituents ("mechanism-free" approach) (*26*).

The mechanistic approach has merits with simple systems such as isolated proteins or enzymes, but leads to problems where the mode of action of agents and other interactions between agent and organism are insufficiently understood. The fragmentary knowledge of the ways in which *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT induce cell proliferation restricted our choice to mechanism-free concepts for defining expected combination effects. Here, only "inputs" (i.e., concentrations of test agents) and "outputs" (i.e., cell proliferation) are regarded as accessible to analysis, while the test system itself (i.e., the processes of uptake of test agents into cells, metabolism, induction of signaling pathways leading to cell division, and the like) is considered a "black box." As long as the effects of individual agents and mixtures are analyzed within the same system and in relation to identical endpoints, mixture effects can be predicted from the potency of individual agents, regardless of the complexities of the system (*26*).

A second aim of our studies was to evaluate whether mixture effects of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT can be accurately predicted on the basis of information about concentration–response relationships of individual agents. A popular and widely used method of calculating expected mixture effects from data about the potency of individual mixture constitutents assumes that the combined additive effect of a mixture should always be equal to the arithmetic sum of the effects of its components (*13,15, 27–30*). Deviations from this expectation are then classified as synergisms or antagonisms. It is frequently overlooked that this concept, termed effect summation, can be applied only to linear dose–response curves. It leads to unreliable predictions when applied to the sigmoidal curves usually seen with estrogenic agents (*26,31,32*). For this reason, we did not adopt effect summation for the present studies.

Instead, we employed concepts that can be applied to sets of compounds with non-linear dose–response curves. The two reference models of concentration addition and independent action are well suited for such purposes. Concentration addition goes back to Loewe and Muischnek (*33*) and rests on the assumption that chemicals act in a similar way. The model states that effects can be produced by replacing one compound totally or partly with other constituents. Each component is thought to contribute to the overall mixture effect by acting in proportion to its concentration, even below threshold concentrations. Independent action was originally developed by Bliss (*34*) on the basis of stochastic considerations and assumes that compounds act on different subsystems in organisms, with different sites of action. When present at subthreshold doses, mixture components will not contribute to the overall mixture effect.

In the past there have been acrimonious disputes about the general applicability of either model, but now both are regarded as equally valid methods for predicting combination effects (*35*). Consequently, we have used both models side by side to calculate expected effects of two mixtures with differing mixture ratios. We then tested the expectations experimentally. This so-called fixed mixture ratio design lends itself to analyzing the effects of multiple mixtures, particularly at low effect concentrations. It was given preference over the "classical" pharmacological design, where the concentration of one agent is held constant and the influence of varying concentrations of a second agent are studied (*36*). With this experimental approach, the study of combination effects at low concentrations of all mixture components is not possible because one mixture constituent is always present at a concentration that produces significant effects.

We measured the stimulation of cell proliferation in MCF-7 human mammary epithelial cells. This assay is regarded as a relevant model system for assessing the effects of estrogenic and estrogen-like agents (*16*).

Materials and Methods

MCF-7 Cell Proliferation Assay. MCF-7 cells were maintained in 75 cm^2 cell culture flasks (Greiner, Frickenhausen, Germany) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Paisley, UK) supplemented with 5% heat inactivated fetal calf serum (FCS; Gibco BRL). These were kept in a humidified incubator, 37°C, 5% CO$_2$ over a maximum of 15 passages and were routinely tested for mycoplasma.

Estradiol was removed from pooled human serum (National Blood Transfusion Service, London, UK) by treatment with charcoal and dextran, as previously described (*17*), and stored at –20°C for up to 6 months.

The assay was carried out using a modification of Soto's E-SCREEN protocol (*17,37*). Briefly, one 70% confluent 25 cm^2 flask of MCF-7 cells was washed with 5 mL phosphate buffered saline (PBS; Sigma, Poole, Dorset, UK) and trypsinized. Cells were resuspended in 20 mL DMEM + 5% FCS, counted and seeded to 12-well plates (Corning, Cambridge, MA, USA) at a density of 1×10^4 cells per well in 1 mL DMEM with 5% FCS (full medium). After 24 hr the cells were washed with 1 mL PBS. The medium was changed to 1 mL estrogen-free medium [phenol red-free DMEM with 5% charcoal-dextran stripped human serum (CDHus)] and left for a further 72 hr. Again, the medium was changed to 1 mL estrogen-free medium, with test compounds added at a range of concentrations. We assessed cell proliferation after 7 days in culture using the method of Skehan et al. (*38*). Briefly, cells were fixed in cold 10% [weight per volume (w/v)] trichloroacetic acid for 30 min, washed 5 times with water and stained with 0.4% (w/v) sulforhodamine B (SRB; Sigma) in 1% acetic acid for 10 min. Unbound SRB was removed by washing in 1% acetic acid, and bound SRB was solubilized with 10 mM Tris pH 10.4. Dye intensity was measured at 510 nm on a plate reader (Labsystems Multiskan, UK). It was shown to increase linearly with cell number (data not shown).

