Characterization of In Vitro Oxidative and Conjugative Metabolic Pathways for Brevetoxin (PbTx-2)

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Brevetoxins are potent marine toxins produced by the dinoflagellate Karenia brevis, the causative organism of Florida red tides. An in vitro metabolism of PbTx-2 was performed using purified cDNA-expressed rat liver cytochrome P-450 (CYP) enzymes and freshly isolated rat hepatocytes. The metabolic activities of six CYP enzymes, CYP1A2, CYP2A2, CYP2C11, CYP2D1, CYP2E1, and CYP3A1, were examined by incubation with PbTx-2 for up to 4 h in the presence of a NADPH-generating system. Further identification of the metabolites produced by CYP1A2 and CYP3A1 was performed using high performance liquid chromatography-mass spectrometry (LC/MS). Both CYP1A2 and CYP3A1 metabolized PbTx-2 to PbTx-3 (MH+: m/z 897), PbTx-9 (MH+: m/z 899), and a newly recorded diol brevetoxin-2 metabolite (MH+: m/z 929). CYP3A1 also produced a considerably higher amount of BTX-B5 (MH+: m/z 911). Subsequent incubation of PbTx-2 with rat hepatocytes produced additional phase 1 metabolites of MH+: m/z 911, 913, 915, 917, and 931, indicating a CYP-catalyzed epoxidation at H-ring (C27,C28-double bond) and a subsequent A-ring hydrolysis of PbTx-2 metabolic products. A conjugation metabolism was identified by the production of a glutathione-brevetoxin conjugate (MH+: m/z 1222) and a cysteine-brevetoxin conjugate (MH+: m/z 1018). Structures of the new metabolites are postulated, and a likely CYP-catalyzed metabolism pathway of PbTx-2 metabolism is discussed.

Key Words: Karenia brevis; red tide bloom; brevetoxin metabolism; in vitro; cytochrome P450; CYP; conjugation.

INTRODUCTION

Brevetoxins are a family of naturally occurring trans-fused cyclic polyether compounds produced by the marine dinoflagellate Karenia brevis (Davis, 1948). Brevetoxins are toxic to marine animals and ultimately to humans. Brevetoxins are also associated with fish and bird mortality, and toxin levels rapidly accumulate and persist in herbivorous fish exposed to K. brevis (Quick and Henderson, 1974; Woofier et al., 2005). Brevetoxins are notably toxic to marine mammals and affect dolphins via consumption of herbivorous fish and manatees via consumption of K. brevis cells associated with seagrass (Flewelling et al., 2005). In humans brevetoxins are the cause of neurotoxic shellfish poisoning (NSP) syndrome (McFarren et al., 1965), and they also produce respiratory and eye irritation via airborne exposure (Woodcock, 1948; Pierce, 1986).

K. brevis produces several brevetoxins that are grouped according to their backbone structures into A- or B-types (Lin et al., 1981; Shimizu et al., 1986). Principal A-types are PbTx-1 and −7, and principal B-types are PbTx-2, −3, and −9 (Poli et al., 1986). PbTx-1 and PbTx-2 are presumed to be the parent molecules containing the precursor backbone of other natural derivatives or metabolites of each type. PbTx-2 is the major natural constituent of a K. brevis toxin extract (approx. 74% of toxin extract); however, it is chiefly metabolized to PbTx-3 and other brevetoxin metabolites in contaminated shellfish (Dickey et al., 1999; Plakas et al., 2002; Wang et al., 2004). Similar metabolites have been described as major contributors to the toxicity in New Zealand shellfish (Nozawa et al., 2003).

Brevetoxin metabolites were also reported in tissues of different animal models used for brevetoxin exposure studies, such as fish (Kennedy et al., 1992; Washburn et al., 1994, 1996; Flewelling et al., 2005), rodents (Poli et al., 1990; Radwan et al., 2005), and Florida red tide exposed dolphin (unpublished observations). Metabolism is an essential step for brevetoxin detoxification; however, certain structural features among brevetoxins, including their natural derivatives or metabolic products, have been found to assign unique physiological activities that modify both potency and the molecular mechanism of action (Jeglitsch et al., 1998; Purkerson et al., 1999; Baden et al., 2005).

In the course of understanding the mechanism of brevetoxin metabolism in mammals using laboratory rats, we recently defined a rapid in vivo metabolism of PbTx-2 (Fig. 1). Within a period of 24 h post-exposure, PbTx-2 was mostly eliminated...
in urine as a water-soluble cysteine conjugate (MH⁺: m/z 1018) (Radwan et al., 2005). Initial CYP-mediated reduction and oxidation are likely mechanisms because PbTx-3 (MH⁺: m/z 897) and a carboxylic brevetoxin BTX-B5 (MH⁺: m/z 911, named by Ishida et al., 2004) were also detected. Nevertheless, additional transitory metabolites were likely produced, but not readily detected in vivo.

