# Inhibition of CYP 1A2–Dependent MROD Activity in Rat Liver Microsomes: An Explanation of the Hepatic Sequestration of a Limited Subset of Halogenated Aromatic Hydrocarbons

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**ABSTRACT:** Many classes of halogenated aromatic compounds (HACs) are highly lipophilic environmental contaminants that exert toxic effects via the Ah receptor signal transduction pathway and whose metabolism generally involves monooxygenase enzymes of the CYP 1A family. Despite their lipophilicity, a high proportion of the body burden of certain polychlorinated dibenzo-p-dioxins and coplanar polychlorinated biphenyls is sequestered in liver, a process believed to involve CYP 1A2. In this work we examined HAC-induced inhibition of the demethylation of 7-methoxyresorufin, a process that is selectively catalyzed by CYP 1A2. 2,3,7,8-Tetrachlorodibenzo-p-dioxin, 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) were found to be strong competitive inhibitors of methoxyresorufin-O-demethylase activity, consistent with the high ability of hepatic tissue to sequester these compounds selectively.  Environ Toxicol 18: 115–119, 2003.

**Keywords:** MROD assay; CYP 1A2; enzyme kinetics; halogenated aromatic compounds; liver sequestration

## INTRODUCTION

Halogenated aromatic compounds (HACs), including polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs), are highly lipophilic environmental contaminants that arise through incineration (PCDDs and PCDFs), improper disposal practices (PCBs), and open use as flame retardants (PBDEs). They are found in the environment as complex mixtures, with food usually the principal route of human exposure (Diliberto et al., 1999). The related compounds, polycyclic aromatic hydrocarbons (PAHs), are released into the environment as a result of incomplete combustion.

The toxicity of the planar or near-planar members of these classes, of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a highly toxic prototype, is mediated through the Ah receptor signal transduction pathway (Wang et al., 1997). One end point of the latter is the induction of monooxygenase enzymes of the cytochrome P-450 (CYP) 1A family (Sutter et al., 1992; Tritscher et al., 1992; Van den Berg et al., 1994; Drahuskuk et al., 1999). CYP 1A1 and 1A2 catalyze the metabolism of many xenobiotics,

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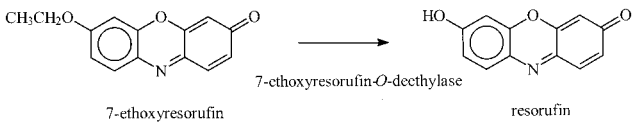


**Fig. 1.** Deethylation of 7-ethoxyresorufin.

including drugs, environmental pollutants, and chemical carcinogens (Guengerich, 1988; Nebert, 1989). The CYP-dependent monooxygenase system, which comprises CYP, NADPH-CYP reductase, and phospholipids, plays a major role in the metabolism of HACs and PAHs, sometimes activating these unreactive contaminants into more reactive, toxic metabolites (Burke et al., 1995).

CYP 1A1 and 1A2 are monooxygenase enzymes with broad and overlapping substrate specificities. In both humans and rodents CYP 1A2 is constitutively expressed in the liver in much greater amounts than CYP 1A1, although the latter is highly inducible by planar xenobiotics such as TCDD and by certain PAHs such as 3-methylcholanthrene (Hu and Bunce, 1999a). Administration of TCDD to male C57BL/6J mice increased both CYP 1A1 and CYP 1A2 hepatic protein levels, whereas the nonplanar PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) increased the levels of CYP 1A2 but not CYP 1A1. Moreover, CYP 1A2 activities (but not protein levels) in mice cotreated with TCDD and PCB 153 were higher than those of mice treated with TCDD alone (De Jongh et al., 1995). CYP 1A1 and 1A2 may be distinguished by immunoblotting or by exploiting their selectivity toward particular substrates. CYP 1A1, for example, preferentially deethylates 7-ethoxyresorufin, whereas CYP 1A2 is selective for demethylation of 7-methoxyresorufin (Sohn et al., 1994)—EROD (ethoxyresorufin-*O*-deethylase) and MROD (methoxyresorufin-*O*-demethylase) activities, respectively. Both EROD and MROD involve oxidation of the substrate at the aliphatic carbon atom $\alpha$ to the ether linkage, affording a hemiacetal that spontaneously dissociates to give resorufin, which can be quantitated by fluorescence spectroscopy (Fig. 1, shown for EROD). Activation of the Ah receptor by a given HAC correlates with the synthesis of the CYP 1A protein and with increased enzyme activity (Petrulis et al., 2000; Chen and Bunce, 2001). Other possible biomarkers for CYP 1A2 induction include induction of estradiol-2-hydroxylase activity (Graham et al., 1988) and acetanilide-4-hydroxylase (ACOH; Liu et al., 1991). Nerurkar et al. (1993) showed that both ACOH and MROD activities correlated well with CYP 1A2 protein levels as determined by Western blot analysis, although MROD activity is preferred because of speed and convenience (DeVito et al., 1997; Nerurkar et al., 1993).

