Cytochrome P450–Mediated Metabolic Activation of Diosbulbin B

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ABSTRACT

Diosbulbin B (DIOB), a furan-containing diterpenoid lactone, is the most abundant component of Dioscorea bulbifera L. (DB), a traditional Chinese medicine herb. Administration of purified DIOB or DB extracts has been reported to cause liver injury in animals. The mechanisms of DIOB-induced hepatotoxicity remain unknown. The major objective of this study was to identify reactive metabolites of DIOB. A DIOB-derived cis-enedial was trapped by N-acetyl lysine (NAL) and glutathione (GSH) or N-acetyl cysteine (NAC) in rat and human liver microsomal incubation systems after exposure to DIOB. Four metabolites (M1–M4) associated with GSH were detected by liquid chromatography coupled to tandem mass spectrometry. Apparently, M1 was derived from both NAL and GSH. M2 and M3 resulted from the reaction of GSH without the involvement of NAL. Two molecules of GSH participated in the formation of M4. M2 and M3 were also detected in bile and urine of rats given DIOB. M5, a DIOB-derived NAC/NAL conjugate, was detected in microsomal incubations with DIOB fortified with NAC and NAL as trapping agents. Biomimetic M1–M5 were prepared by oxidation of DIOB with Oxone for metabolite identification. Microsomal incubation study demonstrated that ketoconazole inhibited the production of the enedial in a concentration-dependent manner, and CYP3A4 was found to be the enzyme responsible for the metabolic activation of DIOB. The metabolism study facilitates the understanding of the role of bioactivation of DIOB in its hepatotoxicity.

Introduction

Dioscorea bulbifera L. (DB), known as Huang-Yao-Zi in Chinese, belongs to a member of the yam family Dioscoreaceae (Li et al., 2000). DB has been widely used in China as a diuretic, antispasmodic, analgesic, aphrodisiac, and rejuvenative tonic agent. It is also used in the treatment of carbuncles, lung abscesses, breast lumps, and goiter (Gao et al., 2003). In addition, DB displays a range of pharmacological properties, including antitumor (Grynberg et al., 1999), antifeedant (Cifuente et al., 2002), anti-inflammation (Demetzos et al., 2001), and antimalarial activities (Teponno et al., 2006). DB and related remedies are used for the treatment of thyroid gland diseases and varieties of tumors (Tang, 1995; Raskiari et al., 2005). Despite this, the safety of DB has been questioned, and ingestion of DB was reportedly associated with high incidence of liver injury (Murray et al., 1984). Two cases of hepatitis were reported in patients after chronic exposure to DB (Liu, 2002). Animal studies showed that oral administration of ethanol extracts of DB caused the elevations of serum alanine transaminase and aspartate transaminase, along with increased lipid peroxide in hepatic tissues in mice (Wang et al., 2010a). Saponins, diterpenoid lactones, tannins, and polysaccharides have been documented as the major components of DB (Wang et al., 2011). Diterpenoid lactones have drawn much attention, and thus far a total of 17 compounds have been identified that oral administration of ethanol extracts of DB caused the elevations of serum alanine transaminase and aspartate transaminase, along with increased lipid peroxide in hepatic tissues in mice (Wang et al., 2010a). Saponins, diterpenoid lactones, tannins, and polysaccharides have been documented as the major components of DB (Wang et al., 2011). Diterpenoid lactones have drawn much attention, and thus far a total of 17 compounds have been identified from DB, namely DIOA–DIOF and DIOH (Teponno et al., 2007, 2008, 2013). DIOB is the most abundant diterpenoid lactone found in DB (Gao et al., 2002; Zhang and Yuan, 2009). Recent studies demonstrated that oral administration of DIOB for 12 consecutive days induced liver injury, along with oxidative stress, in mice (Wang et al., 2010b; Ma et al., 2013). However, the mechanisms of DIOB-induced liver injury remain unknown.

