BIOTRANSFORMATION OF BDE-47 TO POTENTIALLY TOXIC METABOLITES IS PREDOMINANTLY MEDIATED BY HUMAN CYP2B6

Feo ML[1], Gross MS[2]*, McGarrigle BP[3], Eljarrat E[1], Barcelo D[1], Olson JR[3], Aga DS[2]

[1]IDAEA CSIC, Environmental Chemistry Department, Jordi Girona 18-26, 08034 Barcelona, Spain;
[2]Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, New York 14260;
[3]Department of Pharmacology and Toxicology, University at Buffalo, The State University of New York, 3435 Main St., Buffalo, New York 14214

Introduction

Animal and human studies have supported an association between polybrominated diphenyl ether (PBDE) flame-retardants and neurobehavioral / neurodevelopmental disorders, particularly following *in utero* and postnatal exposure.[1-6] While three mixtures of PBDEs were produced globally (penta-BDE, octa-BDE, and deca-BDE), the lower brominated mixtures contain the more bioaccumulative and persistent congeners, with 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) representing the most abundant PBDE detected in human serum.[7]

Hydroxylated metabolites of PBDEs, OH-PBDEs, have been found to accumulate in human serum at levels similar to, and in some cases greater than, that of the parent PBDEs.[8,9] The significance of this finding is heightened by mechanistic studies showing that mono-hydroxylated metabolites of BDE-47 are more potent than the parent BDE-47 in disrupting Ca^{2+} homeostasis, modulating GABA and alpha4beta2 nicotinic acetylcholine (nACh) receptor function, altering spontaneous activity and cell viability in cultured cortical neurons, and competing with thyroxine (T4) for binding to human transthyretin (TTR).[10-16] These and other studies suggest that bioactivation by oxidative metabolism adds considerably to the neurotoxic potential of PBDEs. The purpose of our study was to conduct a qualitative and quantitative characterization of the *in vitro* metabolism of BDE-47, utilizing human liver microsomes (HLMs) and individual recombinant human cytochrome P450s (CYPs). Furthermore, we aimed to identify the major CYP(s) that are active in the oxidative metabolism of BDE-47.

Materials and Methods

CYP-Specific Metabolism of BDE-47

Recombinant human CYPs, including 1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, were individually incubated with 20 μM BDE-47 in a buffer consisting of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), magnesium chloride, bovine serum albumin (BSA), and NADPH at pH 7.4. The assay was initiated with NADPH and incubated at 37 °C for 2 hours. The assay was quenched with an ice bath.

Kinetic Study

Recombinant human CYP2B6 and pooled HLMs were incubated for 60 minutes with concentrations of BDE-47 ranging from 0.1 to 20 μM. Buffer and incubation conditions were the same as previously described.

Extraction

Mass labeled $^{13}C_{12}$-BDE-47 and $^{13}C_{12}$-6-OH-BDE-47 were spiked into the samples and served as recovery surrogates. Hydrochloric acid was added for protein denaturation. Samples underwent liquid-liquid extraction (LLE) with a 75/25 (v/v) solution of hexane/dichloromethane, followed by another LLE with a 50/50 (v/v) solution of hexane/dichloromethane. Samples were then halved. One part was used for the analysis of BDE-47 and possible formation of debrominated metabolites. The second part was derivatized and used for the analysis of hydroxylated metabolites. Derivatization was performed over 18 hours with trimethylsilyl diazomethane. In both cases, BDE-99 was added before analysis for use as an internal standard.

Analysis

Extracts were analyzed using a gas chromatograph (GC; Thermo Scientific Trace GC Ultra), equipped with a Programmable Temperature Vaporization (PTV) injection system and coupled to a triple quadrupole mass spectrometer (MS; Thermo Scientific TSQ Quantum XLS). Separation was achieved on a ZB-5HT Inferno

column (15 m x 0.25 mm id, 0.1 μm film thickness; Zebron) with helium used as a carrier gas. Samples were analyzed under full scan GC-MS for the identification of the metabolites and then by selective reaction monitoring (SRM) GC-MS/MS for the quantification of the derivatized OH-PBDE metabolites.