Most HACs are highly lipophilic, as reflected in octanol–water partition coefficients ($K_{ow} > 10^5$) and therefore tend to accumulate in biota. Indeed, the release of these compounds from adipose tissue may be the rate-limiting step in their elimination (Van den Berg, 1994; Hu and Bunce, 1999b). Sequestration in adipose tissue may also explain the long whole-body half-lives of substances like TCDD in large mammals, for example, adult humans (5–9 years) versus rodents (weeks). The specific problem addressed in this article is the tendency of a small group of HACs, typified by TCDD and the coplanar PCBs, to be sequestered in liver (DeVito et al., 1998; Van Birgelen et al., 1994) and, in PCB 77 (3,3′,4,4′-tetrachlorobiphenyl), to be mainly metabolized by CYP 1A2 (Kramer et al., 2000). Sequestration was hypothesized to be the result of a specific interaction between TCDD—and presumably the coplanar PCBs—and CYP 1A2. In this study we measured the competitive inhibition constants ($K_1$) of several HACs in the MROD assay, using microsomes prepared from the livers of immature male Sprague-Dawley rats, as a measure of their affinities for CYP 1A2.

## MATERIALS AND METHODS

### Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was a generous gift from Wellington Laboratories (Guelph, Ontario, Canada); 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153), 3,3′,4,4′-tetrachlorobiphenyl (PCB 77), 2,2′,4,4′-tetrabromodiphenyl ether (PBDE 47), 3,3′,4,4′-tetrabromodiphenyl ether (PBDE 77), 2,2′,3,4,4′-pentabromodiphenyl ether (PBDE 85), 2,3′,4,4′,6-pentabromodiphenyl ether (PBDE 119), and 3,3′,4,4′,5-pentabromodiphenyl ether (PBDE 126) were synthesized in this laboratory; 1,2,3,6,7,8-hexachlorodibenzofuran (1,2,3,6,7,8-HexaCDF), 2,3,4,6,7,8-hexachlorodibenzofuran (2,3,4,6,7,8-HexaCDF), 2,2′,5,5′-tetrachlorobiphenyl (PCB 52), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), 2,2′,4,4′,6,6′-hexachlorobiphenyl (PCB 155), 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB 156), 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB 169) were purchased from Accustandard Inc. (New Haven, CT); 1-methylphenanthrene (1-MPA), benzo[*a*]pyrene (B*a*P), benzo[*e*]pyrene (B*e*P) were obtained from Supelco Canada (Mississauga, Ontario); dimethyl sulfoxide (DMSO), $MgSO_4$, and tris-(hydroxymethyl)-aminomethane (TRIS) were purchased from Fisher Scientific Co. (Toronto, Ontario, Canada); $\beta$-$NADP^+$ was purchased from CALBIOCHEM (La Jolla, CA); Resorufin was purchased from ICN Biomedicals Inc. (Aurora, OH); sodium pentobarbital (Somnitol®) was obtained from MTC Pharmaceuticals (Cambridge, Ontario, Canada); 3-methylcholanthrene (3-MC), $\beta$-naphthoflavone ($\beta$-NF), Percoll, Hank's balanced salt solution, Dulbelcco's phosphate buffered saline (PBS), DL-isocitric acid, isocitric dehydrogenase, NADH, BSA, and 7-MR were purchased from Sigma Chemical Co. (St. Louis, MO).

