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Toxicity of Mixtures of Polychlorinated Dibenzo-p-dioxins, Dibenzofurans, and Biphenyls Determined by Dose-Response Curve Analysis

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Polyhalogenated aromatic hydrocarbons (PHAH) such as polychlorinated dibenzo-p-dioxins (PCDD), dibenzofurans (PCDF), and biphenyls (PCB) are persistent environmental pollutants, that bioaccumulate throughout the food chain and may pose a health risk to humans and animals (Safe 1994). The most toxic and most studied PHAH is 2,3,7,8-TCDD. PCDD, PCDF, and PCB elicit a number of common biologic and toxic effects in laboratory animals and mammalian cells in culture. The induction of hepatoma microsomal EROD or the associated cytochrome P450 isoenzymes is one of the most characteristic and sensitive response. The toxic mechanism is mediated through combining with the arylhydrocarbon (Ah) receptor (Poland and Knutson 1982; Safe 1990; Whitlock 1993).

Since biota in the environment is exposed to mixtures of these compounds rather than to a single congener, simultaneous or sequential exposure of organism to two or more of these compounds may alter quantitative and qualitative biological responses. Therefore, studies with mixtures could provide more information about the possible interactive effects between these compounds and the fundamental mechanisms behind these interactive effects. The concept of independent action, which is sometimes termed effect multiplication, is based on agents acting on different molecular target sites only. An evaluation of tendency and degree of over or under estimation of mixture toxicity can be done by comparative analysis of dose-additive and independent action. The new approach is the use of dose-response curves (DRC) for evaluating observed combined effects with respect to additive as well as to independence (Pöch 1993; Pöch et al. 1995). This model and analysis method occasionally are applied in pharmacological studies. In general, the simplest cost and time saving approach is to do a dose-response study with a substance A alone and in the presence of a fixed dose of a "similarly" acting substance B. This method is proved can provide a rather simple procedure for obtaining evidence of magnitude and mechanism of action.

The aim of the present study was to evaluate the combined effects of 2,3,7,8-

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TCDD with five selected PHAH by use of DRC analysis. The combined experiments were performed with TCDD in the presence of a few fixed doses of selected PHAH with a micro-EROD assay in vitro using H4IIE rat hepatom cell cultures.

MATERIALS AND METHODS

2,3,7,8-TCDD and other five PHAH compounds were selected for the combined experiments. The five PHAH compounds were 2,3,7,8-polychlorinated dibenzofuran (TCDF), octchlorodibenzo-p-dioxin (OCDD), 3,3',4,4',5,5'-pentachlorobiphenyl (PCB126), 2,2',4,4'5,5'-hexachlorobiphenyl (PCB153) and 3,3',4,4'-tetrachlorobiphenyl (PCB77). 2,3,7,8-TCDD, 2,3,7,8-TCDF, OCDD, PCB126, PCB77 and PCB153 were purchased from Sigma Chemical Co. and diluted in DMSO/isopropanol (4:1) to a desired concentration. Molecular biological reagents were commercially available with high purity.

Rat hepatoma cells H4IIEC3/T (H4IIE) were obtained from Prof. E.B. Thompson (National Cancer Institute, Bethesda, MD, USA). Cells were grown in Dulbecco's Minimum Essential Medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, at 95% relative humidity, 37°C and air containing 5% CO_2 (Deml et al. 1989).

The EROD-activity in intact cells seeded on 96-well plates was determined according to Wu et al. (1996). Cells were seeded at a density of $1x10^4$/well in a 96-well plate. After 3 days the medium was replaced with 100 µl medium containing different concentrations of TCDD or mixtures of TCDD with other five PHAH standards. After 72 h the medium was removed and 100 µl fresh medium containing 8 µmol 7-ethoxyresorufin and 10 µmol dicumarol were added. After incubation for 60 min at 37°C the medium was transferred to a fresh 96-well plate and 130 µl methanol were added. Resorufin-associated fluorescence was measured in these solutions on a multiwell fluorescence reader (Fa. Fluoroskan 11, Fa, Labsystem). The protein content per well was assayed using bicinchoninic acid according to Smith et al. (1985). Standard deviation between triplicates remained less than 0.05.