A potential CYP catalysis and thiol conjugation in brevetoxin metabolism was established about 10 years ago by Washburn and co-workers. They reported that brevetoxin exposure induced CYPs (the oxidation/reduction-mediating enzyme) and also activated glutathione S-transferases (GST) (the conjugation-mediating enzymes) in fish (Washburn et al., 1994; 1996). Washburn and co-workers reported that PbTx-3 induces CYP1A activity in redfish (Scaienops ocellatus); however, PbTx-2 induces the GST in the striped bass (Morone saxatilis). Those findings suggested an H-ring epoxidation of PbTx-2 (C27,C28-double bond, see Fig. 1), which may produce a metabolite similar to the naturally occurring brevetoxin,

![FIG. 1. Chemical structure of brevetoxin-2 (PbTx-2) and its respective LC/MS (MS) product ion spectrum.](image)

![FIG. 2. Metabolic stability profile of PbTx-2 incubated with different rat purified CYP enzymes. Each CYP (50 nmol) was incubated with 50 µM PbTx-2 for 3 min at 37°C prior to addition of the NADPH-generating system. A microsomal preparation was used as a control for native activity. A microsomal preparation was used as a control for native activity.](image)

<table>
<thead>
<tr>
<th>CYP-450</th>
<th>Approx. metabolic rate (pmol/pmol P450/min)</th>
<th>Approx. t¹/₂ (min)</th>
<th>(%) Remainder of substrate at 240 min*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP-control</td>
<td>1.0</td>
<td>&gt;240</td>
<td>79</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>0.5</td>
<td>&gt;240</td>
<td>95</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>3.0</td>
<td>144</td>
<td>33</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>5.0</td>
<td>106</td>
<td>33</td>
</tr>
<tr>
<td>CYP2D1</td>
<td>7.0</td>
<td>69.9</td>
<td>16</td>
</tr>
<tr>
<td>CYP2A2</td>
<td>7.0</td>
<td>69.6</td>
<td>35</td>
</tr>
<tr>
<td>CYP3A1</td>
<td>8.0</td>
<td>63.9</td>
<td>12</td>
</tr>
</tbody>
</table>

Note. A microsomal preparation was used as a control for native activity.

*Approximate percentage of PbTx-2 that remained unchanged at 240 min incubations to controls (at 240 min without CYP). Half-life (t₁/₂) was calculated using time points that fit an exponential regression curve of R² ≥ 0.85. Points 0, 30, and 120 min were used to determine t₁/₂ in the case of CYP2A1.
PbTx-6. Epoxide formation induces GST and microsomal epoxide hydrolase (Washburn et al., 1996). In this study, in vitro metabolism experiments were performed by incubation of PbTx-2 with purified cDNA-expressed rat CYP enzymes and rat hepatocytes. The goal of this study was to characterize the in vitro metabolism of PbTx-2 and to identify the role of CYP-catalyzed metabolic pathway(s) that may produce “short-lived” intermediate metabolites of potential toxicologic significance.

**FIG. 3.** Metabolic stability profile of PbTx-2 incubated with freshly isolated rat hepatocytes. A final incubation mixture containing $0.5 \times 10^6$ cells and 10 µM PbTx-2 was processed under experimental conditions described in Materials and Methods section. Points represent the mean ± SD of triplicate determinations.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compounds incubated with rat hepatocytes</th>
<th>Approx. metabolic rate (pmol min$^{-1}$/10$^6$ cells)</th>
<th>Approx. $t_{1/2}$ (min)</th>
<th>(%) Remainder of substrate at 240 min$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>346</td>
<td>29</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>PbTx-2</td>
<td>313</td>
<td>32</td>
<td>8.0 ± 4.7</td>
</tr>
</tbody>
</table>

Note. Testosterone was used as positive control for assessment of hepatocytes CYP activity.

$^a$Percentage (mean ± SD) of each substrate compound remained unchanged (at 240 min) with respect to control incubation without hepatocytes. $t_{1/2}$ (half-life) was calculated using time points that fit an exponential regression curve of $R^2 \geq 0.85$.

