Interference of polychlorinated biphenyl (PCB) congeners with the CYP1A catalytic activity in flounder (*Platichthys flesus*).

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Introduction

In the aquatic environment, induction of cytochrome P4501A (CYP1A) and associated ethoxyresorufin-O-deethylase (EROD) activity is commonly used as a biomarker for monitoring exposure of fish to environmental pollutants such as PHAHs and PAHs[1,2]. In a previous study, we demonstrated low hepatic CYP1A activity in European flounder (*Platichthys flesus*) upon exposure to the commercial PCB mixture Clophen A50[3]. Furthermore, reduced catalytic CYP1A activity was observed in a number of other fish species exposed to PCBs[4,5]. These observations on low responsiveness and variable results on CYP1A levels and associated EROD activity seriously hamper the further development and use of EROD activity as a reliable biomarker for exposure of fish to PHAHs and related compounds. The reasons for this low responsiveness are not fully understood and may either be a consequence of an "inefficient" Ah receptor pathway for induction of CYP1A activity by PCBs, or may be a consequence of interference of PCB congeners with the catalytic activity of CYP1A in flounder.

In this study we report on interference of PCB congeners with the CYP1A catalytic activity in flounder. We studied and compared the apparent *in vitro* inhibition of hepatic microsomal CYP1A activity by four PCB congeners and by the commercial PCB mixture Clophen A50 in flounder and, as a comparison, in rat.

Material and Methods

Chemicals. Aroclor 1254 was donated by Dr. M. van den Berg (RITOX, University of Utrecht, the Netherlands). Clophen A50 was a gift from Dr. J. P. Boon (NIZO, Den Burg, Texel, the Netherlands). TCDD, PCB-77, PCB-126, PCB-169 and PCB-153 were obtained from CN Schmidt B.V. (Amsterdam, the Netherlands).

Animals. Two-year-old flounder (*Platichthys flesus*) were captured in the Dutch Wadden Sea and housed as described by Besselink *et al*[6]. Wistar rats (10 weeks old) were obtained from the Laboratory Animal Centre (Wageningen Agricultural University, the Netherlands).

Treatment of animals. Flounder (n=12) were orally injected at day 1 and 7 with TCDD (final concentration 10 µg TCDD/kg body weight) using 200 µl gelatin capsules and killed at day 10 as described previously[6]. Male Wistar rat (n=3) were dosed i.p. at day 1 and 2 with 100 mg Aroclor 1254/kg b.w. in corn oil (0.5 ml). At day 3, rats were killed. Livers from both flounder and rats were dissected free from the gall bladder, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C.

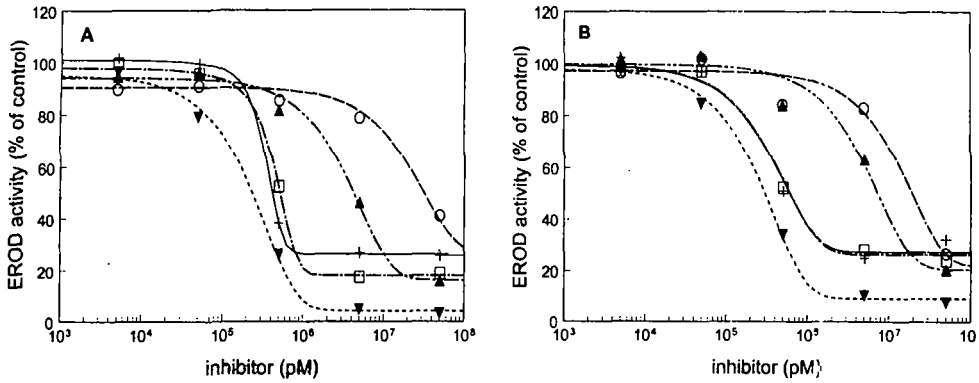

Fig. 1 Inhibition of rat (A) and flounder (B) hepatic microsomal EROD activity by PCB-77 (+), PCB-126 (▼), PCB-169 (□), PCB-153 (O), and CloA50 (▲) with ethoxyresorufin concentration of 0.4 μM for rat and 0.6 μM for flounder. Control EROD activity for rat and flounder are 1.8 and 0.3 nmol/min/mg respectively.