o,p´-DDT (Lancaster, Morecambe, UK; purity > 99%), *p,p´*-DDT and *p,p´*-DDE (Sigma; purity 98%), and β-HCH (J.T.Baker, Milton Keynes, UK; purity 98%) were made up as 1 mM stock solutions in absolute ethanol, diluted in ethanol, and administered to cell cultures so that the final ethanol concentration did not exceed 1% in the media. Solutions of concentrations greater than 1 mM were not made because of concerns about solubility. Nominal concentrations were used. We measured cell proliferation relative to control cultures kept with 1% ethanol in the medium. Ethanol alone, at 1%, had no influence on cell proliferation. We chose phenol red-free DMEM because phenol red

is itself estrogenic (*39*), and we chose pooled human serum over fetal calf serum because of widespread use of growth hormones in cattle, which may interfere with responses.

Data processing. In the MCF-7 assay, absorbance readings of SRB-stained cultures (a direct measure of cell numbers) were compared with readings from untreated controls and expressed as -fold increases. To obtain concentration–response curves with zero effect, we defined control values as 1 and subtracted them from the readings obtained with treated cultures. Thus, a proliferative effect of "0.3" represents a 0.3 + 1 = 1.3-fold increase in cell numbers relative to controls.

Concentration–response analysis and statistical analysis. We constructed scatter plots of relative cell proliferation (MCF-7 assay) versus log concentration and performed concentration–response analyses using the "best fit" approach (*40*). We chose appropriate regression models from a large set of different models, including the commonly used Probit, Logit, Weibull, and Hill models. For each data set we compared the quality of fit and selected the model yielding the best fit for final data analysis. To account for possible heterogeneities in effect variances (heteroscedasticity), we estimated the fit parameters by using the iteratively reweighted least-squares procedure in combination with a nonparametric variance model (*41*). Except for the data for the equimolar mixture of all four agents, the three parameter Langmuir function produced the best fits for all data sets. The data for the equimolar mixture were best fitted by using the four parameter Box-Cox-Logit function (i.e., the logit function with an integrated Box-Cox transformation of the concentration scale; see Table 1).

The 95% confidence belts of estimated mean effects were also determined. All statistical analyses were performed using SAS software (*42*). Table 1 shows a compilation of the non-linear regression models used in this study.

No-observed-effect-concentrations (NOEC) of single agents and mixtures were estimated by using the Dunnett test (*43*).

Mixture testing. We prepared two master solutions of mixtures by diluting 1 mM stocks of each agent, with molar mixture ratios of 1:1:1:1 and 1:10:5:4 (*o,p´*-DDT:*p,p´*-DDE:β-HCH:*p,p´*-DDT). The mixture ratio of the first mixture approximates the ratio of effect concentrations of the individual agents that produce a doubling in cell number. The ratio of the second mixture was chosen to fall in the range of the relative abundance of each of the four organochlorines in human serum in Western industrialized countries (Table 2). We treated MCF-7 cells with serial dilutions of

these master solutions. We ran samples in triplicate and repeated experiments at least twice, so that each concentration–response curve was based on a minimum of 30 observations. We computed predicted mixture effects over a large range of effects and tested the predictions experimentally.

