'Independent' (Additive) *versus* Non-additive Interaction of Ah Receptor Agonists: Experiences from the EROD-Bioassay and from a Liver Tumor-Promotion Model

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Abstract
PCDD, PCDF and PCB are among the most potent liver tumor promoters in rodents. Since the mechanism(s) of action of TCDD and related compounds as liver tumor promoters are not completely understood, a number of hypotheses have been established trying to explain the molecular basis of the promoting effect. Most hypotheses are based on the assumption that activation of the Ah receptor (AhR) represents a necessary but not sufficient initial step for liver tumor promotion. Induction of cytochrome P4501A1 (CYP1A1) is the best understood molecular effect of the ligand activated AhR, and a number of bioassays monitoring the induction of CYP1A1-catalyzed 7-ethoxyresorufin O-deethylase (EROD) activity in mammalian cell culture have been developed. Using the EROD bioassay, the additivity of AhR-mediated CYP1A1 induction was tested for complex mixtures of PCDD or PCB. Using a mixture of 49 PCDD or of six 'dioxinlike' PCB, almost perfectly independent (additive) inducing effects were found, while an about twofold overadditive (synergistic) effect was observed with the complex PCB mixture Arochlor 1254. In a two stage initiation-promotion experiment in female rats, the tissue level-response-relationship of the promoting effect (enhancement of relative mass of preneoplastic liver) of the PCDD mixture was in agreement with the concept of additivity at lower tissue levels, but was lower than expected at higher tissue levels.

Introduction

PCDD and PCDF can differ in their toxic potency by several orders of magnitude, and most if not all toxic effects of TCDD and related compounds including tumor promotion are thought to be mediated *via* a common receptor, the Ah receptor (AhR) [1,2]. The use of congener-specific Toxicity Equivalency Factors (TEFs) and the calculation of Toxicity Equivalents (TEQ) for the risk estimation of mixtures of these compounds has been widely accepted in toxicology for PCDD/PCDF [3-5], and for a number of 'dioxinlike' PCB congeners [6]. An important condition for the use of TEFs is additivity (independence) of action of each individual AhR agonist in a complex mixture. A number of experimental data support the concept of additivity [4,7,8] at least when mixtures of strong agonists were investigated. However, a number of reports also demonstrated synergism or (partial) antagonism between AhR agonists, as well as modulating effects of related compounds which lack affinity to the AhR such as certain PCB [9-11].
Induction of cytochrome P450 (CYP)1A1 is one of the best understood and most sensitive biochemical effects mediated *via* the ligand-activated AhR [3,4]. A very good correlation was found

between the rank order of PCDF congeners relating their *in vivo* subacute toxicity in rats to their CYP1A1-inducing potency [3]. *In vitro*, induction of CYP1A1-catalyzed 7-ethoxyresorufin O-deethylase (EROD) activity has been studied e.g. in rat hepatocytes in primary culture, in rat H4IIE hepatoma cells, and in human HepG2 hepatoma cells [12-15].
In the studies presented here, we investigated if EROD-TEFs of complex mixtures of PCDD or PCB in cell culture can be predicted from the chemical analysis of AhR agonists under the assumption of additivity. Furthermore, we investigated if the TEF concept can be applied to liver tumour promotion by PCDD in rats, a biological endpoint which plays a prominent role in risk assessment for the whole class of compounds.

Experimental Methods

Chemicals and chemical analysis

2,3,7,8-TCDD, and PCBs 77 (3,3',4,4'-tetrachlorobiphenyl), and 118 (2,3',4,4',5-pentachlorobiphenyl) were obtained from Ökometric (Bayreuth, Germany). 1,2,3,4,6,7,8-HpCDD (HpCDD), octachlorodibenzo-*p*-dioxin (OCDD), and all other PCB were from Promochem (Wesel, Germany). Arochlor 1254 was from Monsanto (St. Louis, USA). The PCDD mixture was obtained by catalytic dechlorination/hydrogenation of OCDD. PCDD constituents were analyzed by gas chromatography/mass spectrometry [14], PCB and PCDF were analyzed as described [16].

Cell Culture Bioassay

Hepatocytes from adult male Wistar rats were plated at a density of 100,000 cells/cm^2 on collagen-coated petri dishes in DMEM supplemented with 10 % calf serum, 10 % fetal calf serum, 0.1 μM dexamethasone, 100 units penicillin/ml, and 100 μg streptomycin/ml [14]. After 2 h, medium was replaced by fresh medium, test compounds were added in DMSO, and cells were harvested after 48 h. EROD activity in cell homogenates was determined according to Burke and Mayer [17]. Dose-response curves were calculated using a log-probit procedure (SAS Institute, Cary, USA; Technical report P-179) which also allows calculation of EC_{50}-values and 95 % confidence intervals.

