Lack of Synergy by Mixtures of Weakly Estrogenic Hydroxylated Polychlorinated Biphenyls and Pesticides

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We examined the estrogenicity of binary mixtures of the hydroxylated polychlorinated biphenyls (OHPCBs) 2,4,6-trichloro-4'-biphenylol (2,4,6-TCB-4'-OH) and 2,3,4,5-tetrachloro-4'-biphenylol and the pesticides endosulfan and dieldrin. The OHPCBs and pesticides were tested in both the MCF-7 focus assay and a competitive estrogen-receptor binding assay. Although the individual OHPCBs were estrogenic in both assays, there was no synergy when they were combined at various concentrations as equimolar mixtures. Of the pesticides, only endosulfan was estrogenic. Its weak estrogenicity was seen only in the MCF-7 focus assay at the highest concentration tested—10 μ M. There was no synergy of the equimolar mixture of pesticides. To determine whether OHPCBs might respond synergistically when combined with the natural estrogen 17 β -estradiol (E_2), we tested various concentrations of 2,4,6-TCB-4'-OH in the MCF-7 focus assay in combination with physiologically relevant concentrations of E_2 . There was no synergy between 2,4,6-TCB-4'-OH and E_2 . Pretreatment for 3 or 7 days with 2,4,6-TCB-4'-OH had no effect on subsequent foci induced by a combination of 2,4,6-TCB-4'-OH and E_2 . Although our results showing no synergy between the pesticides or the OHPCBs are in contrast to a recent report that binary mixtures of these same compounds produce synergistic responses in estrogen-sensitive assays, they are in agreement with the results from other assays showing a lack of synergy. Considering all results, it appears that synergy of these weakly estrogenic compounds acting through the estrogen receptor is unlikely. — *Environ Health Perspect* 106(Suppl 4):1041–1046 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-4/1041-1046arcaro/abstract.html>

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Introduction

Exposure to environmental chemicals can modify the endocrine systems of wildlife (1,2). These findings have led some to propose that environmental chemicals, acting as xenoestrogens, are involved in reproductive and developmental problems in both humans and wildlife and in the etiology of breast cancer (3–5). Other researchers have suggested that the low potencies of

environmental or xenoestrogens make it likely that their contribution to an estrogen response will be insignificant in comparison with the natural estrogen 17 β -estradiol (E_2) (6,7). However, relevant environmental exposure usually involves mixtures of compounds, whereas studies on the potency of xenoestrogens have typically addressed individual compounds. Accordingly, the issue

of the potency of mixtures of xenoestrogens needs to be addressed.

Arnold and co-workers (8) examined the estrogenicity of binary mixtures of weak xenoestrogens. They reported that mixtures of weakly estrogenic (or nonestrogenic) pesticides (dieldrin, endosulfan, toxaphene, and chlordane) and hydroxylated polychlorinated biphenyls (OHPCBs) (2,4,6-trichloro-4'-biphenylol [2,4,6-TCB-4'-OH] and 2,3,4,5-tetrachloro-4'-biphenylol [2,3,4,5-TCB-4'-OH]) resulted in synergistic increases in estrogen receptor (ER) binding and reporter gene expression in transfection-facilitated yeast and endometrial carcinoma-derived cell cultures. The magnitude of the synergy varied and was the greatest for a mixture of pesticides (dieldrin and endosulfan)—up to a 1000-fold increase for the mixture as compared to the individual pesticides. The OHPCBs showed far less synergy—a 5- to 10-fold increase for the mixture as compared to the individual OHPCBs. The ER binding studies suggested that the synergy was occurring at the level of the ligand binding with the receptor.

Although the paper by Arnold et al. (8) was subsequently retracted (9), other examples of limited synergy by OHPCBs and pesticides had been reported previously. Using temperature-dependent sex determination in turtles as an estrogen assay, Bergeron et al. (10) found that a single concentration of an equimolar mixture of 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH produced a synergistic estrogen response. The number of female hatchlings at temperatures that normally produce 100% males was significantly greater when the turtle eggs were painted with a mixture of 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH than with the same concentration of either compound alone. However, four more recent reports (11–14) failed to find synergy of equimolar mixtures of pesticides and OHPCBs using a variety of estrogen-sensitive assays.

