BDE-47 and BDE-99 INHIBIT CYP2B6 CATALYTIC ACTIVITY IN HUMAN LIVER MICROSOMES

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Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of flame retardants used in many commercial applications. 2,2,4,4′-Tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) are the two predominant components of the widely used commercial PBDE mixture known as PentaBDE. PBDEs have become widespread environmental pollutants and have been found to bioaccumulate in humans and wildlife. Metabolism and excretion are the primary routes of xenobiotic elimination. Hepatic metabolism is an important determinant of PBDE bioaccumulation and toxicity in humans. The cytochrome P450 (CYP) enzymes are the predominant biotransformation system in mammals and produce oxidative metabolites. In preliminary experiments conducted in our laboratory[1], incubation of BDE-99 with human liver microsomes produced three mono hydroxy and one unidentified di-OH metabolite. A further experiment using a panel of 14 human recombinant CYP enzymes showed that the four OH BDE-99 metabolites were formed by CYP2B6.

CYP2B6 is a relatively minor CYP enzyme in human liver microsomes (2-10% total hepatic CYP content). An increasing number of drugs have been found to be metabolized by CYP2B6. Among them, the antiretroviral drug, efavirenz, the antidepressant and smoking cessation drug, bupropion (BUP) and the alkylating agent, cyclophosphamide, have narrow therapeutic indices and dose-limiting toxicities[2]. Serious adverse effects, which can be due to inhibition of their CYP2B6-mediated metabolism, have been associated with elevated plasma concentrations of these drugs[2]. Inhibition of CYP2B6-mediated metabolism of efavirenz, BUP and cyclophosphamide can be due to drug-to-drug interactions of co-administered drugs but including CYP2B6 substrates such as BDE-99 and possibly BDE-47.

The purpose of the present study was to use human liver microsomes to investigate the inhibitory potency (apparent K_i) of BDE-47 and BDE-99 towards CYP2B6-mediated metabolism of BUP. Moreover, the mechanism through which BDE-47 and BDE-99 inhibit CYP2B6 catalytic activity was also characterized.

Materials and Methods

In vitro bupropion biotransformation assay

In vitro biotransformation assay for BUP was performed as described by Lau and Chang[3]. Briefly, reaction mixtures contained bupropion (25 μM–1.0 mM), human hepatic microsomal protein (0.125–0.5 mg), and 50 mM potassium phosphate buffer containing 3 mM magnesium chloride (pH 7.4) in a final volume of 0.49 ml. Reaction mixtures were preincubated at 37ºC for 5 min in a shaking water bath. Reactions were initiated by addition of 0.01 ml of NADPH solution (1 mM final concentration) and terminated by addition of 0.5 ml ice-cold acetonitrile containing 2 μM of the internal standard, triprolidine (final concentration 1 μM). Each sample was vortex-mixed for 30 sec and then centrifuged at 8,000 g for 10 min. An aliquot of the supernatant was then transferred to UPLC vials for UPLC/MS/MS analysis. Blank, negative and positive control samples were also prepared. Preliminary experiments were conducted to ensure that product (4-OH-BUP) formation was linear with respect to incubation time and protein concentration. Samples were prepared in duplicate in each experiment.

Enzyme inhibition experiments

BDE-47, BDE-99 or the corresponding vehicle (methanol) was added to the reaction mixture described above. Multiple concentrations of BDE-47 or BDE-99 (20–400 nmol/mg protein) and BUP (12.5–100 μM) were used (n=2). The apparent *Ki* value and the mode of action of each inhibitor were determined by nonlinear

regression analysis of the 4-OH-BUP formation data obtained using equations for competitive, noncompetitive, mixed, and uncompetitive inhibition.