Initiation-promotion study

Female Wistar rats weighing 190-210 g received N-nitrosomorpoline in the drinking water (80 mg/l) over 25 days for initiation. After an interval of 2 weeks without treatment, groups of five animals were treated biweekly with corn oil (controls) or PCDD by s.c. injection over 13 weeks. The dosage was equivalent based on EROD-TEFs. Calculated on a daily basis the doses were: 2, 20, or 200 ng TCDD/kg, 100, 1.000, or 10.000 ng HpCDD/kg, or 200, 2.000, or 20.000 ng/kg of a mixture of 49 PCDD congeners as described [18]. After 13 weeks, the animals were sacrificed, liver concentrations of PCDD were analyzed, and enzyme-altered (preneoplastic) liver foci (ATPase-deficient) were quantified as described elsewhere in detail [18]. A four parameter logistic model was applied to investigate the relationship between PCDD liver levels and the relative hepatic volume occupied by preneoplastic foci (relative focal volume; RFV):

$$y = a + (L - a)/\{1 + \exp[\alpha(d_{50} - \log PCDD)]\}$$

where y equals $\sin^{-1}(RFV)^{1/2}$. The parameter L describes the maximal value of y, the three other parameters describe the y value at a PCDD level of zero (a), the log PCDD level at the y value halfway between a and L (d_{50}), and the slope at d_{50} (α). L was preselected out of a series of values corresponding to a range of 7.0 - 13.0 % RFV and tested for best fit [18].

Results and Discussion

The seven 2,3,7,8-substituted PCDD accounted for almost all of the EROD-inducing potency of a complex, defined mixture of 49 PCDD congeners. The TEF value calculated from the chemical composition of the mixture (on mass basis) was in almost complete agreement with the value obtained when the mixture was applied directly to the hepatocytes (Table 1). Thus, the potent congeners showed additive inducing effects irrespective of the presence of 42 non-agonistic PCDDs accounting for about 32 % of the mixture.

Table 1. EROD-TEFs of 2,3,7,8-substituted PCDD in rat hepatocytes in primary culture, calculated EROD-TEQ (sum) and I-TEQ of a mixture of 49 PCDD [14].

Inducer	EROD-TEF	mg/g mixture	mg EROD-TEQ/ g mixture	mg I-TEQ [5]/ g mixture
TCDD	1. 0	1. 1	1. 10	1. 10
1,2,3,7,8-PeCDD	0. 167	12. 0	2. 01	6. 00
1,2,3,4,7,8-HxCDD	0. 074	12. 8	0. 95	1. 28
1,2,3,7,8,9-HxCDD	0. 042	23. 2	0. 97	2. 32
1,2,3,6,7,8-HxCDD	0. 031	18. 2	0. 56	1. 82
HpCDD	0. 018	137. 5	2. 48	1. 38
OCDD	0. 003	128. 9	0. 39	0. 13
Sum		333. 7	8. 46	13. 93

calculated EROD-TEQ : 8. 46 mg/g mixture
experim. EROD-TEQ : 9. 10 mg/g mixture

In a (synthetic) mixture of six PCB congeners including the non-ortho-substituted PCB 77, 126, and 169, a very good coincidence of the calculated and the experimentally determined EROD-TEFs was obtained in rat hepatocytes in primary culture[19] (not shown). These data support the notion that in the bioassay non-ortho- and mono-ortho-substituted PCB also act in an additive manner.

In a technical PCB mixture (Arochlor 1254), the sum of WHO-TEQ based on chemical analysis was about 25 % lower than calculated from the individual EROD-TEFs of the congeners (Table 2). This is probably due to the relatively low estimation of PCB 126 with a WHO-TEF of 0.1 compared to the EROD-TEF of 0.2 in rat hepatocytes. This difference is relevant since PCB 126 contributes more than 90 % to the inducing potency of Arochlor 1254. The contribution of PCDF to the total EROD-TEQ was minor. The experimental EROD-TEQ were about twofold higher than the calculated EROD-TEQ suggesting a synergistic effect of non-'dioxinlike' PCB.

Table 2. EROD-TEFs of 13 PCB attributed with WHO-TEFs [6], and of the sum of 2,3,7,8-substituted PCDF, in H4IIE rat hepatoma cells in culture, and calculated EROD-TEQ and WHO-(I-)TEQ of a technical PCB mixture (Arochlor 1254)[16].