Calculation of predicted mixture effects. The model of concentration addition predicts a concentration of a mixture of agents that produces a predetermined effect. Prerequisites for the calculation of such effect concentrations are information about the relative abundance of an agent in the mixture (mixture ratio) and data on the concentrations of each mixture component that individually produces this same effect. Thus, assuming that the combined effect of the mixture with n components is concentration additive, the following expression will hold for any effect level E:

$$\Sigma\, c_i/EC_i = 1, \qquad [1]$$

where c_i denotes the concentration of agent i in a mixture yielding an effect E, and EC_i the concentration of i needed to produce effect E on its own. Equation 1 can be used to calculate a mixture concentration that produces a predetermined effect, provided the EC_i of the individual mixture components and their relative abundance in the mixture are known. Thus, the concentration c_i of agent i in the mixture is related to the total mixture concentration by

$$c_i = p_i * EC_{mix}, \qquad [2]$$

where p_i is the concentration of the ith compound relative to the total mixture concentration EC_{mix} that is required to produce effect E. Substitution of c_i in Equation 1 gives

$$\Sigma\, (p_i * EC_{mix}/EC_i) = 1, \qquad [3]$$

and rearranging yields

$$EC_{mix} = [\Sigma\, p_i/EC_i]^{-1}. \qquad [4]$$

The effect concentrations EC_i were calculated from the best fits of the concentration–response models of single agents by using the inverse expression of the appropriate regression functions (Table 1).

The model of independent action allows it to calculate the predicted effects e_{mix} of a mixture of known composition by using the expression

$$e_{mix} = 1 - \prod[1 - E(c_i)], \qquad [5]$$

where $E(c_i)$ is the effect E produced by compound i at concentration c, when applied singly. It can be seen from Equation 5 that independent action is a probabilistic model, i.e., $E(c_i)$ is a fraction of a maximal possible effect that cannot exceed 1.

Thus, when this model is applied to proliferative effects $PE(c_i)$, a maximal effect Emax must be defined. For this purpose, the maximal proliferative effect of saturating concentrations of 17β-estradiol was chosen as a reference point and the effects of test agents expressed relative to the maximal effect of 17β-estradiol:

$$E(c_i) = PE(c_i)/E_{max}. \qquad [6]$$

If the concentration–response relationships of all mixture constituents i are described by an appropriate regression model F_i (Table 1), the proliferative effect $PE(c_i)$ can be estimated from the mean effect $F_i(c_i)$ predicted by the regression model. Thus,

$$PE(c_i) = F_i(c_i), \text{ and } E(c_i) = F_i(c_i)/E_{max}. \qquad [7]$$

Substitution of $E(c_i)$ in Equation 5 yields

$$e_{mix} = 1 - \prod[1 - F_i(c_i)/E_{max}]. \qquad [8]$$

Table 1. Nonlinear regression functions employed in this study.

Name	Formula
Langmuir	$P(conc) = 1/(\theta_1 + \theta_2\,(conc)^{(-\theta_3 -1)})$
Logit with Box-Cox transformation	$P(conc) = 1/\{1 + \exp[-\theta_1 -\theta_2(conc^{\theta_3} - 1)/\theta_3]\}$

$P(conc)$ is the mean effect; θ_1, θ_2, and θ_3 are model parameters; and conc is the concentration of the test agent.

Table 2. Levels of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT in human blood serum.

Compound	Range of levels in blood serum (nM)[a]	Levels selected to construct mixture (nM)[b]	References
o,p´-DDT	0.3–2	1	(*44,45*)
p,p´-DDE	8–135	10	(*44–47*)
β-HCH	1–53	5	(*44,45,48*)
p,p´-DDT	0.4–21	4	(*5,44,45,48*)

[a]Data were compiled from the references given. Where necessary, adipose tissue levels were converted to serum levels, assuming that serum levels are typically 250 times lower than adipose tissue levels (*49*). [b]This is equivalent to a 1:10:5:4 molar mixture ratio.

To ensure comparability of the independent action predictions with those of concentration addition, the fractional effects in Equation 8 were rescaled by multiplication with E_{max}, thus:

$$E_{mix} = E_{max} * e_{mix} \text{ and} \quad [9]$$

$$E_{mix} = E_{max} (1 - \prod[1 - F_i(c_i)/E_{max}]). \quad [10].$$

Results

Concentration–response analyses for o,p´-DDT, p,p´-DDE, β-HCH, and p,p´-DDT.