To investigate if inhibition of 4-OH-BUP formation by BDE-47 and BDE-99 was also metabolism-dependent, the effect of different pre-incubation times, NADPH and presence of BDE-47 or BDE-99 was investigated. To investigate the effect of different pre-incubation times, reaction mixture containing human liver microsomes (0.5 mg/ml), BDE-47 or BDE-99 (final concentration 100 µM) and 50 mM potassium phosphate buffer (pH 7.4) were prepared. Reaction mixtures were warmed up in a shaking water bath at 37ºC for 5 min. Reactions were initiated by addition of 5.0 µl of NADPH solution (1 mM final concentration) and allowed to proceed for 15, 30 or 45 min after which BUP (5.0 µl) was added (final concentration 50 µM). BUP metabolism was allowed to proceed for 5 extra min and quenched adding 0.5 ml ice-cold acetonitrile containing 2 µM of triprolidine (final concentration 1 µM). Each sample was then processed as described above. A parallel set of samples was prepared as described above except that 5.0 µl of buffer was added instead of NADPH after the 5 min warm-up period. NADPH (5.0 µl) was then added only to start the BUP metabolism reaction.

Quantification of 4-OH-BUP by UPLC/MS/MS

The amount of 4-OH-BUP formed was quantified according to a previously validated UPLC/MS/MS method[3] using a Waters Acquity Ultra Performance Liquid Chromatograph System (Waters Corp, Milford, MA). 4-OH-BUP was quantified using calibration curves (10 nM – 2.5 µM). The performance of the assay was monitored daily assessing linearity (calibration curve) and accuracy, precision and recovery rates (quality control samples at 75, 400 and 1,500 nM concentrations).

7-Benzyloxyquinoline metabolism assay

Rates of 7-hydroxy-quinoline formation were measured fluorometrically[4]. Preliminary experiments were conducted to ensure that 7-OH-Q formation was linear with respect to incubation time and protein concentration.

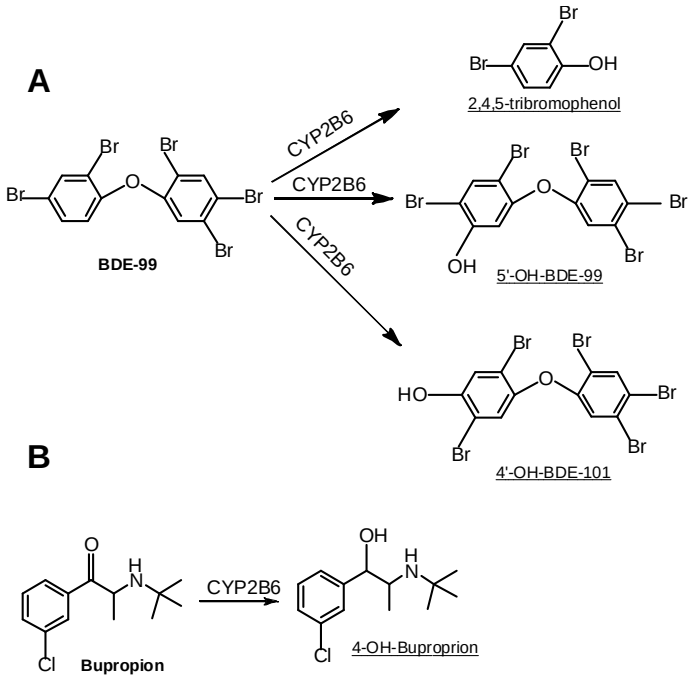

Figure 1. Chemical structure of BUP, 4-OH-BUP, BDE-99 and the hydroxylated metabolites of BDE-99 formed by CYP2B6 in human liver microsomes.

Results and discussion

Recent experiments in our laboratory showed that BDE-99 is oxidatively metabolized in human liver microsomes[1] and that CYP2B6 was the only recombinant CYP enzyme among the 14 CYPs tested that metabolized BDE-99 (Figure 1A). Therefore, the ability of BDE-99 to directly inhibit CYP2B6 metabolism of other CYP2B6 substrates in human liver microsomes was investigated. Formation of 4-OH-BUP (Figure 1B) is a well established specific marker of CYP2B6 activity[5,6] and was used for this purpose. The inhibitory ability of BDE-47 for CYP2B6 catalytic activity was also investigated because of its structural similarity with BDE-99.

