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Human metabolites of brevetoxin PbTx-2: Identification and confirmation of structure

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Abstract

Four metabolites were identified upon incubation of brevetoxin (PbTx-2) with human liver microsomes. Chemical transformation of PbTx-2 confirmed the structures of three known metabolites BTX-B5, PbTx-9 and 41, 43-dihydro-BTX-B5 and a previously unknown metabolite, 41, 43-dihydro-PbTx-2. These metabolites were also observed upon incubation of PbTx-2 with nine human recombinant cytochrome P450s (1A1, 1A2, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5). Cytochrome P450 3A4 produced oxidized metabolites while other CYPs generated the reduced products.

Keywords

brevetoxins; PbTx-2; metabolism; cytochrome P450; human liver microsome

Brevetoxins, produced by the “Florida red tide” dinoflagellate *Karenia brevis*, are a suite of neurotoxins which are responsible for massive fish kills and marine mammal mortalities (Landsberg *et al.*, 2009). In humans, the brevetoxins are responsible for a syndrome known as Neurotoxic Shellfish Poisoning (NSP) which results from the consumption of tainted shellfish (Watkins *et al.*, 2008) as well as respiratory distress in beachgoers upon exposure to aerosolized toxins (Fleming *et al.*, 2005). Brevetoxin PbTx-2 (Fig. 1) is the primary constituent of this suite of toxins.

Several studies of brevetoxin excretion and metabolism in mammals have demonstrated that the hepatobiliary system plays a key role in the detoxification and elimination of brevetoxins. Radwan found that in rats, PbTx-2 is rapidly detoxified and excreted in urine as cysteine conjugates and an unidentified oxidized metabolite. (Radwan *et al.*, 2005) On the other hand, when fed to rats, [³H]-PbTx-3, which is unable to form cysteine conjugates, is eliminated in both the urine and feces. (Poli, *et al.*, 1990; Cattet and Geraci, 1993) Radioimmunoassay of urine from two patients in Florida suffering from NSP showed

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Conflict of interest The authors declare that there are no conflicts of interest.

Supplementary information: Supplementary data associated with this article including experimental details may be found at XXXXXXXX

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brevetoxin-like activity which was later confirmed by LC-MS to be due to the presence of PbTx-3 and other unidentified metabolites. (Poli *et al.*, 2000)

Wang reported the conversion of PbTx-2 to PbTx-3 and the hydrolyzed A-ring lactone by rat liver microsomes. (Wang *et al.*, 2005) Later Radwan examined the metabolic activities of purified cDNA-expressed rat liver cytochrome P-450 (CYP) enzymes toward PbTx-2. All six CYPs studied (CYP1A2, CYP2A2, CYP2C11, CYP2D1, CYP2E1, and CYP3A1) were capable of metabolizing PbTx-2 to varying degrees. The metabolites produced by CYPs 1A2 and 3A1 were assigned on the basis of LC-MS analysis as PbTx-3, PbTx-9, BTX-B5 and the 27, 28-diol of PbTx-2. (Radwan and Ramsdell, 2006) Several additional metabolites were observed upon incubation with rat liver hepatocytes including: the H-ring epoxides of PbTx-2 and PbTx-3; the corresponding diols and two A-ring hydrolyzed products. Recently, Abraham (Abraham *et al.*, 2008) assayed brevetoxin metabolites in urine from seventeen NSP patients and characterized them on the basis of LC-MS/MS as PbTx-3 and 27, 28-epoxy PbTx-3. Additionally, a previously unknown metabolite was tentatively assigned on the basis of MS fragmentation, as 41, 43-dihydro-BTX-B5.

In this communication, we describe the production of 4 metabolites by incubation of PbTx-2 with human liver microsomes and nine human recombinant cytochrome P450s (1A1, 1A2, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5). These metabolites, which include 41, 43-dihydro-BTX-B5 and a previously unidentified metabolite, have been definitively characterized by chemical conversion of PbTx-2.

PbTx-2 was incubated with pooled human liver microsomes (HLM) and the reactions were monitored by LC-MS. The total ion current chromatogram obtained from the positive ion ESI LC-MS analysis of the reaction mixture after incubation of PbTx-2 with HLM for 30, 60, 180 min is shown in Fig. 2b-c. Four new peaks in the LC-MS profile of the reaction mixture were observed, including three (metabolite 1, t_R 11.03 min; metabolite 2, t_R 11.38 min; metabolite 3, t_R 12.53 min) eluting before PbTx-2 (m/z 895, t_R 15.68 min) and one peak (metabolite 4, t_R 15.99 min) eluting after PbTx-2. The positive ESI full-scan mass spectrum of metabolites 1-4 produced molecular ion peaks $[M+H]^+$ at m/z 913, 911, 899 and 897, respectively. This result suggested that metabolites 1 and 2 are oxidation products, while metabolites 3 and 4 result from reduction. Metabolites 2 and 4 were major peaks after 30 minutes of incubation, while metabolite 1 and 3 increased after 60 min. Metabolites 1 and 3 appeared as the major constituents and metabolite 4 was nearly gone at a reaction time of 180 min. This suggested that metabolite 4 may be an intermediate during PbTx-2 metabolism by human liver.