In agreement with previously reported data (17,50) all four agents were able to induce cell proliferation in MCF-7 cells in a typical concentration-dependent manner (Figure 1). The concentration–response plots showed marked differences in shape and position. o,p´-DDT was the most potent of our agents, with a concentration of 0.89 μM yielding an effect of 2 on the effect scale. However, it elicited the smallest maximal effect (2.7) of all four selected chemicals. The largest tested concentration of p,p´-DDE showed an effect of 3.9, but the compound was less potent than o,p´-DDT, with a displacement of the concentration–response curve toward higher concentrations. p,p´-DDT and β-HCH produced concentration–response curves that were almost congruent. Their potency was comparable to that of p,p´-DDE. "Thresholds" were defined as those effect concentrations that correspond to an intersection of the lower 95% confidence interval of mean effects of the regression model with the upper 95% confidence interval of the estimate for untreated control cultures (0 ± 0.035, n = 15). NOECs are also depicted. Because of concerns over limited aqueous solubility of the compounds, we did not test nominal concentrations higher than 10 μM. For this reason, the observed concentration-effect data for p,p´-DDT and β-HCH do not show the leveling off of effects theoretically expected at higher concentrations.

17β-Estradiol was employed as a positive control and produced a maximal response of 5.9, with a median effect concentration EC_{50} of 13.3 pM (37), in excellent agreement with earlier reports (50). Table 3 summarizes key parameters of the relative potency of all four tested agents.

Our results agree broadly with those communicated by Andersen and colleagues (50) and Soto et al. (17), but a lack of published data on quantitative concentration–response relationships with organochlorines precluded us from making more detailed direct comparisons between our observations and previous reports. However, in contrast to the data reported by Shekhar and coworkers (15), we found p,p´-DDT to be a weaker mitogen than o,p´-DDT, mainly because o,p´-DDT

produced higher responses in our hands than those reported by Shekhar et al.; the mitogenic effects observed with p,p´-DDT agree excellently with their data.

To probe the involvement of estrogen receptor activation in MCF-7 cell proliferation, we assessed the effects of the estrogen receptor antagonist tamoxifen. Co-incubations with tamoxifen (1 nM) led to marked reductions in cell proliferation induced by o,p´-DDT and p,p´-DDT, but left the effects of p,p´-DDE and β-HCH unchanged (data not shown). In line with these observations, o,p´-DDT and p,p´-DDT were able to activate estrogen receptors in a yeast-based reporter plasmid system [yeast estrogen screen, performed exactly as described by Routledge and Sumpter (51)], whereas p,p´-

DDE and β-HCH led only to insignificant effects (data not shown).

The effects of mixtures of o,p´-DDT, p,p´-DDE, β-HCH, and p,p´-DDT: prediction and observation.

We used the single agent concentration–response relationships shown in Figure 1 to predict concentration–effect curves for mixtures of all four agents, assuming additive combination effects. The predictions were computed using the models of concentration addition and independent action. Mixtures with two different mixture ratios were investigated, one equimolar (Figure 2A) and the other employing molar mixture ratios that fall in the range of the relative prevalence of the four chemicals in human blood serum, o,p´-DDT: p,p´-DDE : β-HCH : p,p´-DDT = 1:10:5:4 (Figure 2B).

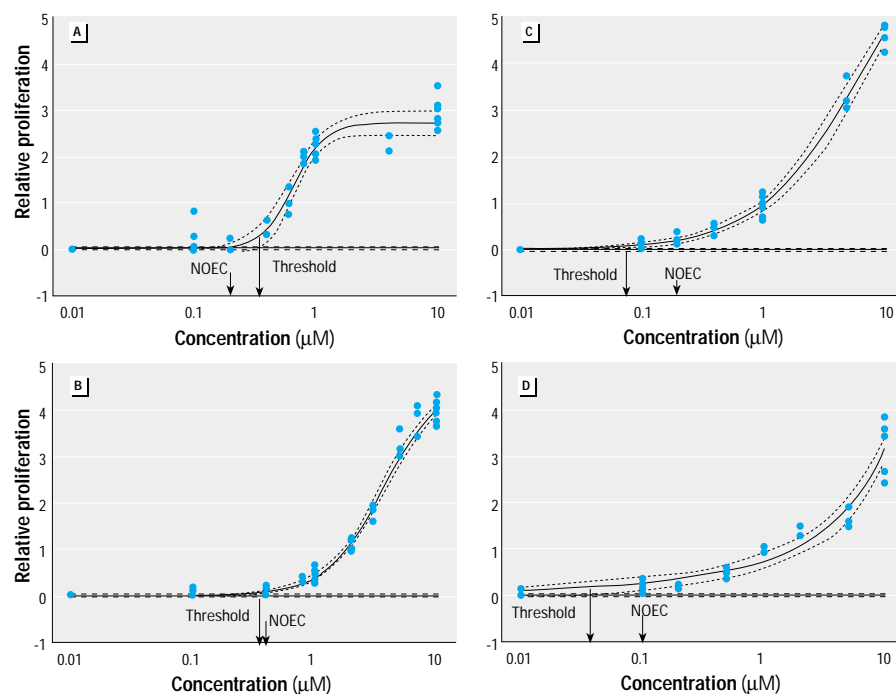**Figure 1.** Concentration–response analyses for o,p´-DDT (A), p,p´-DDE (B), β-HCH (C), and p,p´-DDT (D) in the MCF-7 cell proliferation assay. Data were fitted to the Langmuir function using iteratively reweighted least squares. For p,p´-DDT, least squares were used. Solid curved lines represent the best fits to the Langmuir function; dotted lines are the upper and lower 95% confidence intervals (CIs) of mean responses. The horizontal solid and dashed lines are mean ± 95% CI of the population mean of untreated controls. Arrows represent NOECs estimated using the Dunnett test, or threshold levels as defined in the legend to Table 3. Experiments were performed in triplicate and repeated independently.

