Interactions between chlorinated aromatic hydrocarbons as tumor promoters: Mixtures of 2,3,7,8-TCDD and 2,2´,4,4´,5,5´-hexaCB (PCB153) or α-hexachlorocyclohexane in different *in vitro*-assays.

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INTRODUCTION

Chlorinated aromatic hydrocarbons are ubiquitous environmental contaminants that are present in the environment as complex mixtures of dioxins, furans, biphenyls and organochlorine insecticides, *e.g.*, hexachlorocyclohexanes. For those agents which are Ah receptor agonists, the use of toxic equivalency factors (TEFs) has been proposed[1)]. This approach assumes that minimal interactions between the Ah receptor agonists and other components of the mixtures occur. However, various studies on chronic toxicity (immunotoxicity, teratogenicity and tumor promotion) have shown non-additive interactions between Ah receptor agonists and non-planar agents without any essential receptor binding activity[2-4)]. As the chronic effects are a major concern in risk assessment of contaminated environmental samples, the TEF approach cannot solely be utilized, and more information about the mechanisms of action of the pollutants is urgently required. In this study, the interaction between three model compounds were investigated: 2,3,7,8-TCDD was chosen as a strong Ah receptor agonist, 2,2´,4,4´,5,5´-hexaCB (HxCB) as a relatively non-toxic PCB congener

and α-hexachlorocyclohexane (α-HCH) as a major non-dioxin/PCB-like environmental contaminant. The toxic and tumor promoting activities of these agents were studied in a battery of *in vitro*-assays: dose-response relationships were determined for the ethoxy-resorufin O-deethylase (EROD) induction, cytotoxicity and effects associated with tumor promotion *in vivo, i.e.*, stimulation of hepatocellular growth and enhancement of malignant cell transformation induced by initiating carcinogens. The results of these *in vitro*-studies have shown antagonistic interactions between HxCB and TCDD (at maximally effective concentrations) and synergistic effects between α-HCH and TCDD (at very low concentrations).

MATERIALS AND METHODS

TCDD and HxCB were obtained from Ökometric GmbH (Bayreuth, Germany) and α-HCH from Riedel-de Haen (Seelze, Germany). TCDD and HxCB had a purity greater than 99%, α-HCH was 98% pure. The compounds were dissolved in dimethyl sulfoxide (DMSO).

Hepatocytes were isolated by collagenase perfusion from adult male Wistar rats and cultured as described[5]. On the second day after cell plating, the mitotic index was determined after 3 h exposure to colchicine (0.1 mM). The transformation assay with C3H/M2 mouse fibroblasts was adjusted to determine tumor promotion according to procedures described previously[6]. Briefly, 24 h after cell plating, the cultures were treated for 24 h with the initiating agent MCA (1 μg/ml) or solvent (DMSO, 0.5%). Beginning on day 5 until the end of the experiment, with each of the medium renewals TCDD, HxCB or solvent were added. After 8 weeks, the cells were fixed to determine the transformation yield.

RESULTS

Interaction between TCDD and HxCB

1. The malignant transformation of C3H/M2 mouse fibroblasts induced by a suboptimal concentration of 3-methylcholanthrene (1 μg/ml) was markedly enhanced by treatment with TCDD (1.5×10^{-12} M) or HxCB (3×10^{-8} M). After treatment with defined mixtures of TCDD plus HxCB, the enhancement of the transformation rate was inhibited (by 3×10^{-9} M HxCB) or abolished (by 3×10^{-8} M HxCB; Table 1).

2. The mitotic rates of primary rat hepatocytes were stimulated by TCDD (10^{-14} M, 10^{-12} M) or by HxCB (10^{-8} M to 10^{-7} M). Cotreatment with 10^{-7} M HxCB did not affect the mitotic stimulation by 10^{-14} M TCDD, while HxCB antagonized the effect of 10^{-12} M TCDD.

3. In primary rat hepatocytes, HxCB (10^{-6} M) had no effect on EROD induction or on cytotoxicity (as determined in the neutral red assay as a reduction of dye retention in lysosomes). TCDD ($\geq 10^{-11}$ M) induced concentration-dependent EROD activity and cytotoxicity; cotreatment with HxCB slightly reduced the effects of TCDD (10^{-11} M, 10^{-10} M).

Table 1: Enhancement of malignant transformation by TCDD and HxCB

Results are a summary of 3 experiments. Cultures were pretreated with solvent (DMSO) or with the initiating agent MCA. Thereafter, cells were treated with the tumor promoters.

	DMSO (0.5%)	MCA (1 µg/ml)
Controls (DMSO)	0 /19[a] (0.00)[b]	2 / 25 (0.08)
TCDD (1.5×10^{-12} M)	0 / 28 (0.00)	7 / 34 (0.21)
TCDD + HxCB (3×10^{-9} M)	0 / 30 (0.00)	4 / 33 (0.12)
TCDD + HxCB (3×10^{-8} M)	0 / 16 (0.00)	1 / 18 (0.06)
HxCB (3×10^{-9} M)	0 / 30 (0.00)	3 / 36 (0.08)
HxCB (3×10^{-8} M)	0 / 33 (0.00)	6 / 37 (0.16)

a) Number of transformed foci per treated dishes.
b) Numerical value of the ratio, in parentheses.

Interaction between TCDD and α-HCH in primary rat hepatocytes

1. No interaction on the stimulation of hepatocellular mitosis was found between the maximal effective concentrations of TCDD (10^{-13} M to 10^{-12} M) and α-HCH (10^{-5} M).

2. A clear synergistic effect on the stimulation of mitosis was found at a very low concentration of TCDD (10^{-16} M) and α-HCH (10^{-6} M).

3. A small synergistic effect of α-HCH (10^{-6} M) on the TCDD (10^{-10} M)-induced cytotoxicity (neutral red assay) was observed.

DISCUSSION AND CONCLUSION

The results of this *in vitro*-study show a limited antagonism of TCDD-induced stimulation of growth and promotion of transformation by HxCB at concentration ratios of TCDD/ HxCB of 1 : 10^5 and 1 : 10^4. These results are in accord with reports on an antagonism of TCDD-induced immunosuppression[2)] and teratogenic effects[3)] in mice. The mechanism of inter-

action between TCDD and HxCB remains unclear. It has been suggested that HxCB at high concentrations inhibits the TCDD-induced effects by interference with the Ah receptor[2]. However, TCDD-induced EROD activity, the best studied Ah receptor-mediated response, was only slightly inhibited by HxCB in comparison to the strong antagonism of TCDD-induced enhancement of mitosis and transformation by HxCB. Thus, the latter findings suggest that the antagonism found in the tumor promotion-related assays may involve additional, yet unknown mechanisms of signal transfer modulation. Using the TEF approach, the risk for tumor promotion might be overestimated in case of the interaction between TCDD and HxCB. Conversely, the risk may be understimated in case of the potentiation of TCDD effects by α-HCH: Cotreatment of hepatocytes with an extremely low concentration of TCDD (10^{-16} M) and an uneffective concentration of α-HCH (10^{-6} M) significantly stimulated mitosis. This synergistic interaction is of particular interest for the risk assessment of industrial contaminations where both, TCDD and α-HCH, emerge as by-products of industrial processes. Thus, our results suggest that the chosen *in vitro*-assays might be useful tools to study the complex pattern of interactions of environmental pollutants as tumor promoters.

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