Oxidative metabolism of BDE-99 by rat liver microsomes: metabolites formed and CYP enzymes involved

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Introduction

2,2′,4,4′,5-Pentabromodiphenyl ether (BDE-99) is the major congener in the widely-used commercial penta-brominated diphenyl ether (BDE) mixture and has become a widely distributed environmental contaminant (Hites 2004). Hydroxy-BDEs have been identified in humans and other vertebrate species (Athanasiadou et al. 2008, Marsh et al. 2004, McKinney et al. 2006, Qiu et al. 2009) but the oxidative metabolism of BDE-99 has not been thoroughly investigated *in vivo* or *in vitro*. Recent studies showed that hydroxy metabolites of BDE-99 are formed in rats, mice and humans (Chen et al. 2006, Hakk et al. 2002, Lupton et al. 2009, Stapleton et al. 2009, Staskal et al. 2006). Structural characterization of all the hydroxy metabolites of BDE-99 formed, the identification of the enzymes involved in BDE-99 oxidative metabolism and the characterization of enzyme kinetics (i.e. K_m and V_{max}) have not been undertaken yet. The objectives of the present study were to use rat liver microsomal preparations to investigate the oxidative metabolism of BDE-99, to identify the hydroxy metabolites produced, to determine rates of metabolite formation and apparent K_m and V_{max} values. Ultimately, a panel of recombinant enzymes will be used to determine the degree of participation of individual CYP enzymes in the metabolism of BDE-99.

Materials and Methods

Adult male Long Evan rats were injected intraperitoneally with corn oil (2 ml/kg/day), dexamethasone (DEX, 100 mg/kg/day), 3-methylcolanthrene (MC, 25 mg/kg/day) and phenobarbital (PB, 80 mg/kg/day) for 3 consecutive days and killed 24 h after the last treatment. Liver microsomes were prepared from pooled liver homogenates (Thomas et al. 1983). Rats were treated with DEX, MC or PB because these compounds are inducers of CYP3A (and partially 2B), 1A and 2B enzymes, respectively.

Rates of formation of hydroxy BDE-99 metabolites were determined by incubating BDE-99 with rat liver microsomes using reaction mixtures, which contained 50 mM phosphate buffer, 0-1.0 mg of hepatic microsomal protein and 2.5-200 µM BDE-99 in a final volume of 0.99 mL. Reactions were initiated by addition of 0.01 mL of 100 mM NADPH solution and stopped after 0-30 min by addition of 1.0 mL of 0.5 M NaOH. Metabolites were extracted using 2.0 mL of a mixture of methyl-tert-butyl ether:hexane (1:1 v/v) three times. The organic phases from each extraction were pooled and dried using nitrogen gas and then reconstituted in 250 µl of methanol. Metabolites were detected and quantified using a validated LC/MS method (Erratico et al., submitted). Chromatographic separation was achieved with a C_{18} (2.1 x 100 mm, 1.7 µm) column using a gradient elution program. The mobile phase was composed of water and methanol and the mass spectrometer was operated in negative electrospray ionization mode using selected ion recording, as described (Erratico et al., submitted).

Results and Discussion

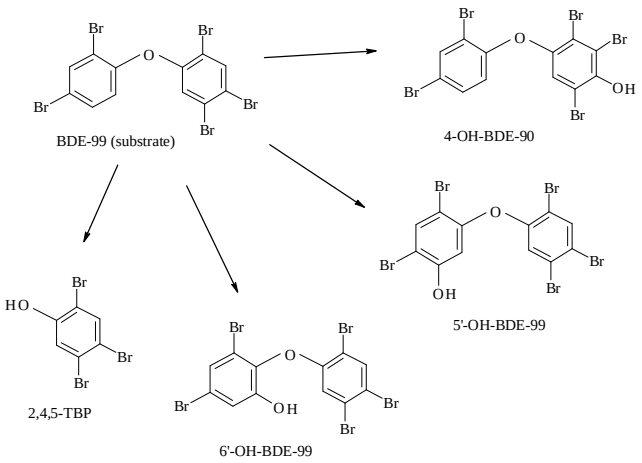

Figure 1. Identified hydroxylated metabolites of BDE-99 formed by rat liver microsomes.

Preliminary experiments indicated that rates of formation of BDE-99 metabolites were highest and lowest in liver microsomes obtained from DEX- and corn oil-treated rats, respectively.

Seven hydroxy metabolites of BDE-99 were formed when BDE-99 was incubated with liver microsomes obtained from PB-treated rats. Four metabolites were identified as 2,4,5-tribromo phenol (2,4,5-TBP), 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99 using authentic standards (Figure 1). The major metabolite formed was 4-OH-BDE-90 in all but MC microsomes. 4-OH-BDE-90 showed increasing rates of formation from control to MC, PB and DEX microsomes (Figure 2). This suggests that mainly CYP2B and 3A enzymes are involved in the formation of 4-OH-BDE-90. 5′-OH-BDE-99 and 2,4,5-TBP showed increasingly higher rates of formation from MC to DEX and PB microsomes. This suggests that CYP2B and 3A enzymes are involved in the formation of 5′-OH-BDE-99 and 2,4,5-TBP metabolites more than what CYP1A enzymes are. Conversely, rates of formation of 6′-OH-BDE-99 showed an increasing trend from DEX to MC and PB microsomes. This suggests that CYP1A and CYP2B enzymes are likely the main enzymes involved in the formation of 6′-OH-BDE-99.

