Pulmonary Toxicity and Metabolic Activation of Dauricine in CD-1 Mice

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ABSTRACT

Dauricine is the major bioactive component isolated from the roots of Menispermum dauricum D.C. and has shown promising pharmacological activities with a great potential for clinical use. However, the adverse effects and toxicity of the alkaloid are unfortunately ignored. The objective of the current study was to evaluate the toxicity of dauricine in vitro and in vivo. Mice (CD-1) were treated intraperitoneally with dauricine at various doses, and sera and lung lavage fluids were collected after 24 h of treatment. No changes in serum aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen were noticed, whereas a dose-dependent increase in lactate dehydrogenase activity was observed in lung lavage fluids. Ethidium-based staining studies showed that remarkable cells lost membrane integrity in the lungs of the animals treated with dauricine at 150 mg/kg. Histopathological evaluation of lungs of mice showed that dauricine at the same dose caused significant alveolar edema and hemorrhage. Exposure to dauricine at 40 μM for 24 h resulted in up to 60% cell death in human lung cell lines BEAS-2B, WI-38, and A549. Ketoconazole showed protective effect on the pulmonary injury in mice given dauricine. A quinone methide metabolite of dauricine was identified in mouse lung microsomal incubations, and the presence of ketoconazole in the microsomal incubations suppressed the formation of the quinone methide metabolite. In conclusion, dauricine produced pulmonary injury in CD-1 mice. The pulmonary toxicity appears to depend on the metabolism of dauricine mediated by CYP3A. The electrophilic quinone methide metabolite probably plays an important role in the pulmonary toxicity induced by dauricine.

Bisbenzylisoquinolines (BBIQ) are a large and diverse family of natural alkaloids found in many plants. The majority of naturally occurring BBIQ alkaloids are mainly identified from families Menispermaceae, Ranunculaceae, Annonaceae, and Moniaceae (Guha et al., 1979; Schiff, 1983, 1987, 1991). Bisbenzylisoquinoline alkaloids have been reported to show a variety of pharmacological activities (Schiff, 1991). Many herbs containing alkaloids have been used as traditional medicines in cultures in China, India, Japan, Chile, and other nations (Chang and Wu, 2005; Martínez et al., 1997; Koh et al., 2006; Amresh et al., 2008). Dauricine (1, Scheme 1) is a bisbenzylisoquinoline alkaloid isolated from the roots of Menispermum dauricum D.C. (Li and Gong, 2007). Dauricine has been reported to show multiple pharmacological actions, including decelerating transmembrane ion shift, inhibiting anoxic depolarization, and scavenging oxygen free radicals, all of which probably affect many of the interconnected pathological processes involved in apoptotic death (Jin et al., 2002). Moreover, this agent has been used for the treatment of various diseases, such as cardiac ischemia, angina, and inflammation (Jin et al., 2002).

Recently, we reported a dauricine-derived quinone methide metabolite (2, Scheme 1) in human liver microsomal incubations (Wang et al., 2009). The quinone methide metabolite was trapped with glutathione (GSH) to form the corresponding GSH conjugate, 3 (Scheme 1). The formation of quinone methide metabolite 2 required NADPH.
present in the incubation system, and the presence of ketoconazole suppressed the generation of the quinone methide metabolite. A selection of individual human recombinant cytochromes P450 were incubated with dauricine, but only CYP3A4 was found to be the P450 enzyme responsible for the formation of the quinone methide metabolite. The dauricine-derived GSH conjugate was also detected in bile from rats given dauricine (Wang et al., 2009). We proposed that the quinone methide metabolite is formed via dehydrogenation mediated by CYP3A4 (Scheme 1).

Quinone methides are well known electrophilic species with high reactivity toward nucleophiles (Monks and Jones, 2002). Many quinone methide metabolites have been documented in metabolism studies of a variety of substances, such as butylated hydroxytoluene (BHT) (Monks and Jones, 2002), p-cresol (Gaikwad and Bodell, 2001), safrole (Monks and Jones, 2002), phencyclidine (Driscoll et al., 2007), troglitazone (Kassahun et al., 2001), tamoxifen (Fan et al., 2000), droloxfene (Yao et al., 2001), and toremifene (Yao et al., 2001). Quinone methide metabolites have been reported to covalently modify nuclear acids (Hecht et al., 2001) and proteins (Monks and Jones, 2002), and the modification of the biomolecules was suggested to correlate toxicities of the quinone methide metabolites (Thompson et al., 2001). Bioactivation of BHT derivatives has been extensively studied, and the reactivity of quinone methides derived from BHT derivatives appears to correlate with their pulmonary toxicity (Monks and Jones, 2002). In this study, we report the pulmonary toxicity induced by dauricine in cultured cells and airways and in mice. In addition, we provide the evidence for the formation of a quinone methide metabolite in lung microsomal incubations with dauricine. This sounds an alarming note for the safe use of dauricine as a potential pharmaceutical agent.