Bergeron et al. (10) suggest that in certain situations weak estrogens can produce synergistic responses. It is not known whether these synergistic responses occur in nontransfected human cells or whether a weak xenoestrogen can produce a synergistic response in combination with low levels of the natural estrogen E_2 . In the present study we address these questions using the MCF-7 focus assay. The MCF-7 cell line, derived from an adenocarcinoma, has been

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Abbreviations used: BCS, bovine calf serum; DMSO, dimethyl sulfoxide; E_2 , 17 β -estradiol; EC₅₀, concentration needed to produce 50% of the maximum response; ER, estrogen receptor; HAP, hydroxyapatite; IC₅₀, concentration needed to inhibit 50% of the maximum response; OHPCB, hydroxylated polychlorinated biphenyl; PCBs, polychlorinated biphenyls; 2,3,4,5-TCB-4'-OH, 2,3,4,5-tetrachloro-4'-biphenylol; 2,4,6-TCB-4'-OH, 2,4,6-trichloro-4'-biphenylol.

studied extensively as an *in vitro* and *in vivo* model of estrogen-dependent breast cancer and displays multiple estrogen-dependent gene expressions (15). Estrogen-dependent preconfluent cell proliferation in specific strains has been used as a marker for estrogenicity (16). We characterized the estrogen-dependent postconfluent development of multicellular nodules or foci on a confluent monolayer in MCF-7 cell cultures (17). This *in vitro* response to estrogens closely mimics the *in vivo* action of estrogen (i.e., an estrogen receptor-mediated induction of cell proliferation in specific target tissue resulting in tissue restructuring), and does not require transfection of ER or reporter constructs. The MCF-7 focus assay is especially relevant to the examination of xenoestrogens because of their proposed association with breast cancer (18).

In the present report, we examined the estrogenicity and potential synergism of the OHPCBs 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH and the pesticides dieldrin and endosulfan in the MCF-7 focus assay and a competitive ER binding assay. We also examined the interaction of various concentrations of 2,4,6-TCB-4'-OH with physiologic levels of E₂. The OHPCBs were estrogenic in both assays. Only one of the pesticides, endosulfan, was weakly estrogenic in the MCF-7 focus assay only. None of the binary mixtures produced a synergistic response. These results demonstrate the usefulness of the MCF-7 focus assay in screening for the potential effects of xenoestrogens.

Materials and Methods

Chemicals

Pesticides and hydroxylated polychlorinated biphenyls (PCBs) (95–99% pure) were obtained from Ultra Scientific (North Kingston, RI), and 5 mM stock solutions were prepared in dimethyl sulfoxide (DMSO). Recombinant human estradiol receptor was obtained from Panvera Inc. (Madison, WI). ³H-labeled estradiol was obtained from NEN Life Science Products (Boston, MA).

Culture medium (DC₅) consisted of Dulbecco's modified Eagle's medium (phenol red-free) supplemented with 5% bovine calf serum (BCS), insulin (10 ng/ml), L-glutamine (2 mM), nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 µg/ml). The complete medium was filter sterilized using 0.2 µm pore-size nalge filter units.

MCF-7 Focus Assay

MCF-7 cells were suspended in DC₅ after treatment with trypsin (0.25%), seeded into 24-well plastic tissue culture plates at a density of 10⁵ cells/ml/well and placed in a 37°C humidified 5% CO₂ incubator. Cells were refed at 24 hr and every 3 to 4 days thereafter with 2 ml DC₅ containing various concentrations of the experimental compounds in DMSO. The final concentration of DMSO was usually ≤ 0.1%. Cells in the 24-well plates were visually inspected (Olympus CK microscope, 10× objective, Olympus Optical Co. Ltd., Tokyo, Japan) for evidence of cytotoxic or cytostatic effects of the test compound. Cytotoxic effects were indicated by cell morphology, e.g., pycnosis, lysis, or detachment; cytostatic effects were indicated by a delay in reaching confluence as compared to the control cultures. After 14 days the cultures were fixed with formalin and stained with 1% rhodamine B. The stained foci were then counted using a New Brunswick automated colony counter (New Brunswick Scientific Co. Inc., Edison, NJ).