Experimental data points representing mean values of four individual experiments were fitted to Sigmoidal curves by the program ALLFIT, which uses the four parameters logistic equation (De Lean et al. 1988).

$$Y=[(a-d)/(1+X/c)b]+d$$

where: **a:** maximum response in curve; **b:** slope; **c:** EC50; **d:** minimum response in curve; **Y:** response; **X:** dose

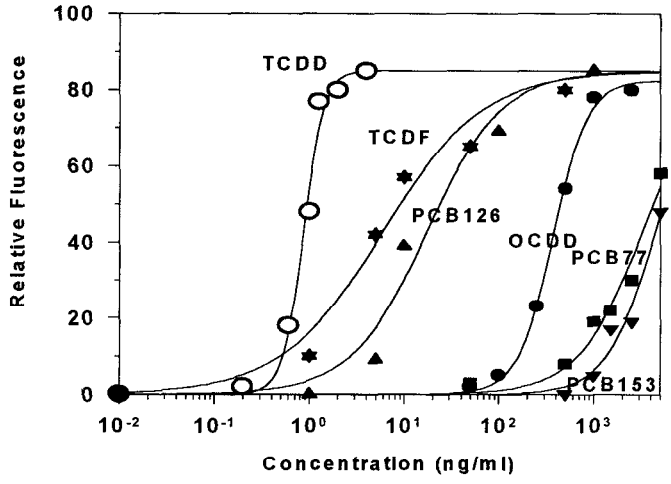


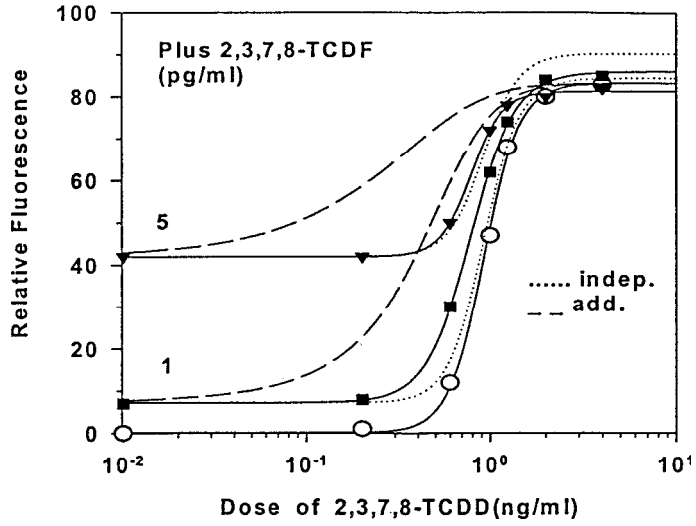
Figure 1. Dose-response relationships of EROD induction of individual polyhalogenated aromatic hydrocarbons (PHAH) used in this study

From the experimental DRC of agent A alone and agent (A+B), the parameters of theoretical DRC for additive and independent combined effects could be calculated with a computer program (Pöch and Panchevn 1995). The comparison of experimental curves with theoretical curves for additive and independent were made with the F-test. Comparison of observed frequencies with expected frequencies above or below median DRC of dose-additive and independent interaction were made with x2 goodness-of-fit statistic. Statistic calculation of x2 and p was done with the aid of a computer program (Statgraphics, Statistic Graphical Corp., Rockville, MD, U.S.A). The significant differences mentioned below represent that $p<0.05$.

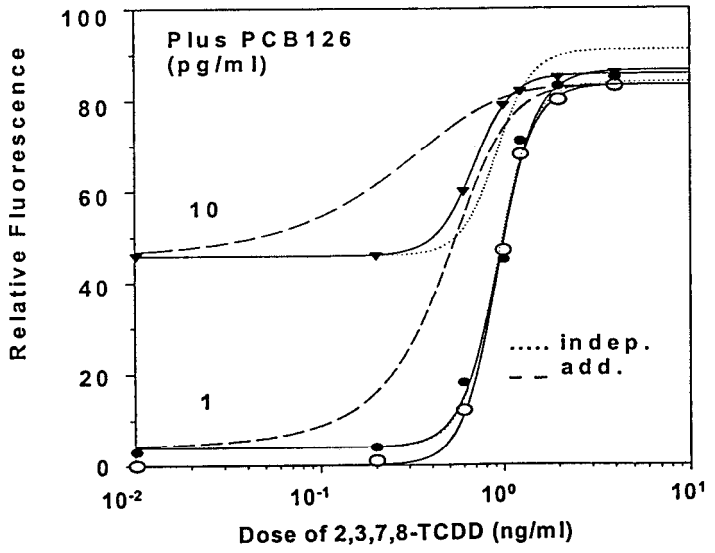
RESULTS AND DISCUSSION

Table 1. Toxic equivalent factors (TEF), half effective concentrations (EC_{50}) and slopes of dose-response curves (DRC) of individual polyhalogenated aromatic hydrocarbons (PHAH) used in this study