DIOB is a furan-containing compound. Many furan compounds are reported to be toxic or carcinogenic (Peterson, 2013), such as 4-ipomeanol (Boyd et al., 1974), furosemide (Mitchell et al., 1974, 1976; Wong et al., 2000; Williams et al., 2007), 3-methylfuran (Haschek et al., 1984; Morse et al., 1984), teucrin A (Larrey et al., 1992), and menthofuran (Sullivan et al., 1979; Anderson et al., 1996). The toxic effects elicited by these furans are suggested to attribute to their cis-enedial oxidative metabolite (Peterson, 2013). We hypothesized that DIOB is metabolized to a cis-enedial, an electrophilic species, which may play an important role in hepatotoxicity induced by DIOB. In this study, we report the successful characterization of cis-enedial metabolites of DIOB and the identification of the cytochromes P450 (P450) responsible for the bioactivation of DIOB. In addition, this article describes the enedial resulting from biomimetic oxidation of DIOB.

Materials and Methods

Chemicals and Materials. Dry rhizomes of DB were obtained from Tong-Ren-Tang Pharmacy (Shenyang, China). DIOB was isolated from DB rhizomes in our laboratory according to previously published literature (Kawasaki et al., 1968), and the structure was confirmed by mass spectrometry (MS) and nuclear characterizated from DB, namely DIOA–DIOF and DIOH (Teponno et al., 2007, 2008, 2013). DIOB is the most abundant diterpenoid lactone found in DB (Gao et al., 2002; Zhang and Yuan, 2009). Recent studies demonstrated that oral administration of DIOB for 12 consecutive days induced liver injury, along with oxidative stress, in mice (Wang et al., 2010b; Ma et al., 2013). However, the mechanisms of DIOB-induced liver injury remain unknown.

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magnetic resonance. The purity of DIOB was >98% as determined by high-performance liquid chromatography (LC) with a diode array detector. Glutathione (GSH), N-acetyl cysteine (NAC), N-acetyl lysine (NAL), N-α-phthalalioflavone, sulfaphenazone, ticlopidine, quinidine, disulfiram, methoxsalen, pilocarpine, ketoconazole, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLMs) and recombinant human P450 enzymes were purchased from BD Gentest (Woburn, MA). All organic solvents were from Fisher Scientific (Springfield, NJ). All reagents and solvents were of either analytical or high-performance LC grade.

Biomimetic DIOB Oxidation. DIOB (5.0 mg) was suspended in acetone (200 µl) and heated until DIOB was completely dissolved. Saturated sodium bicarbonate solution (40 µl) and Oxone (15 mg) were added successively to the solution for the reaction mixture. The mixture was stirred for 15 minutes at room temperature, followed by addition of GSH (60.0 mg) dissolved in 500 µl of saturated sodium bicarbonate solution or NAC (25.4 mg) dissolved in 500 µl of saturated sodium bicarbonate solution, with stirring for 30 minutes. After centrifugation, the supernatants were harvested and evaporated to dryness under a stream of nitrogen gas at 40°C. The resulting samples were reconstituted with 200 µl buffer, followed by addition of NAL (6.0 mg). After further stirring for 30 minutes at 70°C, the reaction was analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Preparation of DB Extracts. Dry rhizomes of DB (5.0 g) obtained from Tong-Ren-Tang Pharmacy were refluxed in 150 ml of 95% ethanol. The resulting crude extracts were concentrated through evaporation to dryness and added into 50 ml distilled water, followed by successive extraction with chloroform (3 × 50 ml). The resultant extracts were pooled and concentrated to dryness. The resultant solid was reconstituted with 5 ml of 50% acetonitrile in water as the stock solution for microsomal incubations.

Animal Experiments. All animal studies were performed according to procedures approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University (Shenyang, China). Male Sprague-Dawley rats (200 ± 20 g), purchased from the Animal Center of Shenyang Pharmaceutical University, were placed in a controlled environment (temperature of 25°C and 12 hour dark/light cycle) and maintained on standard rat chow. After fasting for 12 hours with free access to water prior to the experiment, one group of animals was anesthetized with chloral hydrate, and their bile ducts were cannulated with PE-10 tubing. DIOB dissolved in corn oil was administered intraperitoneally at 100 mg/kg, and bile was collected in Eppendorf tubes 2 hours after dosing. Control bile was collected before the treatment. The other group of rats administered with the same dose of DIOB was placed in metabolism cages. Urine samples were collected from predose to 6 hours and from 6 to 24 hours after the procedure. The control animals treated with vehicle were included. During the experiment, the rats were allowed free access to food and water.