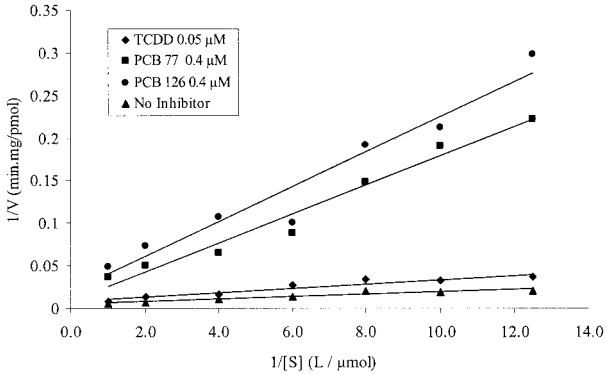


**Fig. 2.** Comparison of competitive inhibition of CYP 1A2 catalytic MROD activity by TCDD, PCB 77, and PCB 126, respectively, in rat hepatic microsomes.

## Animal Treatment and Microsome Preparation

Male Sprague-Dawley rats (100 g) obtained from Charles River Breeding Laboratories (Canada) were administered 100 $\mu$mol 3-MC in corn oil per kilogram by intraperitoneal injection on 4 consecutive days. On the fifth day, the animals were sacrificed by $CO_2$/cervical dislocation. The livers were perfused *in situ* with ice-cold buffer [10 mM HEPES, 1 mM EDTA, 10% (v/v) glycerol, pH 7.6]. The livers were excised, rinsed with buffer, minced with scissors and rinsed again. The tissue was homogenized and spun at 9000 $\times$ g for 20 min at 4°C. Surface lipid was removed by aspiration, and the supernatant was spun at 100 000 $\times$ g for 68 min in a Beckman L7-65 ultracentrifuge at 4°C. The pellet was rinsed, resuspended in buffer, and spun a second time. The microsomal pellet was suspended in buffer and stored in 1-mL aliquots at $-70$°C. Microsomal protein concentrations were determined according to the method of Bradford (1976).

## Kinetics of MROD Inhibition in Rat Hepatic Microsomes

Rat hepatic microsomes (stored with EROD activity of 85 nmol resorufin $min^{-1}$ $mL^{-1}$) were diluted 100-fold with 10 mM TRIS buffer (pH 7.4). Ten microliters was then added to each well of a 48-well microplate containing 450 $\mu$L of NADPH-generating system (5 mM $MgSO_4$, 7.5 mM DL-isocitric acid, 5 mM $\beta$-$NADP^+$, isocitrate dehydrogenase at 0.5 units/mL, NADH at 0.5 mg/mL, and BSA at 0.5 mg/mL in 50 mM TRIS-HCl, pH 7.4). To this, HAC in 2.5 $\mu$L of DMSO was added, followed by incubation for 5 min at 23°C. Inhibition curves were generated by adding various concentrations of the substrate 7-MR, incubating for a further 15 min and assaying for the fluorescent product resorufin by monitoring fluorescence at 590 nm using a Bio-Rad FLUOstar fluorescent plate reader (BMG Lab Technologies, Germany).

## Data Analysis

Through the use of a standard resorufin curve, fluorescence intensities were converted to reaction rates (V, nmol resorufin $min^{-1}$ $mL^{-1}$). Spreadsheet software (Microsoft Excel '97) was used to obtain linear regressions between 1/V and 1/[7-MR] for reactions carried out in the absence and in the presence of at least two concentrations of the inhibitor. The slope of the regression line between 1/V and 1/[7-MR] is $(K_M/V_{max}) \times ([HAC]/K_i)$, from which $K_i$ may be determined.

## RESULTS AND DISCUSSION

Our principal objective was to determine $K_i$ for a wide range of Ah receptor ligands that might interact with CYP 1A2. TCDD and other HACs showed dose-dependent decreases in MROD activity with an increasing concentration of HAC. The slopes of the Lineweaver–Burk plots were calculated using linear regression, and a comparison of the slope in the presence of inhibitor with the slope in the absence of inhibitor was used to calculate the inhibition constant ($K_i$) for each compound. $K_i$ = [Inhibitor]/($\alpha - 1$), where $\alpha$ is the ratio of the slopes in the presence and absence of inhibitor. Figure 2 shows typical examples of the data.

