Cytochrome P450-Mediated Metabolic Activation of Diosbulbin B

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Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CE, collision energy; CXP, cell exit potential; DB, Dioscorea bulbifera L.; DIOB, diosbulbin B; DP, declustering potential; EP, entrance potential; EPI, enhanced product ion; GSH, glutathione; HLMs, human liver microsomes; IDA, information-dependent acquisition; MRM, multiple-reaction monitoring; NAC, N-acetyl cysteine; NADPH, β-nicotinamide adenine dinucleotide 2’-phosphate reduced tetrasodium salt; NAL, N-acetyl lysine; NL, neutral loss; RLMs, rat liver microsomes.
Abstract

Diosbulbin B (DIOB), a furan-containing deterpenoid 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 the present 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 LC-MS/MS. 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 P450 3A4 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 diuretic, antispasmodic, analgesic, aphrodisiac, and rejuvenative tonic agents. It is also used in the treatment of carbuncles, lung abscesses, breast lumps, and goiter (Gao et al., 2003). Additionally, 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 antisalmonellal activities (Teponno et al., 2006). DB and related remedy are used for the treatment of thyroid gland diseases and varieties of tumors (Tang, 1995; Rasikari et al., 2005). Despite this, the safety of DB use 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 study showed that oral administration of ethanol extracts of DB caused the elevations of serum alanine transaminase (ALT) and aspartate transaminase (AST), along with increased lipid peroxide in hepatic tissues in mice (Wang et al., 2010). Saponins, diterpenoid lactones, tannins, and polysaccharides have been documented as the major components of DB (Wang et al., 2011). Diterpenoid lactones have drawn a good attention, and so far a total of seven furanoid norditerpene diosbulbins have been isolated and characterized from DB, namely diosbulbin (DIO) A, B, C, D, E, F, and H (Teponno et al., 2007; Teponno et al., 2008; Teponno et al., 2013). DIOB is the most abundant diterpenoid lactone found in DB (Gao et al., 2002; Zhang
et al., 2009). Recent study demonstrated that oral administration of DIOB for 12 consecutive days induced liver injury, along with oxidative stress, in mice (Wang et al., 2010; Ma et al., 2013). However, the mechanisms of DIOB-induced liver injury remain unknown.

DIOB is a furan-containing compound. Many furan compounds have been reported to be toxic/or carcinogenic (Peterson, 2013), such as 4-ipomeanol (Boyd et al., 1974), furosemide (Mitchell et al., 1974; Mitchell et al., 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 the present study, we report the successful characterization of cis-enedial metabolite of DIOB and the identification of the cytochromes P450 responsible for the bioactivation of DIOB. In addition, this paper 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). Diosbulbin B (DIOB) was isolated from DB rhizomes in our laboratory according to the previous literature (Kawasaki et al., 1968), and the structure was confirmed by mass spectrometry and NMR. The purity of DIOB was > 98% determined by high-performance liquid chromatography (HPLC) with diode array detector (DAD). Glutathione (GSH), N-acetyl cysteine (NAC), N-acetyl lysine (NAL), α-naphthoflavone, sulfaphenazole, 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 HPLC grade.

Biomimetic DIOB oxidation. DIOB (5.0 mg) was suspended in acetone (200 μL) and heated until DIOB was completely dissolved. To the resulting solution were saturated sodium bicarbonate solution (40 μL) and Oxone (15 mg) added successively. The mixture was stirred for 15 min 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 min. 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 of buffer, followed by addition of NAL (6.0 mg). After further stirring for 30 min at 70 °C, the reaction was analyzed by LC-MS/MS.
**Preparation of DB Extracts.** Dry rhizomes of DB (5.0 g) obtained from Tong-Ren-Tang pharmacy (Shenyang, China) was refluxed in 150 mL of 95% ethanol. The resulting crude extracts were concentrated through evaporation to dryness and added into 50 mL of 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 (Shenyang, China), were placed in a controlled environment (temperature of 25 °C and dark/light circle) and maintained on standard rat chow. After fasting for 12 h 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 tube for 2 h following 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 h and from 6 to 24 h post procedure. The control animals treated with vehicle were included. During the experiment, the rats were allowed to 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 min, 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 rat or human liver microsomes (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 min 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 min. The resulting supernatant was injected onto LC-MS/MS for analysis. In a separated 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 P450s 1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5.