Results and Discussion

Metabolite Identification

Incubation of BDE-47 with recombinant human CYP2B6 resulted in the formation of eight hydroxylated metabolites (Figure 1). The six mono-hydroxylated metabolites detected were identified as 2'-OH-BDE-66, 3-OH-BDE-47, 4-OH-BDE-42, 4'-OH-BDE-49, 5-OH-BDE-47, and 6-OH-BDE-47. The formation of 2'-OH-BDE-66, 4-OH-BDE-42, and 4'-OH-BDE-49 metabolites resulted from an NIH-shift of a bromine atom, which proceeds through an arene oxide intermediate.[17] Metabolites were detected as methylated derivatives in the sample extracts. Five mono-hydroxylated metabolites were confirmed through comparison of retention times and full mass spectra between the methylated derivatives and their authentic methoxylated BDE standards (3-MeO-BDE-47, 4-MeO-BDE-42, 4'-MeO-BDE-49, 5-MeO-BDE-47, 6-MeO-BDE-47). No reference standard was available for 2'-OH-BDE-66. The presence of 2'-OH-BDE-66 was reasoned from the full mass spectra of its derivatized metabolite. Furthermore, this metabolite's presence was supported by previous results reported for PBDE metabolites found in human blood samples,[8,18] as well as in mice.[16,19] This also suggests that the pathway for *in vitro* metabolism of BDE-47 by HLMs reflects the *in vivo* biotransformation in humans. Two di-hydroxylated metabolites were identified as di-OH-BDE-47 and di-OH-dioxin. These metabolites were deduced from their mass spectra and were found to co-elute with 5-OH-BDE-47 and 2'-OH-BDE-66, respectively. The di-OH-BDE-47 metabolite has been previously reported,[20] but this study is the first to report di-OH-dioxin formation by CYPs.

Reductive debromination of BDE-47 resulted in the formation of BDE-17 and BDE-28 in all samples. These products were identified based on retention time and full mass spectra matching to their corresponding authentic reference standards. However, debromination was attributed to abiotic processes, as the two BDEs were also present in the blank samples containing no CYPs.

CYP-Specific Metabolism of BDE-47

Incubation of 20 μM BDE-47 with eleven individual recombinant human CYPs (1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4) for 120 minutes clearly shows that CYP2B6 is the predominant enzyme involved in the metabolism of BDE-47 (Table 1). Six mono-hydroxylated metabolites were monitored for formation (2-OH-BDE-66, 3-OH-BDE-47, 4-OH-BDE-42, 4'-OH-BDE-49, 5-OH-BDE-47, 6-OH-BDE-47). Their concentrations were presented as an area percentage to the CYP that produced the highest amount of that specific metabolite. In all cases, this enzyme was found to be CYP2B6. Only CYP2C19 and CYP3A4 showed a metabolite formation of at least one percent of CYP2B6. This occurred in the formation of 3-OH-BDE-47 and was 1.67% for CYP2C19 and 13.33% for CYP3A4. No CYPs other than CYP2B6 appear to contribute significantly in the formation of OH-BDE metabolites.

Kinetic Study

The metabolic formation of 3-OH-BDE-47, 5-OH-BDE-47, and 6-OH-BDE-47 was evaluated for 60 minute incubations of BDE-47, ranging in concentration from 0.1 to 20 μM, with recombinant human CYP2B6 and pooled HLMs. The mono-hydroxylated BDE-47 metabolites were the three major metabolites and were able to be detected at the lower incubation levels of BDE-47. Table 2 reports the kinetic parameters, K_m and V_{max}, obtained from Michaelis-Menten plots of rate of metabolite formation versus incubation concentration of BDE-47. Kinetic studies showed that the formation of 3-OH-BDE-47, 5-OH-BDE-47, and 6-OH-BDE-47 occurs at low BDE-47 concentrations, with apparent K_m values in the range of 2.6-4.1 μM and 7.7-9.7 μM for CYP2B6 and HLMs, respectively. Metabolite 3-OH-BDE-47 showed the lowest K_m values and was the major metabolite produced from BDE-47 in both CYP2B6 (V_{max} = 17.0 pmol/min/nmol CYP2B6) and HLMs (V_{max} = 0.8 pmol/min/mg protein or 1.7 pmol/min/nmol P450).

In conclusion, CYP2B6 was identified as the predominant CYP capable of forming six mono-hydroxylated BDE-47 metabolites. Two di-hydroxylated metabolites, di-OH-BDE-47 and di-OH-dioxin, were also observed. Kinetic parameters were determined for the formation of 3-OH-BDE-47, 5-OH-BDE-47, and 6-OH-BDE-47 metabolites. These results will ultimately better inform future mechanistic and epidemiological

studies investigating the potential of PBDEs and their metabolites to produce neurobehavioral / neurodevelopmental disorders.