Preparation of microsomes. Microsomes from individual livers of TCDD treated flounder and Aroclor 1254 treated Wistar rats were prepared as described by Besselink *et al.*[6], except for the final pellet which was resuspended in TEGD buffer (0.1 M Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 1 mM DTT and 20% (v/v) glycerol).

EROD inhibition studies. Microsomal 7-ethoxyresorufin-O-deethylation (EROD) activity was measured in pooled hepatic microsomes as described by Besselink *et al.*[6]. Incubations with flounder tissue contained 37.5 μg microsomal protein/ml and 1 mg BSA/ml, whereas rat tissue incubations contained 5 μg microsomal protein/ml and 1.03 mg BSA/ml. EROD inhibition studies with PCB-77, PCB-126, PCB-153, PCB-169 or Clophen A50 (0-50 μM in DMSO) were performed at 7 different ethoxyresorufin substrate concentrations (0.1-1.5 μM). All incubations were in duplicate and corrected for a blank without NADPH. Rat microsomal EROD activity was measured at 37^{0}C whereas for flounder microsomal EROD activity assays were carried out at 25^{0}C.

Analysis of inhibition potency. Curve-fitting of the inhibition curves using non-linear regression was carried out at constant ethoxyresorufin concentrations for flounder and rat of 0.6 and 0.4 μM respectively. The IC_{50} value for each competitor was determined by interpolation from the fitted curves. K_i values were calculated using the formula $K_i = IC_{50}/(1+[ER]/K_m)$, with [ER] concentration for flounder and rat of 0.6 μM and 0.4 μM respectively.

Results

In Fig. 1A (rat) and 1B (flounder), the EROD inhibition curves of the tested PCB congeners are shown at ethoxyresorufin concentrations of 0.4 and 0.6 μM for rat and flounder respectively. From these curves, IC_{50} values were calculated which are given in Table 1. The di-ortho substituted PCB-153 was the least potent inhibitor of hepatic microsomal EROD activity in both rat and flounder. PCB-126 had the greatest inhibitory potency of the PCBs tested in rat and flounder. In fact, although the tested PCB congeners showed lower IC_{50} values in rat than in flounder, the order of inhibitory potencies of the PCB congeners was identical in rat and flounder. Calculated IC_{50} values for the commercial PCB mixture were lower than PCB-153 but higher than the 3 non-ortho PCBs tested for both rat and flounder.

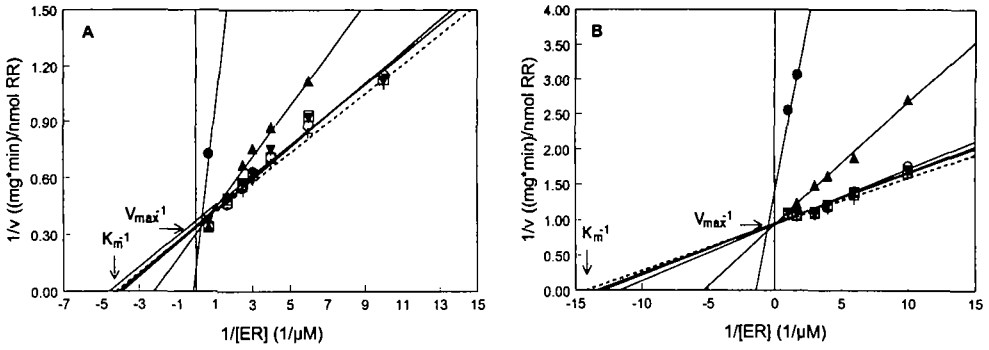

Fig. 2 Lineweaver Burk plots of rat (A) and flounder (B) hepatic microsomal EROD activity with or without addition of inhibitors. The reciprocal values of EROD activity (1/V) are plotted against 1/ER substrate concentrations for incubation without (+) or with 0.05 nM (▼), 0.5 nM (□), 5 nM (O), 50 nM (▲) and 500 nM (●) PCB-126. X-axis intercept for incubation without inhibitor represents K_m^{-1}, while y-axis intercept represents V_{max}^{-1}.