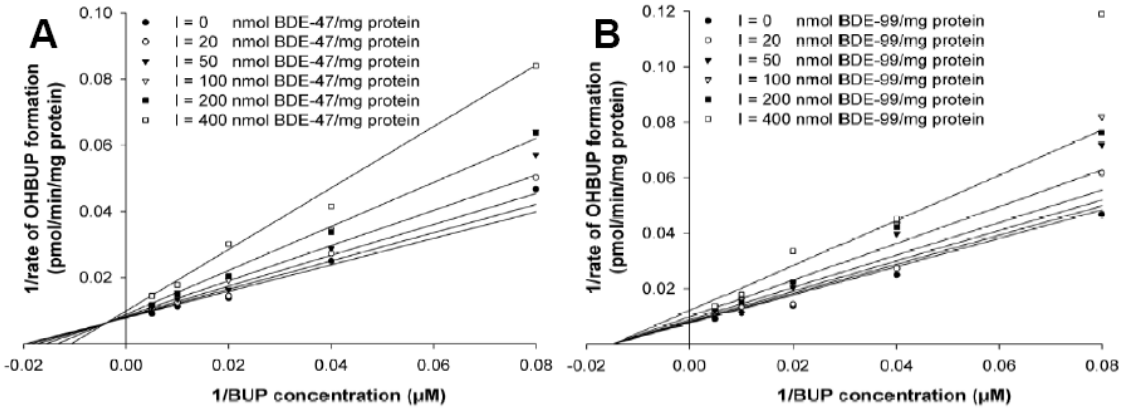

Figure 2. Lineweaver-Burk plot for BDE-47 mixed (A) and BDE-99 noncompetitive (B) inhibition of CYP2B6-mediated formation of 4-OH-BUP in human liver microsomes. Results are the mean of two samples (one experiment).

Direct inhibition of 4-OH-BUP formation in human liver microsomes.

BDE-47 and BDE-99 inhibited CYP2B6 catalytic activity. The inhibition was dose-dependent over the range (20-400 nmol BDE-47 or BDE-99/mg protein) of concentrations tested. As determined by nonlinear regression analysis of enzyme inhibition data and judged by R^2, Akaike Information Criterion and Sy.x index values and visual inspection of the Lineweaver-Burk plot, the mode of inhibition by BDE-47 and BDE-99 was best described as mixed and noncompetitive, respectively (Figure 2). Some natural compounds present in herbs and plants are known to inhibit 4-OH-BUP formation competitively in human liver microsomes[3,7].

The apparent *Ki* values of BDE-47 and BDE-99 were 150 and 335 μM, respectively, suggesting that, in human liver microsomes, BDE-47 is a more potent inhibitor of 4-OH-BUP formation than BDE-99. Comparison of the apparent *Ki* values of BDE-47 and BDE-99 with those of herb and plant ingredients inhibiting CYP2B6 activity in human liver microsomes[3,7] suggests that BDE-47 and BDE-99 are moderate inhibitors of CYP2B6-mediated activity.

Inhibition of 7-hydroxy-quinoline formation in human liver microsomes.

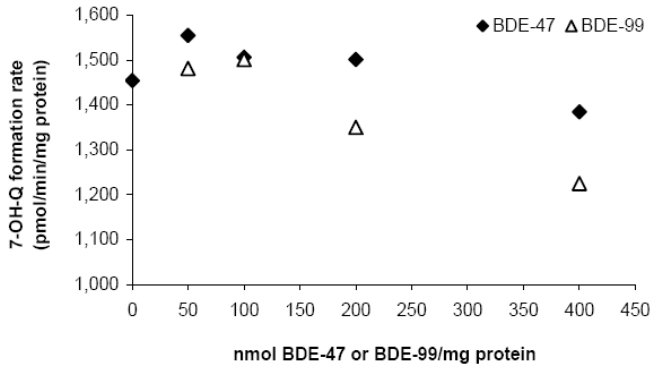

Figure 3. Rates of 7-OH-Q formation by human liver microsomes in the presence of different concentrations of BDE-47 or BDE-99. Results are the mean of two samples (one experiment).