Table 3. Summary of characteristics of single agent- and mixture-concentration response relationships.

	o,p´-DDT	p,p´-DDE	β-HCH	p,p´-DDT	1:1:1:1 mix	1:10:5:4 mix
Number of effect data	41	49	25	31	83	36
Nonlinear regression model	Langmuir	Langmuir	Langmuir	Langmuir	Box-Cox-Logit	Langmuir
Concentration producing effect of 2[a,b]	0.89 μM	3.16 μM	2.45 μM	5.62 μM	1.74 μM	3.31 μM
Maximal effect[a]	2.7	ND	ND	ND	3.1	2.5
NOEC[c]	0.2 μM	0.4 μM	0.2 μM	0.1 μM	0.2 μM	0.7 μM
Threshold level[d]	0.35 μM	0.34 μM	0.075 μM	0.037 μM	0.018 μM	0.50 μM

Abbreviations: Mix, mixture. ND, no data.
[a]Relative cell proliferation as defined in "Materials and Methods." [b]Effect concentrations were estimated by using the best fit regression models. [c]The largest tested concentration that produced effects not significantly different from those of untreated controls. Determined using the Dunnett test. [d]The intersection of the upper 95% confidence interval of the mean response of untreated controls (0 ± 0.0353, n = 15) with the lower 95% confidence interval of the best fit of the regression model.

In the concentration range below 4 μM, the two models yielded very similar predictions, although the responses in the linear portion of the curves expected on the basis of independent action were slightly smaller than those predicted by concentration addition. There were, however, differences in the predicted maximal effects of the equimolar mixture. Although the concentration addition predictions began to plateau off at concentrations exceeding 4 μM, independent action projected a continuous increase with concentration (Figure 2A). Because the concentration addition concept calculates concentrations corresponding to predetermined effects, mixture responses higher than the lowest maximal effect induced by one of the mixture components alone (here, *o,p´*-DDT; see Figure 1) cannot be predicted. Therefore, the line representing the concentration addition prediction in Figure 2B cannot be extended to higher concentrations.

The responses of both mixtures were tested experimentally on the basis of the range of the predicted effect concentrations. As shown in Figure 2, a concentration-dependent increase in cell proliferation was observed. The effects were reproducible, with variabilities comparable to those seen with the single agents.

Assessment of combination effects. We assessed the combined effects of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT on MCF-7 cell proliferation in relation to the effects of the most potent mixture component and in relation to the expected responses, as predicted by the models of concentration addition and independent action.

Assessments of combination effects with respect to the responses of an individual mixture component require the data to be presented not in relation to the total concentration of all mixture components, as in Figure 2, but with re-scaled axes that show the concentration of individual agents in the mixtures. Such plots are presented in Figure 3. In the case of the equimolar mixture, the analysis was performed in relation to *o,p´*-DDT, the most potent of all four compounds. Given its abundance in the 1:10:5:4 mixture, *p,p´*-DDE was deemed the most potent mixture component, and mixture responses were plotted using an axis depicting *p,p*-DDE concentrations in the mixture.