Sample Preparation for LC-MS/MS. An aliquot of 300 µl acetonitrile was added to 100 µl of the bile or urine samples. After centrifugation, the supernatants were harvested and evaporated to dryness under a stream of nitrogen gas at 40°C. The resulting samples were reconstituted with 100 µl of 50% acetonitrile in water, and then centrifuged at 16,000 rpm for 10 minutes, and the supernatants (5 µl) were injected onto LC-MS/MS for analysis.

Microsomal Incubations. Rat (Sprague-Dawley) liver microsomes (RLMs) were prepared as described by our laboratory (Lin et al., 2007). The ethanol extracts from herb DB and DIOB (50 µM) were individually mixed with RLMs or HLMs (1.0 mg protein/ml) supplemented with GSH or NAC and NAL at a final concentration of 1.0 mM. The total incubation volume was 300 µl. The incubation reactions were initiated by the addition of NADPH (final concentration: 1.0 mM). Control samples containing no NADPH were included. After 60 minutes of incubation at 37°C, the reactions were terminated by mixing with equal volumes of ice-cold acetonitrile. The reaction mixture was vortex mixed and centrifuged to remove precipitated protein at 16,000 rpm for 10 minutes. The resulting supernatant was injected onto LC-MS/MS for analysis. In a separate study, the microsomal incubations were performed in the absence of NAL under the similar experimental condition.

Recombinant Human P450 Incubations. Conditions were equivalent to the microsomal incubations except that microsomes were replaced by individual human recombinant P450 enzymes (0.1 nM), including P450 enzymes CYP450Ia2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5.

Metabolizing Enzyme Inhibition Studies. To determine the specific P450 enzymes involved in the formation of reactive metabolites of DIOB, a total of eight P450 inhibitors were tested as follows: α-naphthoflavone (1.0 µM for CYP1A2), sulfaphenazone (20 µM for CYP2C9), ticlopidine (100 µM for CYP2B6 and 100 µM for CYP2C9), quinidine (5.0 µM for CYP2D6), disulfiram (100 µM for CYP2E1), methoxsalen (20 µM for CYP2A13 and 20 µM for CYP2A6), pilocarpine (100 µM for CYP2A1), and ketoconazole (1.0, 10, and 100 µM for CYP3A). Incubation mixtures contained 1.0 mg protein/ml RLMs or pooled HLMS, 50 µM DIOB, 1.0 mM NADPH, 1.0 mM NAC, 1.0 mM NAL, and the individual inhibitors at the designed concentrations. Incubations were performed at 37°C for 60 minutes, and reactions were terminated by mixing with an equal volume of ice-cold acetonitrile. Controls containing no chemical inhibitors were included. Each incubation was performed in duplicate. The production of the NAC/NAL-DIOB conjugate was monitored and quantitated by LC-MS/MS method as below. A comparison was made relative to the controls without inhibitor, and P450 activity was expressed as the percentage of control activity.