**Materials and Methods**

**Chemicals and Materials.** Dauricine was purchased from Shenzhen Medherb Bio-Tech Co., Ltd. (Shenzhen, China). Ethidium homodimer (EtD)-III was purchased from Biotium (Hayward, CA), and 4′-6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Ketocortizone, DNase, and formaldehyde solution were purchased from Sigma-Aldrich (St. Louis, MO). Gentamicin, DMEM/Hams’ F-12, RPMI 1640 medium, and trypsin were purchased from Mediatech (Herndon, VA). Medium LHC-9 was purchased from Invitrogen (Carlsbad, CA). Hematoxylin and eosin (H&E) was purchased from Vector Laboratories (Burlingame, CA). Hematoxytin and eosin (H&E) was purchased from Thermo Fisher Scientific (Waltham, MA). Low-melting-point agarose and β-luciferin were purchased from Promega (Madison, WI). 4-Ipomeanol was kindly provided by the National Cancer Institute (Frederick, MD). Dauricine-derived GSH conjugate 3 was synthesized according to the procedure we reported recently (Wang et al., 2009). Recombinant P450 enzymes CYP1A1, CYP2B6, and CYP3A4 were purchased from BD Gentest (Woburn, MA).

**Animals and Treatment.** Male CD-1 mice (20–22 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and had free access to water and food. Stock solutions of dauricine were prepared as follows. Dauricine (18.0 mg) was suspended in 0.5 ml of water, and the resulting suspension was acidified by adding dilute HCl solution until dauricine was dissolved. The resultant solution was neutralized with dilute NaOH solution, followed by addition of water to appropriate concentrations. For the toxicity test of dauricine, the animals were divided into four groups, and each group contained six mice. One group was administered intraperitoneally with saline as vehicle control, and the other groups were treated with dauricine at dosages of 50, 100, and 150 mg/kg, respectively. The animals were sacrificed 24 h after the administration. After anesthesia with pentobarbital, blood was harvested by cardiac puncture for AST, ALT, and BUN assays. In a separate study, mice were administered intraperitoneal ketoconazole (50 mg/kg). After 1.5 h of pretreatment, the animals were given intraperitoneal dauricine at 150 mg/kg and sacrificed 24 h after the treatment.

**Bronchoalveolar Lavage Fluid Collection.** Mice were anesthetized, and the trachea was exposed and cannulated with a feeding needle tied in place. The lungs were perfused with PBS via right ventricle into the pulmonary artery to remove blood from the lungs. The lungs were lavaged with PBS buffer (two times with 0.8 ml each). The recovered fluids were centrifuged at 1500g for 15 min at 4°C. The supernatants were collected for the measurement of lactate dehydrogenase (LDH) activity.

**ALT, AST, and BUN Assays.** The blood samples collected above were allowed to clot in test tubes at room temperature overnight, followed by centrifuging at 3000 rpm. The resulting sera were collected for ALT, AST, and BUN assays. Serum ALT and AST activities were measured by following the manufacturer’s instructions (Catachem Inc., Bridgeport, CT). BUN was assessed by use of QuantiChrom urea assay kit (Bioassay Systems, Hayward, CA).

**BAL Fluid, LDH Activity, and Protein Assays.** The activity of LDH in BAL fluids harvested was measured by In Vitro Toxicology Assay kit from Sigma-Aldrich. Specific activity was based on protein concentrations as determined using bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

**Histopathological Analysis.** The lung tissues were fixed in 10% neutral buffered formalin, paraffin-processed, and sectioned at 3 μm. For histological analysis, the tissue sections were stained with H&E.