All compounds and mixtures were tested for both their ability to induce foci (estrogenicity) and their ability to inhibit the foci induced by 1 nM E₂ (antiestrogenicity). Any compound that induced

focus formation was retested with 0.1 µM of the ER antagonist LY156758. Four replicates were performed at each concentration in each experiment and at least three experiments were performed with each compound and mixture. An E₂ dose-response curve was generated with each experiment and data were normalized to the maximum E₂ response. SigmaPlot software (version 3.0, Jandel Scientific Software, San Rafael, CA) was used to plot the dose-response curves, fit the data with a linear regression curve, and calculate the concentration needed to produce 50% of the maximum response (EC₅₀). The means and standard deviations from representative experiments are shown.

The MCF-7 focus assay was also conducted with cells that were preexposed to various concentrations of an OHPCB. Normally, before cells are used in the MCF-7 focus assay they are maintained in DC₅ and thus are exposed to very low levels of E₂ (BCS has < 5 pg/ml E₂) (17) and no xenoestrogens. Humans, however, are exposed to low chronic levels of PCBs (19), some of which are estrogenic (20). Because the background level of estrogens can effect the regulation of the ER (21) and consequently modify the response of the body to an exposure of a xenoestrogen or to natural fluctuations in E₂ levels, we

Figure 1. Induction of the formation of foci in MCF-7 cultures by 2,4,6-TCB-4'-OH, 2,3,4,5-TCB-4'-OH, and an equimolar mixture of the two compounds.

examined the effect of preexposure to a xenoestrogen on later focus formation.

Competitive Estrogen-Receptor Binding Assay

Recombinant human ER (1.2 nM) was incubated for 4 hr at room temperature with different amounts (0.1 nM–50 μ M) of cold estradiol, OHPCBs, or pesticides in the presence of 3 H-labeled estradiol (2.5 nM) in a total reaction volume of 100 μ l. After incubation, 100 μ l of 50% hydroxypapatite (HAP) slurry was added to the reaction mixture. After 15 min of incubation, 3 ml wash buffer (40 mM Tris, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, pH 7.4) was added and HAP-bound ER- 3 H-E₂ complex was separated by centrifugation at 200 $\times g$ for 20 min. Radioactivity of the pellet was quantitated in a Beckman liquid scintillation counter (Beckman Instruments, Irvine, CA). The amount of receptor-bound radiolabeled E₂ in the presence or absence of OHPCBs or pesticides was calculated after correcting for nonspecific binding in the presence of 200-fold excess of unlabeled E₂. The nonspecific binding ranged between 5 and 7%. The total specific binding ranged between 25 and 30%. The samples were tested in duplicates and each OHPCB, pesticide, or combination was tested in at least three separate experiments.

SigmaPlot software was used to plot the linear regression for each data set and calculate the concentration needed to inhibit 50% of the maximum response (IC₅₀). Representative experiments are shown.

Results

Hydroxylated Polychlorinated Biphenyls

The OHPCBs were tested for their estrogenicity and antiestrogenicity as measured by their ability to induce the formation of foci or inhibit the foci induced by 1 nM E₂. Both 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH are estrogenic in the MCF-7 focus assay, with EC₅₀s of 0.22 μ M and 0.72 μ M, respectively (Figure 1). The equimolar mixture of the two compounds is not synergistic (EC₅₀ = 0.18 μ M) (Figure 1). The potency of the OHPCBs and their mixture is weak compared to E₂ (Table 1); the EC₅₀ of E₂ is approximately 2000 times less than that of the OHPCBs. Neither of the OHPCBs inhibited the formation of foci induced by 1 nM E₂, thus neither are antiestrogenic at the concentrations tested (Table 1).