Compounds	TEF	EC_{50} (pg/ml)	Slope
2,3,7,8-TCDD	1.0	0.87	4.0
2,3,7,8-TCDF	0.1	5.93	0.8
OCDD	0.001	373	2.4
PCB126	0.1	17.4	1.1
PCB77	0.0005	3235	1.3
PCB153	<0.00001	4436	1.7

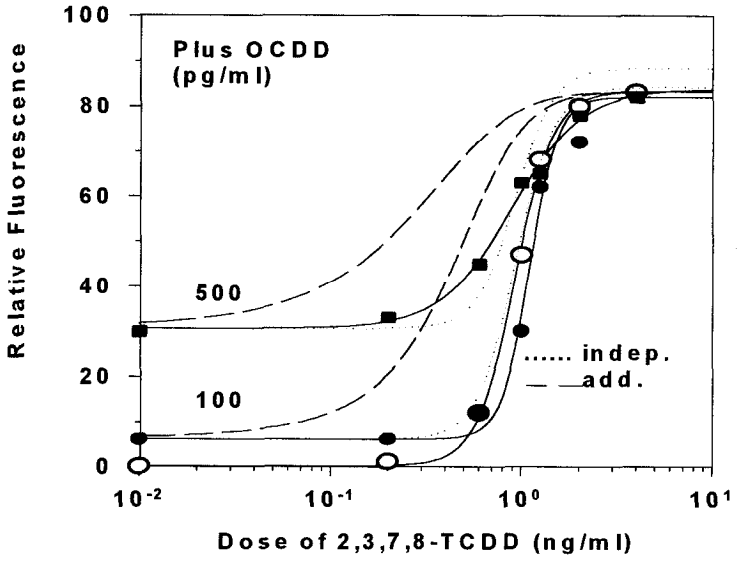


(a)

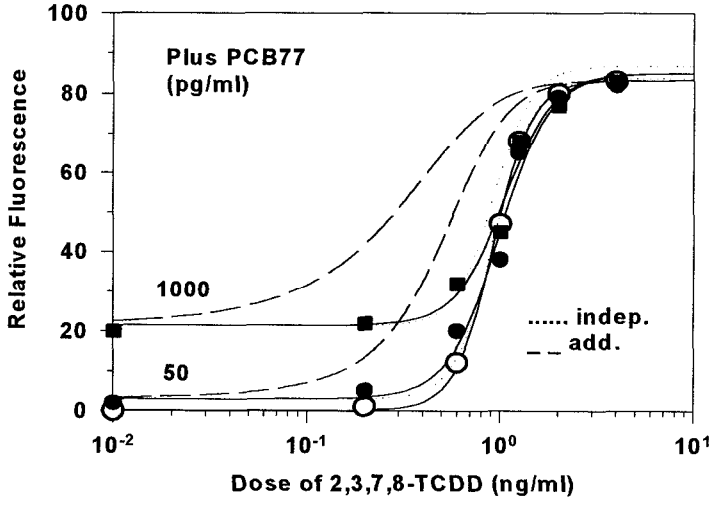


(b)

Figure 2. Dose-response curves (DRC) of EROD induction in combination. (a) DRC of 2,3,7,8-TCDD alone (o) and in the presence of 1 pg/ml (■) or 5 pg/ml (▼) 2,3,7,8-TCDF; (b) DRC of 2,3,7,8-TCDD alone (o) and in the presence of 1 pg/ml (●) or 10 pg/ml (▼) PCB126. Theoretical DRC of additive and independent combinations are shown with point broken line.



(a)



(b)

Figure 3. Dose-response curves (DRC) of EROD induction in combination. (a) DRC of 2,3,7,8-TCDD alone (○) and in the presence of 100 pg/ml (●) or 500 pg/ml (■) OCDD; (b) DRC of 2,3,7,8-TCDD alone (○) and in the presence of 50 pg/ml (●) or 1000 pg/ml (■) PCB77. Theoretical DRC of additive and independent combinations are shown with point broken line.