Fig. 2A (rat) and Fig. 2B (flounder) show Lineweaver Burk plots with PCB-126 as inhibitor. Inhibition of hepatic EROD activity by PCB-126 is competitive in nature for both rat and flounder. In fact, all compounds tested were competitive inhibitors of hepatic EROD activity. V_{max} and K_m values, determined from the Y-axis intercept (V_{max}^{-1}) and X-axis intercept (K_m^{-1}) are given in Table 1. Maximum average CYP1A activity with ER as a substrate was higher in rat than in flounder. The average Michaelis constant for hepatic EROD activity with ethoxyresorufin as a substrate was also higher in rat than in flounder. Using average K_m values, inhibition constants (K_i) for the tested PCB congeners and CloA50 were calculated (Table 1). The inhibition constant K_i was lowest for PCB-126 in rat and flounder whereas highest K_i's were observed for the di-ortho PCB-153. K_i's for the non-ortho PCB were in the same order of magnitude. Intermediate K_i's were calculated for the commercial PCB mixture CloA50.

Discussion

The present results clearly demonstrate the potency of all congeners and CloA50 to inhibit hepatic microsomal CYP1A activity *in vitro* in a competitive way with inhibition constants (K_i) close to the Michaelis constant (K_m) for ethoxyresorufin. Of the PCB congeners tested, the non-ortho PCB-126 was found to be the most potent inhibitor of the CYP1A activity in both rat and flounder, followed by PCB-77, PCB-169 and the di-ortho PCB-153 (Table 2). The order with which the tested PCBs were capable of EROD inhibition reflects their potencies to induce CYP1A[7]. The commercial PCB mixture CloA50 was intermediate in potency, probably due to the fact that CloA50 is composed of non-ortho- and ortho-substituted PCBs. Overall, the concentration of PCB at which 50% inhibition of CYP1A activity was observed was lower in rat than in flounder, except for the di-ortho- substituted PCB-153. Inhibition constants (K_i's) calculated for the various tested PCBs were higher in rat than in flounder, indicating that the cytochrome P4501A system in flounder binds the various PCB congeners with greater affinity than the cytochrome P4501A system in rat.

Table 1 Maximum EROD activity (V_{max}), Michaelis constant (K_m), IC_{50}, and inhibition constant (K_i) of 4 PCB congeners and Clophen A50 in Wistar rat and flounder hepatic microsomal fraction.

Species	Compound	V_{max} [a] (nmol/min/mg protein)	K_m [a] (μM)	IC_{50} [b] (μM)	K_i [c] (μM)
Rat	PCB-77	3.61	0.260	0.42	0.154
	PCB-126	2.90	0.236	0.24	0.088
	PCB-153	2.51	0.306	31.88	11.690
	PCB-169	2.88	0.155	0.52	0.191
	CloA50	2.47	0.201	4.24	1.555
	average ± S.E.M.	2.87 ± 0.20	0.232 ± 0.026		
Flounder	PCB-77	1.11	0.100	0.61	0.084
	PCB-126	1.06	0.070	0.30	0.041
	PCB-153	1.09	0.122	19.46	2.670
	PCB-169	1.02	0.092	0.62	0.085
	CloA50	0.93	0.093	7.39	1.014
	average ± S.E.M.	1.04 ± 0.03	0.095 ± 0.008		

[a]: V_{max} and K_m were derived from Lineweaver Burk plots. [b]: IC_{50} values (molar concentration of inhibitor resulting in inhibition of EROD activity to 50% of control (no inhibitor)) were calculated by non-linear regression curve fitting of inhibition curves (not shown). [c]: K_i's were calculated from the non-linear regression curve fits of the inhibition curves (not shown), using the formula $K_i = IC_{50}/(1+[ER]/K_{m,avg})$, with ethoxyresorufin ([ER]) concentrations of 0.4 μM for rat and 0.6 μM for flounder.

In conclusion, the observed inhibition of EROD activity by PCBs levels near K_m, underscores the necessity of caution when using EROD activity as dependable biomarker for monitoring exposure of fish to xenobiotics such as PCBs.

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