**FIG. 4.** (A, B, and C). Total ion currents representative of metabolic products of PbTx-2 incubated with CYP3A1 and CYP1A2 (A & B). A final incubation mixture contained 50 µM PbTx-2 and 50 pmol of CYP enzyme. Reactions were initiated by adding a NADPH-generating system and were terminated after 240 min. A microsomal preparation was used as a control for native activity (C).
Hepatocytes were isolated by slow-speed centrifugation (~200 g) and dissociated by massaging and filtering through nylon gauze (250 μm). Liver was removed in situ (McQueen, 1993) with some modifications. Briefly, liver was perfused with medium (200 g) by a two-step perfusion procedure (McQueen, 1993) with some modifications. Brieﬂy, liver was perfused with Hanks’ balanced salt solution (HBSS) containing 0.5 mM EGTA (ethyleneglycol-bis (P-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) and 10 mM HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid), pH 7.4, for 5 min, then perfused with a liver digest medium containing collagenase type IV (0.7 U/ml) (Gibco, Invitrogen Corporation) for 10 min. The liver was removed and dissociated by massaging and filtering through nylon gauze (250 μm). Hepatocytes were isolated by slow-speed centrifugation (~50 g for 3 min) and resuspended in incubation medium (William’s Medium E supplemented with 10% [v/v] FBS, L-glutamine [2 mM], HEPES [10 mM], and gentamicin [50 μg/ml]). Cell viability was assessed by trypan blue (0.2%) uptake. Hepatocyte preparations attaining initial cell viability >90% were used. Hepatocytes were counted in a hemocytometer in the presence of 0.04% trypan blue in protein-free medium. All protocols involving animals were carried out in compliance with animal care guidelines identified by laws and regulations.

Incubation of PbTx-2 with CYPs. The standard incubation mixture (final volume 0.5 ml) in a pre-warmed 0.1 M potassium phosphate buffer (pH 7.4) contained 50 pmol CYP and 50 μM PbTx-2 (final mixture contained 1% methanol). After pre-incubation at 37°C for 3 min, reactions were initiated by addition of an NADPH-generating system consisting of 1.3 mM NADPH, 3.3 mM Glc-6-PO 4, 0.4 U/ml G6PDH, and 3.3 mM magnesium chloride. Further incubations were carried out for 0 min, 30 min, 60 min, and 240 min at 37°C with constant shaking. The reaction was terminated by the addition of 1 ml of acetonitrile/methanol mixture (1:1) to each incubate. After centrifugation at 900 × g for 10 min, pellets of each reaction were washed, using another 1 ml of acetonitrile/methanol mixture, and the products formed in the pooled supernatant were evaporated to dryness under a stream of nitrogen and stored at −20°C until use in LC/MS (MS) analysis.

The incubations were carried out in duplicates. Controls were assayed in the same manner using a CYP-inactive microsome preparation as a control for native activity. For those controls established as time 0 min, an NADPH-generating system was added after termination of the reactions.

Incubation of PbTx-2 with rat hepatocytes. PbTx-2 was prepared in methanol and diluted in the incubation medium so that the final methanol concentration was 0.5% (v/v); it was then pre-incubated at 37°C for 10 min. Aliquots of hepatocyte suspension (250 μl; 1.0 × 10^6 cells/ml) were transferred into new incubation wells and pre-incubated at 37°C for 10 min. Metabolism was initiated by adding 250 μl of PbTx-2–enriched medium to a well that contained cells. Testosterone (10 μM) was prepared similarly and used as a positive control to assess the hepatocytes activity of CYPs (Specificity: 2A1, 2C11, 3A1/2; Sonderfan et al., 1987). Also, negative control reactions were performed by incubating PbTx-2 without cells to monitor
FIG. 6. Representatives of positive product ion spectra of CYP-catalyzed metabolites produced by incubation of PbTx-2 with rat CYPs or hepatocytes.
C18 guard cartridge (2.1 mm, 5 μm) (Thermo Electron Co., San Jose, CA). The mobile phase consisted of water (A) and methanol (B) in a binary system, with 0.2% formic acid as an additive. The elution gradient was 45–75% B for 30 min and the flow rate was 0.1 ml/min. Analytes were analyzed with a Sciex API QSTAR quadrupole/time-of-flight (QqTOF) mass spectrometer. The mass spectrometer detected positive ions over the mass range m/z 300–1400 or 100–1400 amu for intense parent molecule ion scan at an orifice potential of 30 V. In some experiments, a negative ion scan was conducted (from 300–1400 amu) to determine acidic hydrogen. Standards of brevetoxin A-ring hydrolysis products for PbTx-2 (MH⁺: m/z 913, MH⁻: m/z 911), PbTx-3 (MH⁺: m/z 915, MH⁻: m/z 913), PbTx-9 (MH⁺: m/z 917, MH⁻: m/z 915) and BTX-B5 (MH⁺: m/z 929, MH⁻: m/z 927) were utilized to identify the presence of similar hydrolytic products of PbTx-2 and its metabolites.