**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 P450 1A2), sulfaphenazole (20 μM for P450 2C9), ticlopidine (100 μM for P450 2B6 and 100 μM for P450 2C19), quinidine (5.0 μM for P450 2D6), disulfiram (100 μM for P450 2E1), methoxsalen (20 μM for P450 2A13 and 20 μM for P450 2A6), pilocarpine (100 μM for P450 2A1), and ketoconazole (1.0, 10, and 100 μM for P450 3A). 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 min, 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 ekspert ultraLC 100 system (Applied Biosystems, Foster City, CA). Samples were subject to chromatographic separation with ekspert ultraLC 100 system. The chromatographic separation was achieved on a Hypersil BDS-C18 column (5 μm, 4.6 × 250 mm, Thermo, Pittsburgh, PA). Mobile phase A was acetonitrile with 0.1% formic acid, and mobile phase B was water with 0.1% formic acid, using gradient
elution profile: 20% solvent A for 2 min, followed by 20–100% A in 8 min, 100–100% A for 2 min and 100–20% A for 3 min. The UPLC flow rate was 0.8 mL/min. LC/MS/MS analyses were performed on a 5 μL aliquot of sample. Multiple-reaction monitoring (MRM) scan of \textit{m/z} 632→503, 939→810 and 820→691 (DIOB-derived GSH/NAL conjugate) or \textit{m/z} 676→553 (DIOB-derived NAC/NAL conjugate) was run in positive ion mode with 0.2 Da step 5.0 ms pause between mass ranges and 2 s scan rate or 200 ms dwell. TurboIonSpray interface for electrospray ionization (ESI) 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 (DP), entrance potential (EP), and cell exit potential (CXP) were set to 70, 10, and 3 V, respectively. Unit mass resolution provided improved sensitivity and selectivity. The information-dependent acquisition (IDA) method was employed to trigger the enhanced product ion (EPI) scans by analyzing MRM. IDA was used to trigger acquisition of EPI spectra for ions exceeding 5000 cps with exclusion of former target ions after three occurrences for 10 s. The EPI scan was run in positive mode at a scan range for product ions from \textit{m/z} 50 to 940. The collision energy (CE) was set at 45 eV with a spread of 15 eV. Data were processed using Applied Biosystems/SCIEX Analyst\textsuperscript{TM} software (versions 1.6 and 1.6.1).

LC-MS/MS analyses were also conducted on an Agilent 1200 Series Rapid Resolution LC equipped with a hybrid quadrupole-time-of-flight mass spectrometer (Bruker microQ-TOF, Germany). 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 min, and increased to 100% B linearly in 10 min, maintained for 6 min and finally decreased to 10% in 2 min to equilibrate the column. Injection volume: 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 L/min; gas temperature: 180 °C. The spectra were acquired at 2 s per spectrum in the range of m/z 50 to 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 (2, Scheme 1), and the resulting electrophilic metabolite is responsible for the hepatotoxicity. DIOB was incubated in RLMs or HLMs supplemented with glutathione (GSH) and N-acetyl lysine (NAL) as trapping agents. The mixture was analyzed by AB SCIEX 4000 Q-Trap MS. A metabolite (M1, Rt = 6.70 min) was detected by the mass spectrometry (Figure 1B) in both RLMs and HLMs. 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 (Figure 1D). The product ions at m/z 745 and 691 were derived from the loss of glycine portion (-75 Da), \( \gamma \)-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 C\(_{37}\)H\(_{50}\)N\(_5\)O\(_{14}\)S (Table 1). No such conjugate was detected in the microsomal incubation system in the absence of NADPH (Fig. 1A), indicating 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, and 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 NMR characterization.

Interestingly, two unexpected metabolites named as M2 and M3 ($R_t = 6.54$ min; $R_t = 6.72$ min) 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), 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 $C_{29}H_{34}N_{3}O_{11}S$ (Table 1). Based on 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 separated microsomal incubation fortified with GSH in the absence of NAL. The mass spectrometry 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 retention time of 6.39 min (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 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 \textit{bis}-GSH-derived DIOB conjugate. To further characterize M2 - M4, we re-analyzed 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 (Fig. 2C, 2E, 3C, and 3E, and Table 1).

