INTERACTIVE EFFECTS OF THREE POLYCHLORINATED BIPHENYLS ON ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY IN VITRO

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Introduction

The 7-ethoxyresorufin-O-deethylase (EROD) assay is widely used to evaluate the activity and induction potency of cytochrome P4501A by polyhalogenated aromatic hydrocarbons such as polychlorinated dibenzo-*p*-dioxins (PCDDs), furans (PCDFs), biphenyls (PCBs) and mixtures of these compounds (1-5). EROD activity is one of the most frequently used measures in comparing toxicological potencies of dioxins e.g. in the experimental data base from which the Toxic Equivalency Factors (TEFs) have been derived (6). The TEF concept (7) has been developed as a tool to estimate the toxicological risk due to environmental exposure of compounds sharing a common mechanism of action with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). It is based on the assumption that binding to the *Ah*-receptor is the first step for all important biological effects and that these compounds act in an additive manner. Nevertheless the additivity of the singular effects for some PCDD, PCDF and PCB mixtures has been questioned in different investigations (2,5, 8-11).

In this study the effect of different combinations of three chlorinated biphenyls (CB) congeners on the EROD induction *in vitro* has been investigated. The investigated CB congeners were chosen to represent different patterns of chlorination: the non-*ortho*-substituted CB 126 (3,3',4,4',5-pentachlorobiphenyl), the mono-*ortho*-substituted CB 105 (2,3,3',4,4'-pentachlorobyphenyl) and the di-*ortho*-substituted CB 153 (2,2',4,4'5,5'-hexachlorobyphenyl). An experimental design previously applied in an *in vivo* study about interactive effects of the same three CB congeners (12) was used for selection of the dose combinations and for the interpretation of the results. Experimental design and modeling (13,14) allow to optimize the information derived from the experiment with regard to interactions between congeners.

Material and Methods

Chemicals. TCDD was supplied by Dow Chemical (Midland, Michigan); the three CB congeners were obtained from Larodan Fine Chemicals AB (Malmö, Sweden). TCDD and CB mixture solutions (Table 1) were prepared in dimethyl sulfoxide.

EROD assay. Rat hepatoma MH1C1 cells were grown as a continuos cell line in Dulbecco´s Modified Eagle Medium supplemented with 10% foetal bovine serum, sodium pyruvate (0.6 mM) and L-glutamate (3.8 mM) at 37°C in a humidified air/carbon dioxide (95/10%) atmosphere. Cells were seeded into 96-well plates at a density of $20*10^3$ cells per well in 0.2 ml of medium. After 24 hours, the plates were treated with medium containing either TCDD or PCB mixtures. After 24 hours of exposure, each well was washed and plates were stored at -80°C until the enzyme analysis was performed. After thawing the cells were incubated at 37°C for 15 min containing 0.6

mM NADPH, and 4.1 mM $MgSO_4$ in Hepes buffer (0.1 M, pH 7.8). The reaction was started by adding ethoxyresorufin and the plates were incubated for an additional 15 minutes. The reaction was stopped by adding methanol. Formation of resorufin was determined fluorimetrically (15).

Planning of PCB mixtures and data analysis. The fifteen individual dose-combinations of the three CB congeners used in the experiment are given in Table 1. Each CB congener was tested at three dose-levels which were varied simultaneously in a systematic way following a face-centered central composite (CCF) design. CB 126 and 105 were tested separately by the EROD bioassay in order to select the doses to use in the experimental design. The doses of CB 153 were chosen 10 time higher than the doses of CB 105 since this approximately was the ratio between these two congeners in human fat (16). TCDD was used as positive control in each experiment and EROD induction in the mixtures was expressed as relative induction in comparison to the TCDD. Every combination was tested using 12 wells. Results were evaluated with multivariate modeling by Multivariate Linear Regression (MLR) using the statistical package Modde 4.0 (Umetri AB, Umeå, Sweden).

Table 1. Combinations of CB 126, 105 and 153 tested by the EROD assay.