With the equimolar mixture, the presence of *p,p´*-DDE, β-HCH, and *p,p´*-DDT led to increases of the effects of *o,p´*-DDT (Figure 3A). Although particularly marked in the median effect range, increases were also discernible at low effect concentrations. Except in the very low effect range, there was no overlap of the 95% confidence intervals of the mixture- and the single-agent regression models. We therefore became interested

in assessing whether the threshold level of *o,p´*-DDT was altered by the presence of the other mixture components. Thus, for *o,p´*-DDT administered individually, a threshold concentration of 0.35 FM can be estimated. This value changed to 4.4 nM in the equimolar mixtures (Figure 3A).

Similar trends became apparent with the 1:10:5:4 mixture. The presence of the remaining mixture components exacerbated the effects of *p,p´*-DDE. There was a small change of the threshold of *p,p´*-DDE from 0.34 FM when applied individually to 0.25 FM in the mixture.

Assessments in relation to the model predictions showed that the combination effects of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT did not deviate significantly from the additivity expectations of the models of concentration addition and independent action (Figure 2). Applying the criterion of overlap between the prediction curves and the 95% confidence belts of the best fit

regression models of observed effects, no marked deviations could be identified. However, both models slightly overestimated the effects of the 1:10:5:4 mixture in the low effect range. At maximum effect concentrations the observed mixture responses began to plateau off, a feature modeled well by concentration addition for the equimolar mixture (Figure 2A). With both mixtures the performance of independent action was poor in this effect range.

Mixture effects at low effect concentrations of individual mixture constituents. We also considered whether *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT in combination were able to produce effects at low effect concentrations. To this end, we analyzed combination effects for mixture concentrations where each agent was present at or below its individual NOEC. At a concentration of 0.4 FM of the equimolar mixture, each agent was present at 0.1 FM. This concentration equals the NOEC for *p,p´*-DDT
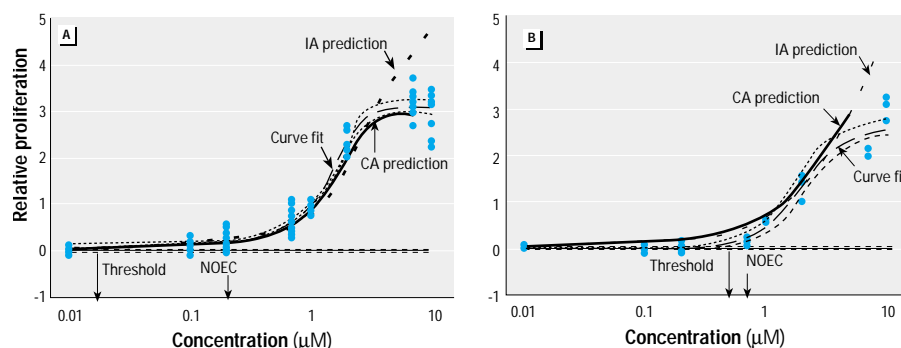

Figure 2. Predicted and observed mixture effects of a 1:1:1:1 (molar ratio) mixture (*A*) and a 1:10:5:4 (molar ratio) mixture (*B*) of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT. Observed mixture effects are shown as blue circles and are triplicates from two to three independent experiments. Predicted effects were calculated using the models of concentration addition (CA) and independent action (IA) and are shown as solid and dotted lines, respectively. The dashed curves are the best fit of the data to the regression model (1:1:1:1 mixture: Box-Cox-Logit function; 1:10:5:4 mixture: Langmuir function) and dotted lines are the upper and lower 95% CIs of mean responses. Arrows represent NOECs estimated using the Dunnett test, or threshold levels as defined in Table 3.
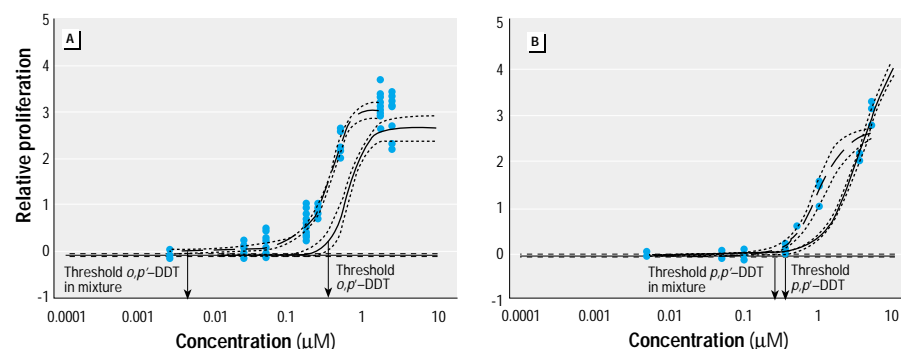