Figure 1: Metabolic products of BDE-47 formed by CYP2B6

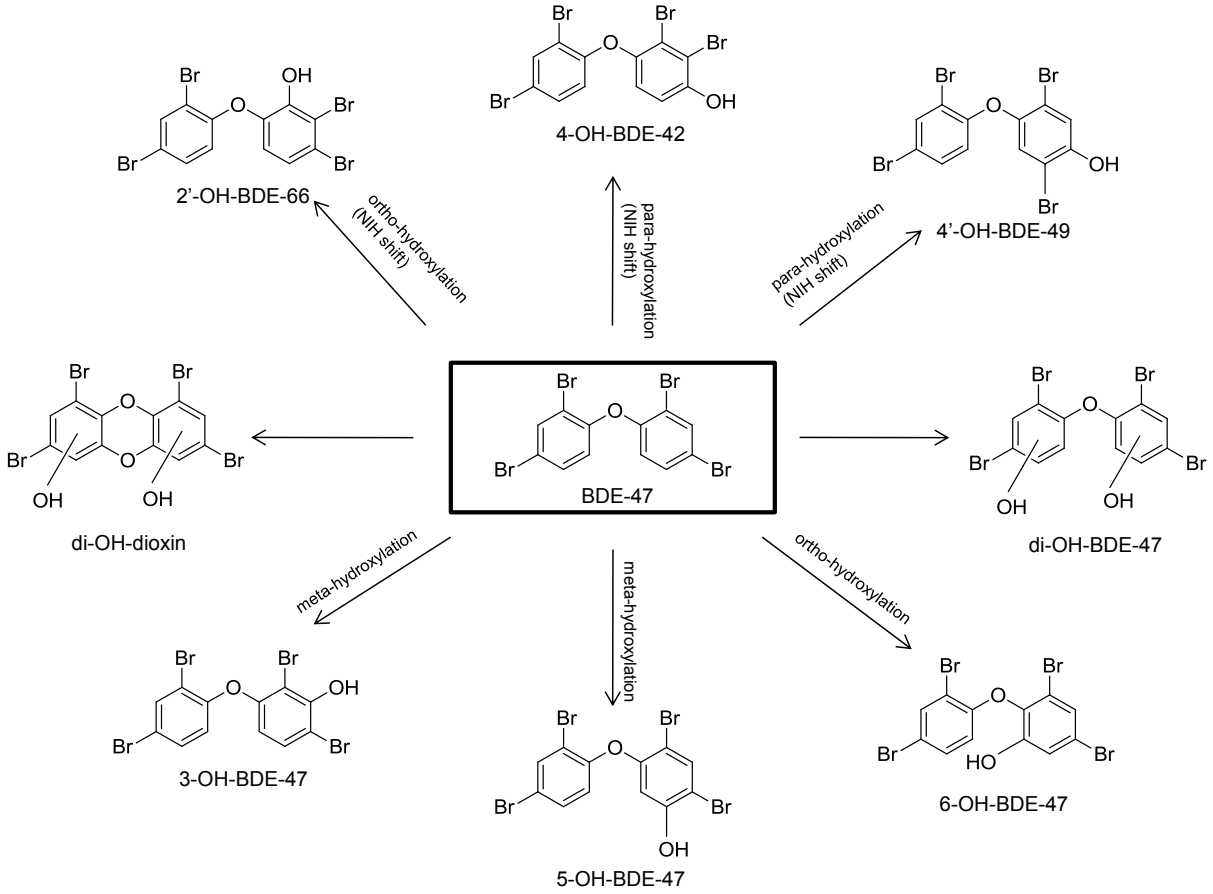

Table 1: Screening of CYP-Specific Metabolism of BDE-47 (20 μM) over 120 minutes

CYPs	**3-OH-BDE-47** (% area relative to CYP2B6)	**5-OH-BDE-47** (% area relative to CYP2B6)	**6-OH-BDE-47** (% area relative to CYP2B6)	**4'-OH-BDE-49** (% area relative to CYP2B6)	**4-OH-BDE-42** (% area relative to CYP2B6)	**2'-OH-BDE-66** (% area relative to CYP2B6)
1A1	nd*	nd	nd	nd	nd	nd
1A2	nd	nd	nd	nd	nd	nd
1B1	nd	nd	nd	nd	nd	nd
2A6	nd	nd	nd	0.75	nd	0.17
2B6	100	100	100	100	100	100
2C8	nd	nd	nd	nd	nd	0.29
2C9	nd	nd	nd	nd	nd	0.08
2C19	1.67	nd	nd	nd	nd	0.05
2D6	nd	nd	nd	nd	nd	0.09
2E1	nd	nd	nd	nd	nd	0.01
3A4	13.33	0.23	nd	nd	nd	nd