**RESULTS**

In Vitro Metabolic Stability of PbTx-2

FIG. 2 shows that all CYPs used in our experiments, except CYP2E1, yield substantial catalysis of PbTx-2 metabolism. Table 1 summarizes the metabolic activities for each of the individual CYPs. CYP3A1 and CYP2D1 caused the highest depletion of PbTx-2 at the 240 min incubation period. For example, CYP3A1 metabolized 88% of PbTx-2 within 240 min at a metabolic rate of 8 pmol/pmol CYP/min and a half-life (t₁/₂) 63.9 min (Table 1). A subsequent analysis (particularly of 30–120 min incubations) suggests that rat CYP catalysis may fall into two patterns of metabolic activity, one including both CYP1A2 and CYP2C11 and the other including CYP2A2, CYP2D1, and CYP3A1 (Fig. 2). From among these patterns, we selected CYP1A2 and CYP3A1 to pursue identification of metabolic products produced after 240 min of incubation with PbTx-2.

FIG. 7. Representatives of positive product ion spectra and postulated fragmentation patterns of PbTx-2 metabolites of the conjugation phase, including a potential PbTx–glutathione (GSH) conjugate (m/z 1222) (A), and the PbTx–cysteine conjugate (m/z 1018) (B).
Incubation of PbTx-2 with rat hepatocytes revealed a metabolic rate of 313 pmol min⁻¹/10⁶ cells, which occurred primarily in the first 60 min of toxin exposure (Fig. 3). This rate of metabolism accounted for an approximate depletion of about 92% of PbTx-2 within 240 min (Table 2).

**Metabolite Identification**

Characteristic positive ion products of parent molecule PbTx-2 used in our experiments, including combinations of precursor/product ions (MH⁺: m/z 895/877/473/455), were next determined (see Fig. 1). Positive ions m/z 455, 473, 753, and/or 779 in B-type brevetoxin were typically observed in further mass fragmentation spectra. Metabolites were defined as they contained all or part of the above characteristic product ions. A metabolic pathway was established according to LC/MS ion products (examined in both positive and negative modes) and the common LCMS/MS fragments shared by a metabolite and its potential precursor molecule(s).

Figure 4 shows the LC/MS analysis of PbTx-2 metabolic products caused by the catalysis of CYP3A1 and CYP1A2. Both CYPs produced PbTx-3 (MH⁺: m/z 897), PbTx-9 (MH⁺: m/z 899), and a newly recorded MH⁺: m/z 929 metabolic product that has a similar positive ion mass, but a different LC/MS(MS) characteristics than BTX-B5 hydrolytic standard (i.e., retention, fragments and negative ion). CYP3A1 also catalyzed the production of substantial amounts of the carboxylic brevetoxin BTX-B5 (MH⁺: m/z 911, MH⁻: m/z 909) with an estimated concentration equal to 64.7% of the total metabolites produced in PbTx-2 equivalents. Further LC/MS(IMS) fragmentation of each metabolic product was performed. Representatives of positive product ion spectra are included in Figure 5.

Several additional brevetoxin metabolites were detected in rat hepatocyte in vitro metabolism of PbTx-2 (Fig. 6). Those metabolites are a combination of CYP-catalyzed metabolism (activation phase) and subsequent thiol conjugation (conjugation phase). The activation phase metabolism was characterized by subsequent epoxidation of the H-ring conjugate bond, producing unique metabolic products such as m/z 911 (PbTx-6) and m/z 913. The metabolite m/z 913 has similar positive ion mass of PbTx-2 hydrolytic product standard, but different LC/MS(IMS) characteristics. Hydrolysis of the epoxide products m/z 911 and m/z 913 by the microsomal epoxide hydrolase, likely produces new metabolites, including m/z 929 and m/z 931; the former of which was previously identified using purified CYPs (Fig. 5 and Fig. 6 for MS/MS spectra). The PbTx-2 reduction metabolites PbTx-3 (m/z 897) and PbTx-9 (m/z 899) were hydrolyzed in the