LC-MS/MS Method. LC-MS/MS analyses were performed on an AB SCIEX Instruments 4000 Q-Trap (Applied Biosystems, Foster City, CA) interfaced online with an expeditor ultraLC 100 system (Applied Biosystems). Samples were subject to chromatographic separation with an expeditor ultraLC 100 system. The chromatographic separation was achieved on a Hypersil BDS-C18 column (5 µm, 4.6 × 250 mm; Thermo Fisher Scientific, Inc., Pittsburgh, PA). Mobile phase A was acetonitrile with 0.1% formic acid, and mobile phase B was water with 0.1% formic acid, using the following gradient elution profile: 20% solvent A for 2 minutes, followed by 20–100% A for 8 minutes, 100–100% A for 2 minutes, and 100–20% A for 3 minutes. The ultra-performance LC flow rate was 0.8 ml/min. LC-MS/MS analyses were performed on a 5-µl aliquot of sample. Multiple-reaction monitoring (MRM) scans of m/z 632 → 503, 939 → 810, and 820 → 691 (DIOB-derived GSH/NAL conjugate) or m/z 676 → 553 (DIOB-derived NAC/NAL conjugate) were run in positive ion mode with a 0.2-Da step 5.0-millisecond pause between mass ranges and a 2-second scan rate or 200-millisecond dwell. The TurbolonSpray interface for electrospray ionization was operated in positive ion mode. Ion spray voltage was set at 5500 V and source temperature at 650°C. Curtain gas, ion source gas 1, and ion source gas 2 were set at 20, 50, and 50 psi, respectively. Declustering potential, entrance potential, and cell exit potential were set to 70, 10, and 3 V, respectively. Unit mass resolution provided improved sensitivity and selectivity. The information-dependent acquisition method was utilized to trigger the enhanced product ion (EPI) scans by analyzing MRM. Information-dependent acquisition was used to trigger acquisition of EPI spectra for ions exceeding 5000 cps with exclusion of former target ions after three occurrences for 10 seconds. The EPI scan was run in positive mode at a scan range for product ions from m/z 50 to 940. The collision energy was set at 45 eV with a spread of 15 eV. Data were processed using Applied Biosystems/SCIEX Analyst software (version 1.6 and 1.6.1).

LC-MS/MS analyses were also conducted on an Agilent 1200 Series Rapid Resolution LC system equipped with a hybrid quadrupole time-of-flight (Q-TOF) MS system (microQ-TOF; Bruker Corporation, Billerica, MA). The equipment was coupled with the same column described above. Mobile phase A was acetonitrile with 0.1% (v/v) formic acid, and mobile phase B was water with 0.1% (v/v) formic acid. The flow rate was set at 0.8 ml/min, and the column temperature was maintained at 25°C. The mobile phase consisted of linear gradients of acetonitrile (A) and 0.1% formic acid (B). The gradient elution was started from 10% solvent B and maintained for 2 minutes, and increased to 100% B linearly in 10 minutes, maintained for 6 minutes, and finally decreased to 10% in 2 minutes to equilibrate the column. The injection volume was 20 µl. The mass spectrum data were acquired in positive ion mode. The mass spectrometric parameters were optimized as follows: end plate offset, −500 V; capillary voltage, −4500 V; nebulizer gas pressure, 1.2 bar; dry gas, high-purity nitrogen (N2); dry gas flow rate, 8.0 liters per minute; and gas temperature, 180°C. The spectra were acquired at 2 seconds per spectrum in the range of m/z 50–1500. The data were analyzed by Bruker Daltonics Data Analysis 3.4 software.

Results

In Vitro Metabolic Activation of DIOB. We proposed that the furan group of DIOB played an important role in DIOB-induced liver injury, and specifically that DIOB is metabolized to the corresponding cis-enedial (Scheme 1, enedial 2), and the resulting electrophilic...
metabolite is responsible for the hepatotoxicity. DIOB was incubated in RLMs or HLMs supplemented with GSH and NAL as trapping agents. The mixture was analyzed by an AB SCIEX 4000 Q-Trap mass spectrometry system. A metabolite (M1; retention time = 6.70 minutes) was detected by the mass spectrometry (Fig. 1B) in both RLMs and HLMs. The tandem mass spectrometry (MS/MS) spectrum of M1 was obtained by MRM-EPI scanning (ion transition m/z 820 → 691), and the spectrum showed the indicative characteristic fragment ions associated with the cleavage of the GSH moiety (Fig. 1D). The product ions at m/z 745 and 691 were derived from the loss of glycine portion (−75 Da) and the γ-glutamyl portion (−129 Da) from m/z 820, respectively. The mixture was also analyzed by LC/Q-TOF MS. M1 showed its protonated molecule ion [M + H]+ at m/z 820.3032 in positive ion mode, which matches the elemental composition of C37H50N5O14S (Table 1). No such conjugate was detected in the microsomal incubation system in the absence of NADPH (Fig. 1A), indicating that metabolism was mediated in the formation of M1. To further characterize M1, we oxidized DIOB with Oxone in acetone, followed by mixing with NAL and GSH. A product formed in the reaction showed the same chromatographic and mass spectrometric identities (Fig. 1, C and E; Table 1) as that for the product (M1) generated in microsomal incubations. Unfortunately, the yield of the biomimetic oxidation was so low that we were unable to obtain enough amount of the product for nuclear magnetic resonance characterization.