To determine whether the estrogenicity observed in the presence of 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH involved an ER-mediated mechanism, the focus assay was performed with 5 μ M of an OHPCB in combination with various concentrations of the ER antagonist LY156758. At 0.1 μ M, LY156758 suppressed all foci induced by 5 μ M of either of the OHPCBs (data not shown), indicating an ER-mediated mechanism. This is supported by the finding that both 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH inhibit 3 H-E₂ binding to ER with IC₅₀s of 79 and 15 nM,

respectively (Figure 2 and Table 1). However, in contrast to the results of Arnold et al. (8), the equimolar mixture of the two OHPCBs did not bind synergistically to ER (IC₅₀ = 15 nM).

To determine whether 2,4,6-TCB-4'-OH interacts synergistically with the natural estrogen E₂, we tested five concentrations of 2,4,6-TCB-4'-OH in combination with increasing concentrations of E₂. At all concentrations the combined effect of 2,4,6-TCB-4'-OH and E₂ was only additive (Figure 3). The maximal effect elicited by 1 nM E₂ was not increased with

Table 1. Estrogenic and antiestrogenic responses.

Compound	Induction of foci EC ₅₀ , μ M	Inhibition of foci induced by 1 nM E ₂ IC ₅₀ , μ M	Inhibition of ER binding IC ₅₀ , μ M
E ₂	0.0003	ND	0.0005
2,4,6-TCB-4'-OH	0.22	ND	0.079
2,3,4,5-TCB-4'-OH	0.72	ND	0.015
2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH	0.18	ND	0.015
α -Endosulfan	>10	ND	ND
β -Endosulfan	>10	ND	ND
Dieldrin	ND	ND	ND
α -Endosulfan and dieldrin	>10	ND	ND
β -Endosulfan and dieldrin	>10	ND	ND
LY156758	ND	0.01	0.0008

ND, not detectable. EC₅₀ values and IC₅₀ values were not calculated for these compounds because they did not show either the estrogenic or antiestrogenic response at any of the concentrations tested. The highest concentration tested in the MCF-7 focus assay was 10 μ M with 0.2% DMSO. The highest concentration tested in the ER binding assay was 10 μ M with 1.0% DMSO.

Figure 2. Inhibition of 3 H-E₂ binding to ER by 2,4,6-TCB-4'-OH, 2,3,4,5-TCB-4'-OH, and an equimolar mixture of the two compounds. An E₂ binding curve is shown for comparison.

Figure 3. Induction of the formation of foci in MCF-7 cultures by various concentrations of 2,4,6-TCB-4'-OH alone or in combination with increasing concentrations of E₂. A dose-response curve for E₂ alone is also shown.

any concentration of 2,4,6-TCB-4'-OH (Figure 3).

Effects of Preexposure

To examine whether chronic low-level exposure to a xenoestrogen, as is found in human and animal populations, affects the estrogenic response to a subsequent exposure of a xenoestrogen in the presence of physiologic E₂ levels, we pretreated MCF-7 cells with various concentrations of 2,4,6-TCB-4'-OH before testing them in the MCF-7 focus assay. MCF-7 cells were pre-exposed to 0.1 to 1.0 μ M 2,4,6-TCB-4'-OH for 3 or 7 days. E₂ dose-response curves of the pretreated cells, both alone and in combination with 2,4,6-TCB-4'-OH, were the same as the dose-response curves for the control cells (data not shown) and were similar to those shown in Figure 3. Again, there was no synergism with any of the combinations. This indicates that pretreatment with 2,4,6-TCB-4'-OH for up to 7 days had no effect on the formation of foci induced by 2,4,6-TCB-4'-OH or E₂ alone or in combination.

Pesticides

We tested the pesticides dieldrin and endosulfan, alone and in combination, for their ability to induce or inhibit the formation

of foci and bind ER. Previous studies examining the potential estrogenicity of endosulfan have used α -endosulfan, β -endosulfan, technical grade endosulfan, or a combination of 60% α -isomer and 40% β -isomer. Therefore, we tested both α - and β -endosulfan in each assay and an equimolar mixture of the α - and β -isomers in the MCF-7 focus assay. Figure 4 shows the results from the MCF-7 focus assay for dieldrin, α -endosulfan, and a mixture of dieldrin and α -endosulfan. Dieldrin exhibits no estrogenicity, whereas α -endosulfan exhibits weak estrogenicity at the highest concentration (10 μ M). The mixture was not synergistic. β -endosulfan was weakly estrogenic at 10 μ M and a mixture of dieldrin and β -endosulfan was not synergistic (Table 1). The equimolar mixture of α - and β -endosulfan was also weakly estrogenic at 10 μ M (data not shown). The weak estrogenicity observed for endosulfan is at a concentration 10,000 times greater than the concentration needed to produce the maximum E₂ response.