Inducer	EROD-TEF	µg/g mixture	ng EROD-TEQ/ g mixture	ng WHO(I)-TEQ/ g mixture
PCB 77	1×10^{-4}	389	39	195
PCB 126	2×10^{-1}	205	41 000	20 500
PCB 169	3×10^{-3}	1	3	10
PCB 105	1×10^{-5}	21 800	218	2 180
PCB 114	7×10^{-5}	1 300	91	650
PCB 118	4×10^{-6}	34 000	136	3 400
PCB 123	1×10^{-4}	3 800	380	380
PCB 156	6×10^{-5}	5 800	348	2 900
PCB 157	5×10^{-5}	970	49	485
PCB 167	8×10^{-6}	6 770	54	68
PCB 189	$< 1 \times 10^{-6}$	250	-	25
PCB 170	n.d.	5 280	-	528
PCB 180	n.d	7 800	-	78
sum of 2378-PCDFs[16]		5. 4	950	712
Sum			43 268	32 111

calculated EROD-TEQ : 43. 3 µg/g mixture
experim. EROD-TEQ : 84. 2 µg/g mixture

In a two stage initiation-promotion-experiment in female Wistar rats, TCDD, HpCDD and a mixture of 49 PCDD congeners acted as promoters of preneoplastic liver foci. In all cases a hypolinear tissue level-effect-relationship was found, in agreement with previous reports using TCDD as a liver tumor promoter [20,21]. Furthermore, the concept of TCDD equivalency apparently was not completely valid for HpCDD which, at higher tissue levels, showed a lower potency than expected from I-TEF or EROD-TEF values (Fig. 1). For the mixture a rough coincidence with the expected promoting potency based on chemical composition, I-TEF or EROD-TEF values, and additive effects of 2,3,7,8-substituted constituents was obtained at lower tissue levels. Interestingly, the efficacy of the mixture at higher doses (liver levels) was considerably lower than that of TCDD.

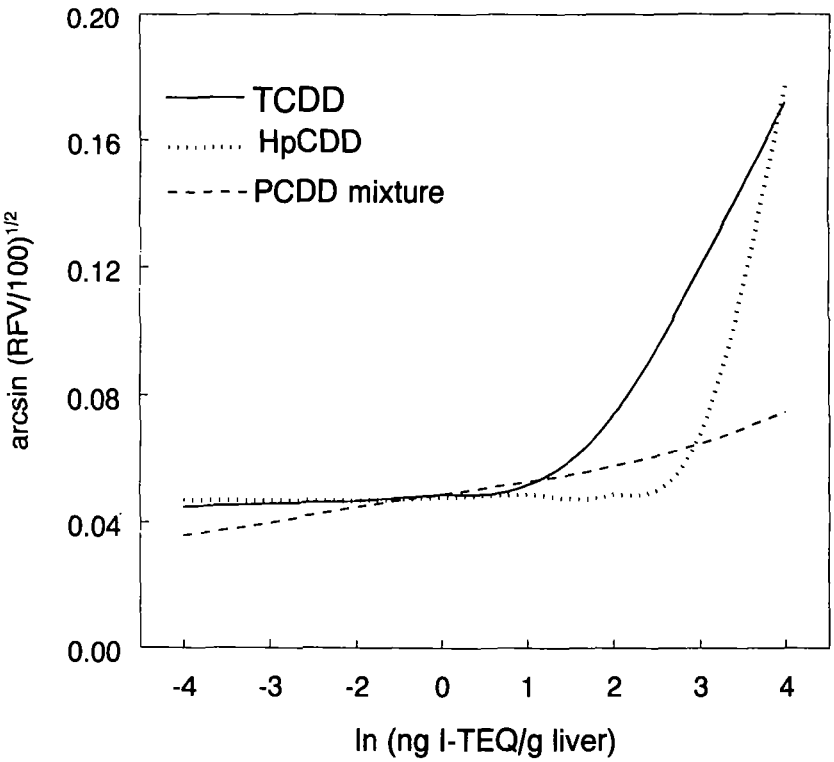

Fig. 1. Relationship between hepatic PCDD level (ng I-TEQ/g liver) and RFV of ATPase-deficient preneoplastic liver tissue using a four-parameter logistic model [18] as described under 'Experimental methods'. Animals initially received N-nitrosomorpholine, and were subsequently treated with various doses of TCDD, HpCDD, or a complex mixture of 49 PCDD congeners.

In conclusion, 2,3,7,8-substituted congeners were found to be almost exclusively responsible for the CYP1A1-inducing and liver tumor promoting effects of a complex PCDD mixture. In the EROD bioassay, these congeners acted in an independent (additive) manner, which is not influenced by non-2,3,7,8-substituted congeners.
In the case of PCB, AhR agonists attributed with WHO-TEFs also acted independently in the EROD bioassay, whereas, in a complex PCB mixture, a slight synergism with non-'dioxinlike' PCB was observed. In an initiation-promotion experiment in rat liver using TCDD, HpCDD or a PCDD mixture, equivalency and additivity of the promoting effects were found at lower (< 100 ng I-TEQ/g liver) but not at higher tissue levels.

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