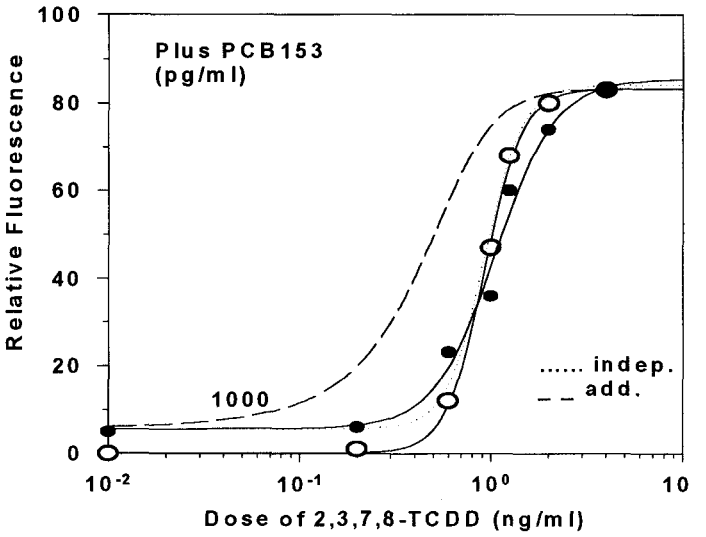


Figure 4. Dose-response curves (DRC) of EROD induction in combination of 2,3,7,8-TCDD alone (o) and in the presence of 1000 pg/ml PCB153 (•). Theoretical DRC of additive and independent combinations are shown with point broken line.

The EROD activity of individual compounds was dose-dependent with the 2,3,7,8-TCDD being the most active compound, whereas, PCB153 was relatively inactive (Fig. 1). Comparing the EC50 values of these compounds with their TEF values, a good agreement between structure-activity and structure-toxicity was obtained. In addition, a significant difference ($p<0.05$) was found in slopes of DRC between TCDD and the other five compounds (Fig. 1, Table 1). Dose-additive occurs if substances have common target sites and similar modes of action, one agent like a dilution of the other (Greco et al. 1992). In present study, the five PHAH compounds did not exhibit a dilution of TCDD. Thus, the result implied that the interaction was not additive.

Similarly, when 2,3,7,8-TCDD was combined with OCDD and PCB77, the cumulative dose-response curves reflected significantly ($p<0.05$) antagonistic response (Fig. 3).

PCB153 was relatively inactive and non-toxic compared with the other four PHAH compounds. However, the combined effects of TCDD in the presence of 1000 pg/ml PCB153 were not significantly different compared with the combined effects of TCDD in the presence of the other four compounds. The interaction of TCDD and PCB153 was significant ($p<0.05$) under the theoretical effects of an additive combination and response an independent interaction (Fig. 4).

It was clear that effects of TCDD combined with higher toxic compounds, lower toxic compounds, or even with inactive compounds, were significantly different from a single additive interaction, and were antagonism which matched with DRC of an independent interaction. Consequently, two possible explanations may contribute to our understanding of the mechanisms responsible for this non-additive interaction: a) TCDD and PHAH (such as PCB77 and PCB153) might bind to different receptors and act through a different mechanism of action, therefore, the combined effects showed significantly non-additive interaction; b) TCDD and related PHAH (such as TCDF, PCB126) probably bind to the same Ah-receptor but act at different sites and induce different conformations of the Ah-receptor complex which exhibit different activities as nuclear transcription factors. The interactions of both potent and weak Ah receptor agonists to give non-additive interactions has previously been reported from multiple laboratories and summarised in a recent review (Safe 1998). The non-additive antagonistic interactions between various PHAH are also consistent with results obtained for other ligand-induced transcription factors such as the estrogen receptor (ER) where PHAH such as PCB153: exhibit tissue specific weak ER aginist/antagonist activities (Ashby et al. 1997; Gaido et al. 1997; Ramamoorthy et al. 1997).

Our results showed that EROD activity of TCDD, TCDF, OCDD, PCB126, PCB77, and PCB153 were dose-dependent. The difference of slopes between TCDD and the five selected PHAH implied that the interaction between TCDD and these compounds may be a non-additive response. This non-additive response may be an important consideration in development of a toxic equivalent factor approach for hazard and risk assessment. Besides the importance at measuring and expressing the antagonistic response seen in combination, the DRC analysis may also contribute to our understanding of the mechanism of interaction,

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