Pesticides and their combinations were also tested in the MCF-7 focus assay with 1 nM E₂ to determine whether any of the pesticides were antiestrogenic. None of the pesticides or their combinations inhibited the foci induced by 1 nM E₂ (Table 1).

Although dieldrin was not estrogenic in the MCF-7 focus assay and endosulfan was only weakly so, we tested the pesticides and their binary combinations in a competitive ER binding assay because previous reports (8,16) suggested that the pesticides do bind the ER. Dieldrin, α -endosulfan, β -endosulfan, and their binary combinations did not inhibit ³H-E₂ binding to the receptor at any concentration; the highest concentration tested was 10 μ M (Table 1).

Discussion

We found that 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH are estrogenic in the MCF-7 focus assay and in the competitive ER binding assay. However, there is no synergy in either assay by equimolar mixtures of the two compounds at various concentrations. The estrogenicity of 2,4,6-TCB-4'-OH and 2,3,4,5-TCB-4'-OH has been demonstrated previously in a competitive binding assay (8,14,22), a variant of the estrogen-responsive preconfluent growth assay referred to as the E-SCREEN assay (16), a temperature-sensitive sex determination assay (10), reporter gene assays (7,8,14), and a mouse uterotrophic assay (2,4,6-TCB-4'-OH only) (14,22). To date, one study has reported estrogenic synergy of 2,4,6'-TCB-4'-OH and 2,3,4,5-TCB-4'-OH [the present study and Ramanmoorthy et al. (14)] failed to find synergy. Because of the great differences among the assays, e.g., the turtle sex determination assay, the yeast reporter gene assay, and the MCF-7 focus assay (which relies on the physiologically relevant estrogen-dependent induction of postconfluent cell proliferation resulting in the *in vitro* equivalent of *in vivo* tissue restructuring), lack of total agreement is not surprising. However, the lack of agreement with the same assay, i.e., the competitive binding assay, is more problematic.

Of the pesticides, dieldrin was not estrogenic and α - and β -endosulfan were weakly estrogenic in the MCF-7 focus assay. There was no synergy between the binary mixtures. The pesticides alone did not bind ER and again, combinations of the pesticides were not synergistic.

Circulating levels of E₂ vary greatly during a woman's ovulatory cycle (10–440 pg/ml), whereas the levels in children, postmenopausal women, and men are restricted to the relatively low adrenal and adipose secretion. This difference in circulating levels of E₂ may result in different sensitivities and responses to environmental exposures of xenoestrogens. To examine

Figure 4. Induction of the formation of foci in MCF-7 cultures by α -endosulfan, dieldrin, and an equimolar mixture of the two compounds. A dose-response curve for E_2 alone is shown for comparison.

the effects of a range of physiologic levels of E_2 in combination with a xenoestrogen, we tested mixtures of E_2 and 2,4,6-TCB-4'-OH in the MCF-7 focus. There was no synergy at any of the concentrations tested. This lack of synergy addresses two important questions: Do xenoestrogens produce synergistic responses within the range of relevant levels of the natural estrogen E_2 ? Do nonequimolar combinations of a xenoestrogen and E_2 show synergistic responses? Although only 2,4,6-TCB-4'-OH was tested in this study [and it may not be an environmentally relevant estrogen because 2,4,6-TCB-4'-OH and its parent congener are not found in the environment or in animals (23)], these results may generalize to other synthetic environmental estrogens. These findings suggest that environmental estrogens may not produce synergistic responses in combination with physiologic levels of E_2 .

However, further research using mixtures of relevant environmental xenoestrogens alone and in combination with submaximal levels of E_2 in other test systems are needed to place health risk concerns into appropriate perspective.

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