**FIG. 8.** Proposed schematic of a plausible in vitro CYP-catalyzed metabolic pathways of PbTx-2 in rat (including proposed names, positive ion mass, and combinations of characteristic ion fragments). PbTx-2 is thought to be initially metabolized by CYPs to its prominent BTX-B5, PbTx-3, and PbTx-9 conjugates. Additional metabolic reactions, including H-ring epoxidation, yield several biologically active products. Further conjugation steps, mostly with GSH, are essential to trap some of those intermediates into readily eliminated water-soluble GSH and cysteine conjugates.
presence of rat hepatocytes, producing products of positive ions
\( m/z \ 915 \) (MH\(^+\) \( m/z \ 913 \)) and \( m/z \ 917 \) (MH\(^+\) \( m/z \ 915 \)) that
matched the retention characteristics of the respective A-ring
hydrolytic standards. They may result from either a catalytic
or a spontaneous hydrolysis reaction. The new metabolites are
listed according to their approximate ion mass abundances at
180 min incubation as follows MH\(^+\): \( m/z \ 917 \geq m/z \ 915 > m/z \ 929, 911(PbTx-6) > m/z \ 931 > m/z \ 913 \).

Conjugation phase metabolism was characterized by the
detection of two (GSH) PbTx conjugates (MH\(^+\): \( m/z \ 1204 \) and
\( m/z \ 1222 \)). The former is analogous to PbTx-type B-GSH in
shellfish (MH\(^+\): \( m/z \ 1204 \)) (Wang et al., 2004); whereas the
mass of later is consistent with GSH conjugation of PbTx-2
after H-ring epoxidation. A PbTx-cysteine conjugate (MH\(^+\):
\( m/z \ 1018 \)) has been detected that is similar to a cysteine con-
jugate previously recovered from rat urine extracts (Radwan et al., 2005). The fragmentation patterns of both GSH and
cysteine conjugates are shown in Figure 7.

**DISCUSSION**

Although purified CYPs have the advantage to investigate
metabolism selective to individual CYP enzymes, the advan-
tages of using intact hepatocytes include the existance of using
interacting enzyme systems, physiological cofactor concentra-
tions, and three to four times higher enzyme activities than
those recognized in microsomal preparations (Wortelboet et al.,
1990; Donato, 1993). Consistent with our results, the PbTx-2
metabolic rate as well as CYP-catalyzed metabolic products
varied with respect to the CYP preparation studied, whether
purified or natively found in hepatocytes. Also, catalysis of
BTX-B5 production by CYP3A1 clearly indicates a unique
CYP selective metabolism.

High amounts of \( m/z \ 929 \) generated by purified CYP3A1 and
CYP1A2 were consistent with CYP-catalyzed H-ring epoxidation
of PbTx-2 to an epoxide intermediate – (a likely PbTx-6 conger of a positive ion mass \( m/z \ 911 \)), followed by an
epoxide hydrolysis that likely was catalyzed by the microsomal
epoxide hydrolase enzyme. Epoxidation in the presence of
intact hepatocytes, however, results in the formation of detect-
able amounts of intermediate metabolic products such as
\( m/z \ 911 \) (a metabolic product similar to the naturally occurring
PbTx-6) and \( m/z \ 913 \) (27,28 epoxy-PbTx-3 (Fig. 5)). However,
a subsequent epoxide breakage results in the formation of \( m/z \ 929 \) and \( m/z \ 931 \) metabolites. Hydrolysis of both PbTx-3 and
PbTx-9 was identified by the detection of A ring hydrolytics \( m/z \ 915 \) and \( m/z \ 917 \), respectively. The metabolic pathway and the
fragmentation patterns of new metabolites are postulated in
Figure 8.

In summary, PbTx-2 is metabolized by two separate path-
ways; initial CYP-dependent activation and a GST-catalyzed
GSH conjugation that was followed by metabolic cleavage of
the peptide to leave only the PbTx-cysteiny1 residue. The
present findings, in conjunction with our previous in vivo
metabolism studies (Radwan et al., 2005), show that PbTx-2 is
metabolized predominantly by oxidation of the aldehyde group
(C\(_{42}\)) forming BTX-B5 and by reduction, but to lesser extent, to
hydroxyl forming PbTx-3 (as confirmed by CYP incubations
at 240 min). Additional reduction of the C\(_{41}–C_{50}\) double bond
yields PbTx-9. A CYP-catalyzed activation was more efficient
in producing reactive brevetoxin epoxide precursors via
oxidation at the C\(_{27}–C_{28}\) double bond, which occurred to the
greatest extent in hepatocytes/toxin incubations. The substan-
tial amounts of \( m/z \ 929 \) produced by CYP3A1 and CYP1A2-
catalysis suggest an important role of these enzymes in
triggering an initial toxin epoxidation which could be neutral-
ized by the formation of H-ring diol metabolites. Epoxide
metabolites are strong electrophilic species that may not last
long in biological compartments without binding to cellular
micro- or macromolecules, although they can impose an
ultimate genotoxic activity.

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