PbTx-2 was also incubated with nine recombinant human cytochrome P450 enzymes (CYP1A1, 1A2, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5). LC-MS analysis revealed that all nine enzymes are capable of metabolizing PbTx-2. Fig. 2d shows that CYP3A4 converts PbTx-2 to metabolites 1, 2 and 4, while the other enzymes convert PbTx-2 to metabolites 3 and 4 only. This result suggested that CYP3A4 is involved in the oxidation of PbTx-2 while the other CYPs catalyzed two reduction reactions.

Metabolite 4 is an important intermediate which appeared in all reactions. A difference of 2 in the m/z ratios for the molecular ion peaks of metabolite 4 when compared to PbTx-2 (m/z 895) is suggestive of a reduction of the PbTx-2 molecule. PbTx-3 (m/z 897) has been identified as a metabolite in the incubation of PbTx-2 with rat liver microsomes (Wang *et al.*, 2005), rat hepatocytes (Radwan and Ramsdell, 2006) and in human urine (Abraham *et al.*, 2008). However, the HPLC retention time of metabolite 4 did not correspond to that of PbTx-3 (m/z 897, t_R 11.81 min, data not shown) which was slightly shorter than PbTx-2 (t_R 15.68 min). Whereas the retention time of metabolite 4 (m/z 897, t_R 15.99 min) was slightly

longer than PbTx-2. A standard of 41, 43-dihydro-PbTx-2 was prepared by reduction of PbTx-2 with Pd on BaSO₄ according to the method described by Lin. (Lin *et al.*, 1981). Comparison of metabolite 4 with the semi-synthetic 41, 43-dihydro-PbTx-2 by LC-MS indicated that the two were identical. Furthermore, metabolite 4 may be converted to PbTx-9 by sodium borohydride reduction. These observations confirm that metabolite 4 is 41, 43-dihydro-PbTx-2. This metabolite has not been reported in earlier studies either in rat or humans. Metabolite 3 was identified as PbTx-9 by comparison with standard which was prepared by the reduction of PbTx-2 with NaBH₄ in acetonitrile. As we did not observe any PbTx-3 in these reactions, we conclude that metabolite 3 (PbTx-9) was generated from the reduction of metabolite 4.

Two additional metabolites, 1 and 2 which produced molecular ion peaks [M+H]⁺ at *m/z* 913 and 911, were presumed oxidation products. Metabolite 2 has difference of 16 in the *m/z* ratio when compared to PbTx-2. Metabolite 2 was identical to BTX-B5 (Ishida *et al.*, 2004) which was generated by the oxidation of PbTx-3 with NaClO₂ according to the method described by Michelliza (Michelliza *et al.*, 2007).

Metabolite 1 has same *m/z* as that of hydrolyzed A ring PbTx-2 which was reported earlier by Wang (Wang *et al.*, 2005). However, the retention time of metabolite 1 differed from that of synthetic open A ring PbTx-2 (*m/z* 913, *t_R* 10.04 min, data not shown) which was prepared as previously described (Roth *et al.*, 2007). However, the oxidation of 41, 43-dihydro-PbTx-2 (metabolite 4) with NaClO₂, yielded 41, 43-dihydro-BTX-B5, which was found to be identical to metabolite 1 and was tentatively identified by Abraham (Abraham *et al.*, 2008).

This is the first study of metabolism of brevetoxins using human cytochrome P450 enzymes. Earlier studies were performed using rat enzymes (Radwan and Ramsdell, *et al.*, 2006 and Wang, *et al.*, 2005) and one study (Abraham, *et al.*, 2008) identified metabolites in human urine. This work links a human xenobiotic metabolizing enzyme, with a specific metabolite. CYP3A4 was involved in the oxidation of the substrate, while other CYPs catalyzed two reduction reactions. A previously unknown metabolite (41, 43-dihydro-PbTx-2), has been identified which is incapable of forming the types of conjugates described by Abraham (Abraham, *et al.*, 2008) and would thus not be readily excreted. Moreover, 41, 43-dihydro-BTX-B5 was confirmed as a metabolite. The characterization of all metabolites produced from a xenobiotic is crucial to understanding its range of toxicity, because diet, environment, gender, age, health, prior exposure to xenobiotics and genetic polymorphisms in liver xenobiotic metabolizing enzymes in humans will influence metabolism and susceptibility in different populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Enzymatic and chemical conversion of PbTx-2. a) $\text{H}_2/5\%$ Pd-BaSO₄, THF, 0 °C, 32 psi, 2 h; b) NaBH₄, CH₃CN, rt, 0.5 h; c) NaClO₂, Na₂HPO₄, 2-methyl-2-butene, *t*-BuOH, RT, 4 h; d) NaClO₂, Na₂HPO₄, 2-methyl-2-butene; *t*-BuOH, RT, 4 h; e) NaBH₄, CeCl₃·6H₂O, MeOH, rt, 0.5 h.

FIG. 2.

Total ion chromatogram (TIC) of metabolic products of 50 μ M PbTx-2 incubated with pooled human liver microsomes (1 mg/mL) (a) 30 min. (b) 60 min. (c) 180 min. (d) Total ion chromatogram (TIC) of metabolic products of 50 μ M PbTx-2 incubated for 30 min with 50 pmol CYPs.