O-dealkylation of 7-benzyloxy quinoline to 7-hydroxy-quinoline is a specific marker for CYP3A4 activity and was used to determine the inhibitory activity of BDE-47 and BDE-99 towards CYP3A4. Rates of 7-hydroxy-quinoline formation were not affected by either BDE-47 or BDE-99 across a large range of BDE-47 and BDE-99 concentrations tested (Figure 3). This result suggests that BDE-47 and BDE-99 do not inhibit CYP3A4 catalytic activity but tend to specifically inhibit CYP2B6 activity. More experiments are ongoing to assess the inhibitory effect of BDE-47 and BDE-99 towards methoxyresorufin *O*-dealkylation activity, a specific marker for CYP1A2 activity.

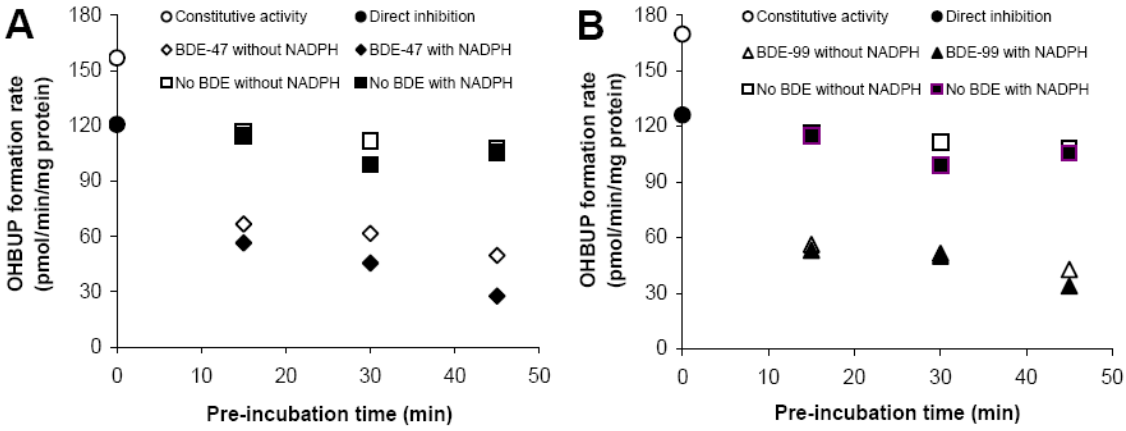

Figure 4. Rates of 4-OH-BUP formation in human liver microsomes after different pre-incubation times with BDE-47 (A) or BDE-99 (B). The BDE-47 and BDE-99 concentration used was 100 µM (200 nmol BDE/mg protein). The BUP concentration used was the apparent K_m (50 µM). Results shown are the mean of two samples and one experiment only.

Involvement of BDE-47 and BDE-99 metabolism in inhibiting 4-OH-BUP formation.

Pre-incubation of BDE-47 and BDE-99 with human liver microsomes caused a time-dependent decrease in 4-OH-BUP formation. The decrease of 4-OH-BUP formation was observed in the presence or absence of NADPH (Figure 4). These results suggest that metabolism of BDE-47 and BDE-99 could be involved in the inhibition of 4-OH-BUP formation. In the samples lacking NADPH, inhibition of 4-OH-BUP formation may be due to non-NADPH dependent metabolism (possibly reductive debromination of BDE-47 and BDE-99) or to a chemical interaction of BDE-47 or BDE-99 with CYP2B6. In the samples containing NADPH, rates of 4-OH-BUP formation were lower than those in samples lacking NADPH and pre-incubated for the same amount of time. This effect occurred when BDE-47 but not BDE-99 was incubated with human liver microsomes. Further experiments are underway to confirm these preliminary results and to conclusively evaluate the inhibitory ability of BDE-47, BDE-99 and their metabolites towards CYP2B6 activity in human liver microsomes.

Acknowledgments

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