Each experiment was replicated twice; values in Table I are the means of the replicates; overall variation in all cases was $< 5\%$. TCDD and PCB 126 bound strongly to CYP 1A2, with $K_i \sim 3 \times 10^{-8}$ M, with PCB 169, $K_i \sim 1 \times 10^{-7}$ M, consistent with their sequestration in the liver, where CYP 1A2 is particularly abundant (DeVito et al., 1997; Chen CY et al., 2001). PCB 77 also bound strongly to CYP 1A2, with $K_i \sim 3 \times 10^{-8}$ M, but it has not been reported to sequester in the liver because of rapid metabolism (Chen CY et al., 2001). 2,3,4,7,8-Pentachlorodibenzofuran has also been reported to be sequestered in hepatic tissue (DeVito et al., 1997), but we did not have a sample of this compound available.

**TABLE I. Competitive inhibition constants (*Ki*) of aromatic compounds to MROD activity (CYP 1A2)**

| Congeners | *Ki* (nM) | Congeners | *Ki* (nM) |
|---|---|---|---|
| TCDD | 33 | 1-MPA | 320 |
| 1,2,3,6,7,8-HexaCDF | 620 | 3-MC | 92 |
| 2,3,4,6,7,8-HexaCDF | 610 | $\beta$-NF | 50 |
| | | B*a*P | 46 |
| PCB 52 | 80 | | |
| PCB 77 | 31 | B*e*P | 65 |
| PCB 126 | 26 | PBDE 47 | 800 |
| PCB 153 | 80 | PBDE 77 | 400 |
| PCB 155 | 1200 | PBDE 85 | 1200 |
| PCB 156 | 220 | PBDE 119 | 2000 |
| PCB 169 | 110 | PBDE 126 | 400 |

Among the other compounds studied, attention may be drawn to β-naphthoflavone, B[*a*]P, B[*e*]P, and 3-MC, all with $K_i$ values in the range of 5–9 × $10^{-8}$ M; despite being Ah receptor agonists, they do not sequester in the liver because they are readily metabolized (Gonzalez et al., 1994; Zhang et al., 1997; Petrulis et al., 2000). Hepatic sequestration by CYP 1A2 is thus limited to Ah receptor agonists that resist Phase I metabolism. We also studied several PBDE congeners, which are flame retardants that are increasingly found in the environment and in biota (Darnerud et al., 2001). These HACs are only weak Ah receptor agonists (Chen G et al., 2001); none of them was a significant inhibitor of MROD activity.

Unlike CYP 1A1, CYP 1A2 is constitutively expressed in hepatic tissues, and its induction occurs through tissue-specific mechanisms (Goldstein and Linko, 1983; Tukey and Nebert, 1984). That CYP 1A2 is the binding protein for dioxin in the liver is shown by the lack of sequestration of TCDD in the livers of the CYP 1A2 knockout mice (Diliberto et al., 1997). Also in contrast to CYP 1A1, CYP 1A2 is not highly inducible by PAHs (Hu and Bunce, 1999a). The tendency of TCDD, PCB 77, and PCB 126 to bind CYP 1A2 is thus of potential toxicological significance, in that such an association may deplete the pool of CYP 1A2 available for metabolizing other xenobiotics. Whether this is positive or negative from the perspective of toxicity depends on the substrate. Some xenobiotic substrates for CYP 1A, such as PAHs, aromatic amines, and aflatoxins, are bioactivated into carcinogenic species (Crespi et al., 1989, 1991; Shimada et al., 1989; Gonzalez et al., 1994; Eaton et al., 1995; Rendic et al., 1997). In these cases the inhibition of CYP 1A2 by HACs might be expected to exert a protective effect.

Recent work indicates widening toxicological implications for CYP 1A2–sequestering HACs such as TCDD and PCBs 77 and 126. PCB 126 was already known as the most toxic PCB congener in the context of Yusho poisoning, an incident of human poisoning in Japan resulting from consumption of PCB-contaminated rice oil (Yoshimura et al., 1979). PCB 126 is also known to depress the hepatic concentrations of class 1 alcohol dehydrogenase (Ishii et al., 2001), aldolase B, carbonic anhydrase III, glucose-regulated protein 78 (GRP78), GRP94, calreticulin, and calnexin (Ishii et al., 1997; Tasaki et al., 1999; Ikeda et al., 2000; Yoshioka et al., 2001).

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