In a parallel incubation, \textit{N}-acetyl cysteine (NAC) in place of GSH was used to trap DIOB-derived 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 retention time of 5.85 min (Fig. 4B). The metabolite was further analyzed by LC-Q-TOF MS. M5 showed its protonated molecule ion \([M + H]^+\) at \(m/z\) 676.2480 in positive ion mode (Table 1), which matches the elemental composition of \( \text{C}_{32}\text{H}_{42}\text{N}_{3}\text{O}_{11}\text{S} \) (Table 1). The MS/MS spectrum of M5 showed the indicative characteristic neutral loss (NL) of 129 Da. The produced fragment ions at \(m/z\) 658, 634, 616, 588, 571, and 553 were assigned to \([\text{MH- H}_2\text{O}]^+\), \([\text{MH-CH}_2\text{CO}]^+\), \([\text{MH-CH}_2\text{CO-H}_2\text{O}]^+\), \([\text{MH-CH}_2\text{CO-H}_2\text{O-CO}]^+\), \([\text{MH-CH}_2\text{CO-H}_2\text{O-CO-NH}_3]^+\), \([\text{MH-CH}_2\text{CO-2H}_2\text{O-CO-NH}_3]^+\), respectively (Fig. 4D). The formation of M5 was also found to be NADPH-dependent (Fig. 4, A and B). To further characterize M5, DIOB was oxidized with Oxone in acetone, followed by reaction with NAL and NAC. A product formed in the reaction showed the same chromatographic and mass spectrometric identities as that for the product (M5).
generated in microsomal incubations (Fig. 4, C and E, and Table 1).

**Biliary and Urinary Excretion of DIOB GSH Conjugates.** In order to investigate the bioactivation of DIOB *in vivo*, biliary and urinary excretion of GSH conjugates were monitored by a designed MRM template, using AB SCIEX 4000 Q-Trap MS after an intraperitoneal injection of DIOB at 100 mg/kg in SD rats. M2 and M3 were found in the bile and urine obtained from the animals after given DIOB (Fig. 5, B and D), but no such metabolites were observed before the treatment with DIOB (Fig. 5, A and C). The two metabolites showed the same retention times as those for the metabolites produced in microsomal incubations (Fig. 2B). Interestingly, M4 (the bis-GSH-derived DIOB conjugate) was detected in the urine (Fig. 5F) but not in the bile.

**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 described as above. The participation of P450 enzymes in bioactivation of DIOB was probed by coincubation with individual P450 enzyme-selective inhibitors, including ketoconazole (P450 3A), α-naphthoflavone (P450 1A2), sulfaphenazole (P450 2C9), ticlopidine (P450 2B6 and P450 2C19), quinidine (P450 2D6), disulfiram (P450 2E1), methoxsalen (P450 2A13 and P450 2A6), and pilocarpine (P450 2A1). 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. Similar observation was obtained in the HLM incubations (Fig. 6B). This indicates that there was not species difference in the bioactivation of DIOB. In a separate study, DIOB was incubated with individual recombinant human P450 enzymes, including P450 1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, mixed with NAC and NAL as trapping agents. M5 was detected in the incubation with P450 3A4. 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 P450 3A4 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 et al., 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

Diosbulbin B (DIOB), the major component in Dioscorea bulbifera L. widely used in traditional Chinese medicine, was reported to cause liver injury in mice (Wang et al., 2010; 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 75 Da (glycine portion) and 129 Da (γ-glutamyl portion) derived from GSH moiety and the characteristic precursor ion at m/z 231 (Fig. 1D, 2D, and 3D) resulting from DIOB moiety. The obtained mass spectrometric date indicate that M1 – M4 were derived from DIOB and GSH.

Based on the high resolution molecular ion and MS/MS spectrum, 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 the further evidence that the formation of M2 and M3 did not necessarily require NAL.

Similar cyclic molecules derived from cis-enedial and GSH have been documented previously (Chen et al., 1995; Peterson et al., 2005; Peterson et al., 2011; Gates et al., 2014). Additionally, we performed a separated 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 N-acetyl-cysteine has no alpha-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. The reason for that could again attribute 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.