Figure 3. Comparison of mixture effects with the responses of the most potent mixture component. Mixture effects shown in Figure 2 were replotted with concentration axes showing the concentrations of *o,p´*-DDT in the 1:1:1:1 mixture (*A*) and of *p,p´*-DDE in the 1:10:5:4 mixture (*B*). Observed mixture effects are shown as blue circles, with dashed solid lines and dotted lines depicting the best fit and the upper and lower 95% CIs, respectively. Solid sigmoidal lines are the best fit regression models for *o,p´*-DDT (*A*) and *p,p´*-DDE (*B*). Vertical arrows are threshold levels as defined in Table 3.

and is well below the NOECs estimated for the remaining three agents (Table 3). For a concentration of 0.4 FM of the equimolar mixture, the regression model predicts an effect of 0.31, with a 95% confidence interval of ± 0.07 (Figure 2A). This effect is well above the responses seen with untreated control cultures (0 ± 0.035). Because the mixture ratio of the 1:10:5:4 mixture was too dissimilar from the ratio of the NOECs of the mixture components, a similar analysis could not be performed in this case.

Discussion

The assessment of combination effects of estrogenic and estrogen-like agents has in the past been fraught with experimental and conceptual problems [reviewed in Kortenkamp and Altenburger (*31,32*)]. The events leading to the withdrawal of Arnold and colleagues' paper (*11,14*) have polarized views even further. Although it is widely acknowledged that the potential for interactions between xenoestrogens exists (*8,10*), conclusive experimental evidence was lacking. To fill this gap we present here an assessment of the combined effects of the persistent organochlorines *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT in the induction of cell proliferation in MCF-7 cells. We have underpinned our evaluation with extensive concentration–response analyses. Our results show clearly that the four tested agents act together to produce combination effects.

In assessing combinations of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT we compared the observed mixture responses with the effects of the most potent individual mixture constituent on the one hand, and with model expectations on the other. Both approaches are well recognized in pharmacology (*21–23,26*).

Our results show clearly that the observed mixture responses were larger than the proliferative effects of the most potent mixture component. In the equimolar mixture, concentrations of *o,p´*-DDT that on their own would have failed to produce measurable effects led to significant cell proliferation in the presence of the remaining three mixture components. This was accompanied by a large shift of the individual threshold of *o,p´*-DDT toward lower concentrations. The magnitude of this change is related to the curvature of the mixture concentration–response curve, which is shallower in the low effect range than the *o,p´*-DDT curve. Similarly, the proliferative responses of the 1:10:5:4 mixture were stronger than the individual effects of *p,p´*-DDE, although due to the mixture ratio the differences were not as pronounced as with the equimolar mixture in relation to *o,p´*-DDT. The small shift of the individual threshold of *p,p´*-DDE toward lower concentrations was deemed insignificant.

When evaluated in relation to the additivity expectations of the models of concentration addition and independent action, the observed mixture effects of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT agreed well with the predicted effects. Although the model of concentration addition was better suited to predict the leveling off of effects seen at higher mixture concentrations, both models performed equally well in the low and median effect range. Thus, for the first time we demonstrate that the proliferative effects of mixtures of estrogenic and estrogen-like agents can be predicted on the basis of concentration-effect data of individual mixture components. These predictions appeared to be valid over a large range of effect levels for two different mixture ratios.