*nd, not detected

Table 2: Comparison of the kinetic parameters K_m and V_{max} between recombinant CYP2B6 and pooled human liver microsomes (HLMs)

Metabolites	**Recombinant CYP2B6**		**Pooled HLMs**		
	K_m (μM)	V_{max} (pmol/min/nmol CYP2B6)	K_m (μM)	V_{max} (pmol/min/mg protein)	V_{max} (pmol/min/nmol P450)
3-OH-BDE-47	2.6	17.0	8.6	0.8	1.7
5-OH-BDE-47	4.1	5.0	7.7	0.3	0.6
6-OH-BDE-47	3.4	11.3	9.7	0.6	1.4

Acknowledgements

This work was supported in part by the National Institute of Environmental Health Sciences (NIEHS, grant # ES021554). The content is solely the authors' responsibility and does not necessarily represent official views of NIEHS.

References:

1. Darnerud PO, Eriksen GS, Jóhannesson T, Larsen PB, Viluksela M. (2001) *Environ. Health Perspect.* 109: 49-68
2. Eriksson P, Jakobsson E, Fredriksson A. (2001) *Environ. Health Perspect.* 109: 903-908
3. Costa LG, Giordano G. (2007) *NeuroToxicology.* 28: 1047-1067
4. Suvorov A, Girard S, Lachapelle S, Abdelouahab N, Sebire G, Takser L. (2009) *Neonatology.* 95: 203-209
5. Roze E, Meijer L, Bakker A, Van Braeckel KN, Sauer PJ, Bos AF. (2009) *Environ. Health Perspect.* 117: 1953-1958
6. Herbstman JB, Sjödin A, Kurzon M, Lederman SA, Jones RS, Rauh V, et al. (2010) *Environ. Health Perspect.* 118: 712-719
7. Birnbaum LS, Staskal DF. (2004) *Environ. Health Perspect.* 112: 9-17
8. Qiu X, Bigsby RM, Hites RA. (2009) *Environ. Health Perspect.* 117: 93-98
9. Athanasiadou M, Cuadra SN, Marsh G, Bergman A, Jakobsson K. (2008) *Environ. Health Perspect.* 116: 400-408
10. Kim KH, Bose DD, Ghogha A, Riehl J, Zhang R, Barnhart CD, Lein PJ, Pessah IN. (2011) *Environ. Health Perspect.* 119: 519-526
11. Dingemans MM, de Groot A, van Kleef RG, Bergman A, van den Berg M, Vijverberg HP, et al. (2008) *Environ. Health Perspect.* 116: 637-643
12. Dingemans MM, Heusinkveld HJ, Bergman A, van den Berg M, Westerink RH. (2010) *Environ. Health Perspect*. 118: 519-525
13. Dingemans MM, van den Berg M, Bergman A, Westerink RH. (2010) *Toxicol. Sci.* 114: 302-309
14. Dingemans MML, van den Berg M, Westerink RHS. (2011) *Environ. Health Perspect.* 119: 900-907
15. Hendriks HS, Antunes Fernandes EC, Bergman A, van den Berg M, Westerink RH. (2010) *Toxicol. Sci.* 118: 635-642
16. Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Visser TJ, Van Velzen MJM, Brouwer A, Bergman A. (2008) *Mol. Nutr. Food Res.* 52: 284-298
17. Boyd DR, Sharma ND. (1996) *Che. Soc. Rev*. 25: 289-296
18. Athanasiadou M, Marsh G, Athanassiadis I, Aplund L, Bergman A. (2006) *J. Mass Spectrom.* 41: 790-801
19. Qiu X, Mercado-Feliciano M, Bigsby RM, Hites RA. (2007) *Environ. Health Perspect.* 115: 1052-1058
20. Lupton SJ, McGarrigle BP, Olson JR, Wood TD, Aga DS. (2010) *Rapid. Commun. Mass Spectrom.* 24: 2227-2235.