Interestingly, two unexpected metabolites named as M2 and M3 (retention time = 6.54 minutes and 6.72 minutes, respectively) were observed by the Q-Trap MS (Fig. 2B). The MS/MS spectra acquired through MRM-EPI scanning (ion transition m/z 632 → 503) showed two major fragment ions, including m/z 557 (loss of 75 Da) and 503 (loss of 129 Da), indicating the participation of GSH in the formation of M2 and M3 (Fig. 2D). Further analysis by LC/Q-TOF MS demonstrated that both M2 and M3 showed their protonated molecular ions at m/z 632.1897 in positive ion mode, corresponding to the formula of C29H34N3O11S (Table 1). On the basis of the observed mass spectrometric data, we speculated that the two metabolites were derived from GSH solely, without the involvement of NAL.

To verify the sole role of GSH in the formation of M2 and M3, we conducted a separate microsomal incubation fortified with GSH in the absence of NAL. The MS analysis demonstrated that little M1 was formed in the mixture and that more M2 and M3 were generated (data not shown). In addition to M2 and M3, another metabolite (M4) was detected at a retention time of 6.39 minutes (Fig. 3B). The MS/MS spectrum acquired through MRM-EPI scanning (ion transition m/z 939 → 810) showed three major fragment ions, including m/z 864 (loss of 75 Da), 810 (loss of 129 Da), and 632 (loss of 307 Da) (Fig. 3D), indicating the participation of GSH in the formation of M4. The metabolite was further analyzed by LC/Q-TOF MS, and M4 showed its molecular ion at m/z 939.2747. The protonated molecular ion observed was consistent with the molecular weight of the elemental

Scheme 1. Proposed pathway for the formation of DIOB-derived cis-enedial by P450- and Oxone-mediated oxidation of DIOB.

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composition of \( \text{C}_{39}\text{H}_{51}\text{N}_{6}\text{O}_{17}\text{S}_{2} \) (Table 1), suggesting that M4 was derived from two molecules of GSH, a bis-GSH–derived DIOB conjugate. To further characterize M2–M4, we reanalyzed the mixture of the biomimetic oxidation of DIOB described above. As expected, M2–M4 were all detected, based on their retention time, molecular ion, and MS/MS spectra (Figs. 2, C and E, and 3, C and E; Table 1).