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. Additionally, 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 P450 3A4 catalyzed the metabolism of
DIOB to enedial 2 (Supplemental Fig. 2). The enzyme inhibition and individual recombinant enzyme studies demonstrated that P450 3A4 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 \textit{in situ} formation of \textit{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 \textit{in vivo} that further react with GSH. This is consistent with the reported GSH depletion in mice treated with DIOB (Wang et al., 2010; 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, the present study provided a clear evidence for the formation of a \textit{cis}-enedial metabolite from DIOB both \textit{in vitro} and \textit{in vivo}. The electrophilic metabolite reacted with glutathione to produce four glutathione-derived conjugates. P450 3A4 was the primary enzyme responsible for the metabolic activation of DIOB. The observed bioactivation of DIOB will assist us to investigate the correlation of the \textit{cis}-enedial metabolite formation with the hepatotoxicity induced by DIOB.
Authorship contributions

Participated in research design: Zheng, Gao.

Conducted experiments: Lin, Li, and Peng.

Performed data analysis: Lin.

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

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

Figure Legends

Figure 1. Extracted ion (m/z 820 → 691) chromatograms obtained from LC-Q-Trap MS analysis of rat liver microsomal incubations containing DIOB, GSH, and NAL in the absence (A) or the presence (B) of NADPH. C: Extracted ion (m/z 820 → 691) chromatogram obtained from LC-Q-Trap MS analysis of biomimetic M1. D: MS/MS spectrum of M1 generated in microsomal incubation. E: MS/MS spectrum of biomimetic M1.

Figure 2. Extracted ion (m/z 632 → 503) chromatograms obtained from LC-Q-Trap MS analysis of rat liver microsomal incubations containing DIOB, GSH, and NAL in the absence (A) or the presence (B) of NADPH. C: Extracted ion (m/z 632 → 503) chromatogram obtained from LC-Q-Trap MS analysis of biomimetic M2 and M3. D: MS/MS spectrum of M2 and M3 generated in microsomal incubation. E: MS/MS spectrum of biomimetic M2 and M3.

Figure 3. Extracted ion (m/z 939 → 810) chromatograms obtained from LC-Q-Trap MS analysis of rat liver microsomal incubations containing DIOB and GSH in the
absence (A) or the presence (B) of NADPH. C: Extracted ion (\(m/z\) 939 → 810) chromatogram obtained from LC-Q-Trap MS analysis of biomimetic M4. D: MS/MS spectrum of M4 generated in microsomal incubation. E: MS/MS spectrum of biomimetic M4.

**Figure 4.** Extracted ion (\(m/z\) 676 → 553) chromatograms obtained from LC-Q-Trap MS analysis of rat liver microsomal incubations containing DIOB, NAC, and NAL in the absence (A) or the presence (B) of NADPH. C: Extracted ion (\(m/z\) 676 → 553) chromatogram obtained from LC-Q-Trap MS analysis of biomimetic M5. D: MS/MS spectrum of M5 generated in microsomal incubation. E: MS/MS spectrum of biomimetic M5.

**Figure 5.** 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. 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.

**Figure 6.** Inhibitory effects of P450 inhibitors on the formation of M5 in rat liver microsomal (A) and human liver microsomal incubations (B) containing NADPH, DIOB (50 μM), NAC, and NAL. The concentrations of α-naphthoflavone, sulfaphenazole, ticlopidine, quinidine, disulfiram, methoxsalen, pilocarpine, and ketoconazole were 1.0, 20, 100, 5.0, 100, 20, 100 and 1.0 μM, respectively.
Table

Table 1. Summary of metabolite profiling data obtain from LC-Q-TOF mass spectrometry for diosbulbin B incubated with rat liver microsomes in the presence of NADPH, N-acetyl lysine, and GSH or N-acetyl cysteine. M1-M5 were the metabolites detected in microsomal incubations, and M1'-M5' were the biomimetic DIOB oxidation products.

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<th>measured mass</th>
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<td>[M+H]^+</td>
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</table>
Figure 2

A

- NADPH

B

+ NADPH

C

Biomimetic oxidation

D

E
Figure 3

(A) -NADPH

(B) +NADPH

(C) Biomimetic oxidation

(D) Mass spectrum of the substrate

(E) Mass spectrum of the product
Figure 4

(A) -NADPH

(B) +NADPH

(C) Biomimetic oxidation

(D) Molecular structure

(E) Mass spectrum
Figure 5

(A) Control (bile)

(B) Treated (bile)

(C) Control (urine)

(D) Treated (urine)

(E) Control (urine)

(F) Treated (urine)
Figure 6