**Cell Permeability.** The evaluation of lung cell membrane integrity was conducted according to the method developed by Van Winkle and coworkers (Van Winkle et al., 1999; Phimister et al., 2005), with some modifications. In brief, mice were anesthetized with pentobarbital. The trachea was exposed and cannulated with a stainless steel feeding needle tied in place. The lungs were lavaged two times with a solution of fluorescent dyes EthD-III (2.0 μM) and DAPI (300 μM) and inflated with the same solution for 20 min. The unbound dyes were then removed by lavaging with PBS buffer twice. The lungs were harvested, embedded in Tissue-Tek
O.C.T. (Sakura Finetek USA Inc., Torrance, CA) and quickly frozen in liquid nitrogen. The frozen lung tissues were cut into 5-μm-thick slices and placed on glass slides. After fixing in cold acetone (−20°C) for 2 min, the cryostat slices were mounted with water-based mounting media under glass coverslips. Representative areas were photographed with an Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany).

**Microdissection of Airway Segments and Cultures.** Microdissection of airway segments and tissue cultures were performed as described by Van Winkle et al. (1996). In brief, animals were anesthetized with pentobarbital, and the lungs were inflated with 1% low-melting agarose gel. The lung tissues were immersed in DMEM/Ham’s F-12 medium during dissection. The distal airway branches were dissected starting from their branchpoint with the major daughter airway to the most distal terminal bronchioles. The resulting dissected lung airways were gently washed with PBS buffer to remove agarose possibly remaining within the tissues and then incubated with 4-iodoamphetamine or dauricine in serum-free DMEM/Ham’s F-12 supplemented with gentamicin (50 μg/ml). After 24-h incubation, the media were harvested for LDH assay, the airways were homogenized, and the lysates were collected for protein assay.

**Preparation of Primary Pulmonary Cells.** Primary pulmonary cells were prepared by following the protocol described by Kim et al. (2005). In brief, mice were anesthetized, and lungs were perfused with 10 ml of PBS buffer, followed by intratracheal instillation with 1.0 ml of 1% low-melting agarose. The resulting lung tissues were minced on ice, incubated in digest solution containing 0.001% DNase and 0.25% trypsin in PBS buffer for 45 min at 37°C, filtered through 100- and 40-μm cell strainers, and centrifuged at 800 rpm for 5 min at 4°C. The collected cell pellets were resuspended in red blood cell lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 4 min, washed, and resuspended in DMEM/Ham’s F-12/10% fetal bovine serum for CYP3A assays.

**Cell-Based CYP3A Assays.** CYP3A assays were performed using a P450-Glo luciferin-PFBE assay kit (Promega), according to the manufacturer’s protocol. In brief, cells were seeded (1 × 10⁵ cells/well) in a 24-well plate and cultured in RPMI 1640 medium for 24 h. The plate was washed three times with PBS buffer, followed by addition of 300 μl of fresh medium and 7.5 μl of luminoenic-pentafluorobenzyl ester substrate agent. After 4-h incubation at 37°C, an aliquot (50 μl) of the resulting medium was mixed with luciferin detection reagent (50 μl) in a well of a 96-well opaque white luminometer plate. The mixture was incubated at room temperature for 20 min, and luminescence was read in a VICTOR3 multilabel plate reader. The mixture was vortexed and centrifuged at 11,000g for 5 min. The supernatant was transferred into a glass tube, evaporated to dryness under a stream of nitrogen at 40°C, and then reconstituted in 100 μl of methanol with 5 mM ammonium acetate [30:70, v/v]. A 20 μl aliquot of the reconstituted solution was injected to LC/MS* for analysis.

**Human Recombinant Cytochromes P450 Incubation.** Human recombinant P450 enzymes (supersomes), including P450s 1A1, 2B6, and 3A5, were carefully thawed on ice before the experiment. Dauricine (10 μM) was mixed with the individual human P450 enzymes (50 nM) in 200 μl of 100 mM potassium phosphate buffer, pH 7.4, supplemented with GSH at final concentration of 5 mM. After 3 min of preincubation at 37°C, the incubation reactions were initiated by addition of NADPH (1.0 mM). After 60-min incubation, the reactions were terminated with equal volume of ice-cold acetonitrile. In a separated incubation, ketoconazole (10 μM) was incorporated before the addition of NADPH. Control samples containing no NADPH were included. Each incubation was performed in duplicate. Protein of the incubation samples was precipitated by addition of 400 μl of methanol. The mixture was vortexed and centrifuged at 11,000g for 5 min. The supernatant was transferred into a glass tube, evaporated to dryness under a stream of nitrogen at 40°C, and then reconstituted in 100 μl of methanol with 5 mM ammonium acetate [30:70, v/v]. A 20 μl aliquot of the reconstituted solution was injected to LC/MS* for analysis.