In a parallel incubation, NAC in place of GSH was used to trap DIOB-derived enedial (enedial 2). No such adducts similar to M2, M3, and M4 found in GSH-fortified microsomal incubations were detected in the NAC-supplemented microsomal incubation system. Instead, we detected a metabolite (M5) most likely associated with NAC/NAL. M5 was detected at a retention time of 5.85 minutes (Fig. 4B). The
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Identification of P450 Enzymes Responsible for Bioactivation of DIOB. To determine which P450 enzymes preferentially catalyze the oxidation of DIOB, DIOB was incubated with RLMs or HLMs, using the NAC/NAL trapping system as described above. The participation of P450 enzymes in bioactivation of DIOB was probed by coincubation with individual P450 enzyme selective inhibitors, including ketoconazole (CYP3A), α-naphthoflavone (CYP1A2), sulfaphenazole (CYP2C9), ticlopidine (CYP2B6 and CYP2C19), quinidine (CYP2D6), disulfiram (CYP2E1), methoxsalen (CYP2A13 and CYP2A6), and pilocarpine (CYP2A1). The formation of M5 was monitored in the microsomal reactions in the presence or the absence of the individual inhibitors. The presence of ketoconazole significantly reduced the formation of M5 in the rat microsomal mixtures (Fig. 6A), and the inhibition increased with the increase in the concentration of ketoconazole applied (Supplemental Fig. 1). No or minor inhibition was observed in the microsomal incubations in the presence of the other inhibitors. A similar observation was obtained in the HLM incubations (Fig. 6B). This indicates that there was no species difference in the bioactivation of DIOB. In a separate study, DIOB was incubated with individual recombinant human P450 enzymes, including CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5, mixed with NAC and NAL as trapping agents. M5 was detected in the incubation with CYP3A4. Apparently, little M5 was detected in the incubations with the other P450 enzymes (Supplemental Fig. 2). The results were consistent with the observation obtained in the microsomal enzyme inhibition studies. The experiments illustrated that CYP3A4 was the principal enzyme responsible for the bioactivation of DIOB.

Microsomal Incubation of Extracts of DB. DIOB was reported to be the major component in DB (Gao et al., 2002; Zhang and Yuan, 2009). We examined the bioactivation of DIOB present in herb extracts. Similar microsomal incubation was performed except for mixing with an ethanol extract from herb DB in place of pure DIOB. As expected, M1–M5 were all detected in the corresponding incubation systems (Supplemental Fig. 3).

Discussion

DIOB, the major component in D. bulbifera L. widely used in traditional Chinese medicine, was reported to cause liver injury in mice (Wang et al., 2010b; Ma et al., 2013), but the mechanisms of DIOB-induced hepatotoxicity remain unknown. We proposed that DIOB was metabolized to the corresponding cis-enedial metabolite and the resulting electrophilic metabolite may play a role in DIOB-induced toxicity. As an initial effort, we performed microsomal incubations with DIOB in the presence of GSH and NAL to trap the enedial metabolite. A total of four GSH-derived conjugates (M1–M4) were detected in the incubations by LC-MS/MS. The four metabolites showed the characteristic neutral losses of 75 Da (glycine portion) and 129 Da (γ-glutamyl portion) derived from DIOB moiety and the characteristic precursor ion at m/z 231 (Figs. 1D, 2D, and 3D) resulting from DIOB moiety. The obtained mass spectrometric data indicate that M1–M4 were derived from DIOB and GSH.

On the basis of the high-resolution molecular ion and MS/MS spectra, we considered M1 as a GSH/NAL-DIOB conjugate. However, it is true that the mass spectrum is unable to tell where GSH is located on the pyrrole ring. M1 was generated apparently from the reaction of enedial 2 with the sulfur of GSH and the nitrogen of NAL through respective addition and condensation. A separate experiment showed that the absence of NAL in the microsomal incubation system supplemented with GSH stopped the formation of M1. This implies that NAL participated in the production of M1.

The observed high-resolution molecular ions of M2 and M3, along with their MS/MS spectra, allow us to propose that the two metabolites are derived from GSH solely without the involvement of NAL. In other words, the two metabolites were produced by the reaction of enedial 2 with the nitrogen (glutamate residue) and the sulfur (cysteine residue) of the same molecule of GSH, respectively. To ensure the
lack of NAL participation in the formation of M2 and M3, similar incubation was conducted, and the microsomal reaction excluded NAL. It appears that the absence of NAL did not stop the production of the M2 and M3. Instead, the formation of M2 and M3 was enhanced. This provided further evidence that the formation of M2 and M3 did not necessarily require NAL.

Similar cyclic molecules derived from cis-enedial and GSH were previously documented (Chen et al., 1995; Peterson et al., 2005, 2011; Gates et al., 2014). In addition, we performed a separate microsomal incubation in which GSH was replaced with NAC, and no such metabolite formed by the intramolecular cyclization occurring in the GSH-fortified incubation was detected. This may be explained by that
NAC has no α-amino group; it is an acetamido nitrogen that is not reactive, since the acetyl group attached decreases the nucleophilicity of the nitrogen.