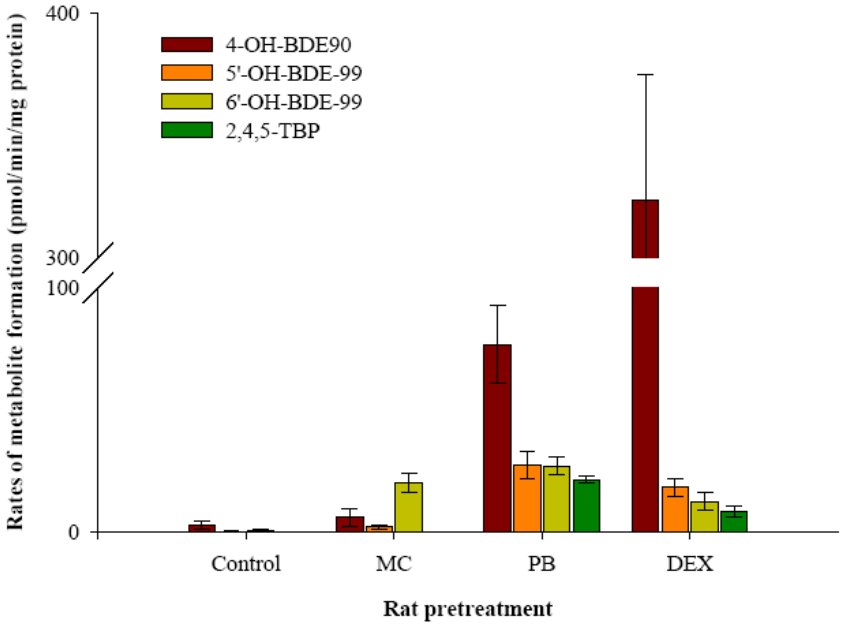

Figure 2. Rates of formation of hydroxy metabolites of BDE-99 by liver microsomes prepared from rats pretreated with corn oil, DEX, MC, or PB. Results are expressed as mean±S.D. of three independent experiments (samples were prepared in duplicate in each experiment).

Kinetic experiments showed that the formation of all the known metabolites obeyed the Michaelis-Menten kinetic model, with the only exception of 2,4,5-TBP which obeyed a substrate inhibition kinetic model (Table 1). In control microsomes, only two hydroxy metabolites were formed: 4-OH-BDE-90 and 6′-OH-BDE-99. Apparent V_{max} values confirmed the former to be the major metabolite. In DEX microsomes, 2,4,5-TBP, 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and two unknown OH-penta-BDE metabolites were formed (unknowns not reported). Again, 4-OH-BDE-90 showed the highest apparent V_{max} value. Data obtained using DEX microsomes confirmed that CYP3A and CYP2B enzymes are likely involved in the formation of all of the six hydroxy metabolites formed and especially of 4-OH-BDE-90.

Table 1. Mean values±S.D. (n=3) of apparent V_{max} and K_m of BDE-99 metabolites formation by liver microsomes obtained from rats pretreated with corn oil or DEX

Metabolite	Apparent V_{max} pmol/min/mg protein		Apparent K_m (μM)	
	Corn oil	DEX	Corn oil	DEX
2,4,5-TBP[a]	N.A.	8.7 ± 0.6	N.A.	1.5 ± 0.8
4-OH-BDE-90[b]	16.6 ± 5.9	402.0 ± 44.5	947.3 ± 467.3	23.2 ± 8.1
5′-OH-BDE-99[b]	N.A.	17.3 ± 1.9	N.A.	16.0 ± 2.0
6′-OH-BDE-99[b]	1.1 ± 0.4	20.9 ± 1.8	7.4 ± 5.5	4.5 ± 0.8

N.A., not available.

[a] Kinetic parameters for 2,4,5-TBP were calculated using a substrate inhibition kinetic model. Mean K_i value was 482.7 ± 95.7 μM (n=3).

[b] Kinetic parameters for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99 were calculated using Michaelis-Menten model.

Further experiments are underway to identify the individual CYP enzymes responsible for the formation of the hydroxy metabolites of BDE-99. A panel of rat recombinant enzymes including CYP1A1, 1A2, 1B1, 2A1, 2A2, 2B1, 2B2, 2C6, 2C7, 2C11, 2C12, 2C13, 3A1, 3A2, 2D1, 2E1, 4A1 will be used. The outcomes of these experiments will be used to confirm the kinetic dataset obtained using microsomal preparations.

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