Our results are relevant to evaluations of mixture effects of estrogenic or estrogen-like agents at low effect concentrations. At 0.4 FM of the equimolar mixture each single mixture component was present at 0.1 FM, a concentration equal to (*p,p´*-DDT) or below (*o,p´*-DDT, *p,p´*-DDE, β-HCH) their individual NOECs (see Table 3). At this point, the best fit regression model predicts effects well above those of untreated controls (Figure 2). Although mixture effects were not tested experimentally at 0.4 FM, these data strongly suggest that *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT can induce cell proliferation when present at or below their individual NOECs. This is further underlined by considering the lowest tested concentration (0.7 FM) that induced significant responses. Here, the concentration of each agent was 0.175 FM—i.e., below the NOECs of *o,p´*-DDT, *p,p´*-DDE, and β-HCH, but higher than the NOEC of *p,p´*-DDT, although the effects of *p,p´*-DDT were small in this range. Because the composition of the 1:10:5:4 mixture was not in proportion to the NOECs of its components, it is hard to demonstrate that combination effects occurred at low effect concentrations in this case. To establish conclusively whether there are combination effects when all individual mixture constituents are present at concentrations that induce unmeasurable, statistically insignificant effects, it will be necessary to conduct studies of mixtures with far more than four components.

It is informative to consider the differences among the concentrations of single agents that are needed to elicit effects in the *in vitro* cell proliferation assay and their levels in human tissues. A graphic representation of key data is given in Figure 4. It shows that the concentrations of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT that individually produce observable effects in the MCF-7 cell proliferation assay are 100- to 1,000-fold

higher than their levels in human blood serum. Thus, it appears unlikely that the four tested compounds can individually induce MCF-7 cell proliferation at the concentrations found in serum. Crucially, however, the gap between physiological levels and concentrations effective *in vitro* reduces to about 10-fold when comparisons are made with the concentration (1 FM) that produced measurable effects in the 1:10:5:4 mixture of all four agents (Figure 4). Further work with mixtures composed of a larger number of xenoestrogens is required to probe whether there are effects at physiological levels. However, this issue is complicated by uncertainties as to what constitutes relevant "physiological levels" of persistent organochlorines in a breast cancer context. Shekhar and colleagues (*15*) have pointed out that the levels of xenoestrogens detected in adipose tissue of human breast cancers can be about 1,000-fold higher than their levels in serum. Given the close proximity between the epithelial cells that line the milk ducts of the female breast and the surrounding adipocytes, it may be possible that epithelial cells are exposed to higher levels of xenoestrogens than suggested by blood serum levels.

Taken together, our results highlight the importance of taking mixture effects into account when assessing the effects of estrogenic and estrogen-like agents. If it is problematic to explain human health risks in terms of exposure to individual agents, the challenge lies in assessing the effects of multiple mixtures of xenoestrogens. It is hard to see how the health risks potentially associated
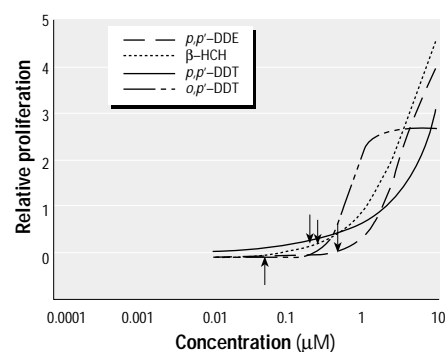
Figure 4. Comparison of the levels of *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT in blood serum (horizontal lines) with the range of observed effects of the single compounds in the MCF-7 cell proliferation assay (curved lines). The same symbols were used for each agent: *o,p´*-DDT (dot-dashed lines), *p,p´*-DDE (dashed lines), β-HCH (dotted lines), and *p,p´*-DDT (thin solid lines). The vertical arrows pointing to the respective single agent curves depict the concentrations at which each agent was present in 1 μM of the 1:10:5:4 mixture. This was the lowest tested concentration that produced effects significantly different from untreated controls (Figure 2B).

with xenoestrogens can be evaluated without taking mixture effects into consideration.

In conclusion, our study shows that *o,p´*-DDT, *p,p´*-DDE, β-HCH, and *p,p´*-DDT act together to produce proliferative effects in MCF-7 cells, and that the combined effect of the four agents can be predicted on the basis of data about single-agent concentration–response relationships. With respect to quantitative expectations based on the potencies of individual mixture components, the combined effect of the four organochlorines can be called additive. In relation to the most potent component of either of the two mixtures, the combination effect can be classed as synergistic. Our findings strongly suggest that there are mixture effects even when each mixture component is present at concentrations that individually produce insignificant effects.

One crucial step to establishing whether xenoestrogens contribute to breast cancer risks is to probe whether they can exert an impact on the already strong effects of endogenous steroidal estrogens when combined at physiological concentrations. Our work provides a rationale for the design of experimental studies addressing this problem.

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