M4 was the metabolite with the highest molecular ion detected in the microsomal incubation supplemented with GSH. This indicates that the reaction of enedial 2 can take place by reaction with GSH both intramolecularly and intermolecularly. It is most likely that the formation of M4 resulted from the reaction of enedial 2 with the nitrogen (glutamate residue) of a molecule of GSH and the sulfur (cysteine residue) of the other molecule of GSH. However, this does not apply to that observed in microsomal reactions fortified with NAC, since no such conjugate as M4 was detected in the incubation system.
reason for that could again be attributed to the poor nucleophilicity of the α-amino group of NAC supplemented in the microsomes.

M2 and M3 found in microsomal incubations were also detected in bile of the animals given DIOB. It appears that M2 and M3 were the primary DIOB-derived GSH conjugates secreted from the bile. However, M4, the bis-GSH-derived DIOB conjugate, was found in the urine but not in the bile. This indicates that M4 was formed in vivo, but for some reason it was excreted only in urine and not bile.

An epoxide-derived metabolite resulting from furan ring oxidation could also be formed. If the epoxide was generated, we would see the GSH conjugates via the opening of the epoxide by a molecule of GSH and/or its sequential dehydration. However, we failed to detect the two GSH conjugates in the microsomal incubations, indicating that the epoxide metabolite either was not formed or was instantly rearranged to enedial 2.

Coincubation with ketoconazole in microsomes significantly decreased the formation of enedial 2 (Fig. 6), but no or minor inhibition was observed in the presence of the other P450 inhibitors. In addition, the reactive metabolite formation decreased with the increase in the concentration of ketoconazole applied (Supplemental Fig. 1). Furthermore, bioactivation studies with individual recombinant enzymes demonstrated that only CYP3A4 catalyzed the metabolism of DIOB to enedial 2 (Supplemental Fig. 2). The enzyme inhibition and individual recombinant enzyme studies demonstrated that CYP3A4 dominated
the bioactivation of DIOB. The findings facilitate the investigation of the biochemical mechanisms of DIOB-induced hepatotoxicity. Our recent work demonstrated that ketoconazole reversed the liver injury induced by DIOB (to be published separately).

A number of furanoid compounds have been linked to adverse events, including hepatotoxicity, pulmonary toxicity, and carcinogenesis, possibly in part resulting from the in situ formation of *cis*-enedial (Peterson, 2013). These reactive species are capable of alkylating key cellular proteins and/or DNA. The observation of M2 and M3 in bile and urine of rats given DIOB indicates the formation of enedial 2 in vivo that further reacts with GSH. This is consistent with the reported GSH depletion in mice treated with DIOB (Wang et al., 2010b; Ma et al., 2013). The identification of enedial 2 allows us to anticipate the involvement of the reactive metabolite in DIOB-induced hepatotoxicity.

In conclusion, this study provided clear evidence for the formation of a *cis*-enedial metabolite from DIOB both in vitro and in vivo. The electrophilic metabolite reacted with glutathione to produce four glutathione-derived conjugates. CYP3A4 was the primary enzyme

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**Fig. 5.** (A–D) Extracted ion (*m/z* 632 → 503) chromatograms obtained from LC/Q-Trap MS analysis of bile and urine of rats before (A and C) and after (B and D) treatment with DIOB. (E and F) Extracted ion (*m/z* 939 → 810) chromatograms obtained from LC/Q-Trap MS analysis of urine of rats before (E) and after (F) treatment with DIOB.
responsible for the metabolic activation of DIOB. The observed bioactivation of DIOB will assist us in investigating the correlation of the cis-enolized metabolic formation with the hepatotoxicity induced by DIOB.

### Authorship Contributions

**Participated in research design:** Gao, Zheng.

**Conducted experiments:** Lin, Li, Peng.

**Performed data analysis:** Lin.

**Wrote or contributed to the writing of the manuscript:** Lin, Zheng.

### References


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