Dose Combination (CB126/105/153)	**CB 126** (ng/well)	**CB 105** (ng/well)	**CB 153** (ng/well)
LLL	0.001	5	50
HLL	0.02	5	50
LHL	0.001	100	50
HHL	0.02	100	50
LLH	0.001	5	1000
HLH	0.02	5	1000
LHH	0.001	100	1000
HHH	0.02	100	1000
LMM	0.001	22.36	224
HMM	0.02	22.36	224
MLM	0.00447	5	224
MHM	0.00447	100	224
MML	0.00447	22.36	50
MMH	0.00447	22.36	1000
MMM	0.00447	22.36	224

legend: L= low dose; H= high dose; M = medium dose.

Results and Discussion

When tested separately, the non-*ortho*-CB 126 and the mono-*ortho*-CB 105 showed EROD induction about 10 and 10^5 times weaker than TCDD, respectively, based on the equivalent EROD induction value in the linear part of the dose response curve. CB 153 was not tested *per se* since previous investigations showed no EROD activity *in vitro* (2,5).

Results of the CB combinations were evaluated using MLR in order to investigate the relationship between the exposure to the three CB congeners and the EROD induction. An interaction model was created. The R^2 and Q^2 of the model (0.91 and 0.65, respectively) show that the model had good predicting capacity. The relationship between the CB doses in logarithmic scale and the EROD activity did not deviate significantly from linearity. Figure 1 shows the regression coefficients of the individual congeners and their combination in the model created. At the tested doses CB 126 was the most important variable for the EROD induction, followed by CB 105, while the di-*ortho*-substituted CB 153 did not show any significant effect on the EROD induction. Interaction terms were significant between the non-*ortho*-substituted CB 126 and the mono-*ortho*-substituted CB 105 but not between the di-*ortho*-substituted CB 153 and CB 105 or CB 126 (Figure 1).

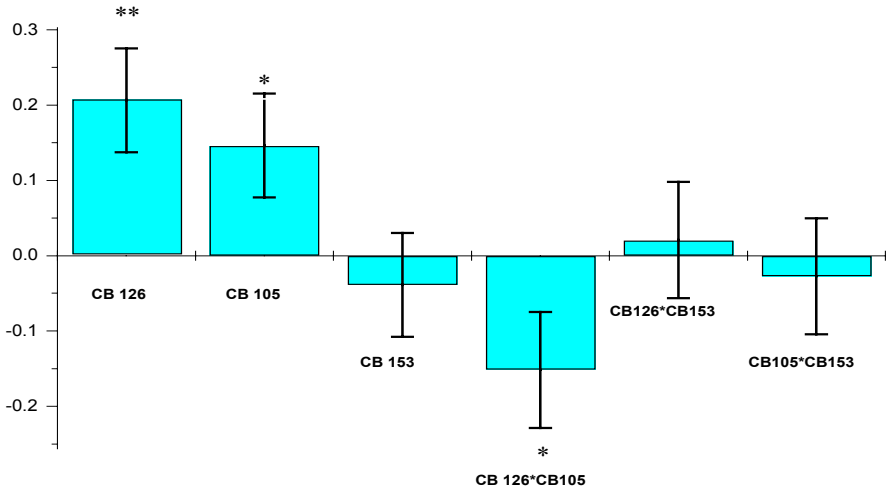

Figure 1. Scaled and centred regression coefficients with confidence intervals of the Multivariate Linear Regression (MLR) model representing the effects of the individual congeners and of the interactions between congeners (*P<0.05, **p<0.001).

These results indicate that there is an antagonistic effect between CB 126 and the less potent CB 105 at the dose-levels investigated, probably due to the competitive binding to the *Ah*-receptor. The di-*ortho*-substituted CB 153 did not interact with EROD activity induced by CB 126 or CB 105. Inhibition of EROD induction in mixtures containing CB 105 and TCDD has recently been seen by van der Plas *et al.* (5). Antagonistic effects have also been observed with combinations of

TCDD and CB 153 (2,5) and in a previous investigation antagonistic effects were found in mixtures containing CB 126 and the di-*ortho*-substituted CBs 138 and 128 (17). Results of the present study seem to confirm the presence of non-additive interactions between PCB congeners.

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