**Mass Spectrometry Instrumentation.** The mass spectrometry analyses were carried out on a 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with a 6330 LC/MSD Trap XCT Ultra (Agilent Technologies). Separation was achieved using a Zorbax SB-C18 column (150 × 4.6 mm i.d., 5 μm; Agilent Technologies, Wilmington, DE) protected by a 4.0 × 3.0-mm i.d. Security-Guard (5 μm) C₁₈ column (Phenomenex, Torrance, CA). The mobile phase was a gradient of a mixture of methanol (A) and 5 mM ammonium acetate (B) programmed as follows: initial 20% A maintained for 5 min, then increased to 30% in 0.5 min, maintained at 30% for 10 min, then increased to 40% in 0.5 min, maintained at 40% for 5 min, then increased to 60% in 0.5 min, maintained at 60% for 5 min and finally decreased to 20% A in 0.5 min, maintained at 20% A for 10 min. The flow rate was 0.6 ml/min and the injection volume was 20 μl. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 5 min after sample injection. Major operating parameters for the ion-trap electrospray ionization-MS method are as follows: nebulizer pressure, 40 psi (N₂); dry gas, 12 ml/min (N₂); dry gas temperature, 350°C; spray capillary voltage, 3500 V; skimmer voltage, 40 V; ion transfer capillary exit, 124 V; scan range, m/z 150 to 1300; spectra average, 3; and target, 200,000. For MS* spectra, the fragmentation amplitude varied between 0.4 and 0.9 V. Other parameters, including the potentials of octopole offset and the tube lens offset, were also optimized for maximal abundance of the ions of interest by the automatic tune procedure of the instrument. The MS* product-ion spectra were produced by collision induced dissociation of the molecular ion [M + H]⁺ of all analytes at their respective HPLC retention times. Data acquisition was performed in full-scan LC/MS and MS* modes (n = 2 or 3). All data acquired were processed by ChemStation Revision B. 01.03 software (Agilent Technologies, Santa Clara, CA).
**Tissue Distribution Study.** Mice were anesthetized, and liver, lung, and kidney were harvested. In total, 200 mg of individual organ tissues were homogenized in 1.0 ml of methanol, followed by centrifuge at 3000 rpm for 5 min. The resulting supernatants (200 µl) were mixed with 20 µl of tetrandrine solution (1.0 mg/ml) as internal standard. The resultant mixtures were deproteinized with 200 µl of methanol, and the precipitate removed by centrifugation at 3000 rpm for 5 min. The supernatants were dried as described above, reconstituted in 200 µl of methanol with 5 mM ammonium acetate (30:70, v/v), and subjected to LC/MS analysis.

**Data Analysis.** All results are given as means ± S.E. The results were analyzed by Student’s *t* test (GraphPad Software Inc., San Diego, CA). A value of *p* < 0.05 was considered significant, and *p* < 0.01 was highly significant compared with the corresponding control.

**Results**

**LDH Activity in BAL Fluids.** To evaluate the pulmonary toxicity by dauricine, LDH activity in BAL fluids was monitored at 24 h after administration of dauricine. As shown in Fig. 1A, intraperitoneal administration of dauricine produced a dose-dependent increased LDH activity in BAL fluids and an approximately 3-fold elevation of LDH activity in BAL fluids in animals at 150 mg/kg.

**Permeability and Histology.** To image the pulmonary injury by dauricine at a tissue/cell level, the lungs were stained with EthD-III and DAPI. Remarkable ethidium-positive (shown in red in Fig. 1B) cells were observed in the lungs obtained from the animals treated with dauricine at the dose of 150 mg/kg, whereas lungs of the control animals showed no detectable ethidium-labeled cells. The ethidium labeling of lung cells exhibited dose dependence of lung injury after intraperitoneal exposure to dauricine (Fig. 1B). To examine lung toxicity induced by dauricine, the histopathological changes of the lungs of mice were also monitored. Alveolar edema along with hemorrhage was observed in the animals given dauricine at dose of 150 mg/kg (Fig. 1C).

**Distal Airway Incubation.** To examine the toxic effect of dauricine on distal airway tissues, distal airways were dissected, harvested, and incubated with dauricine, followed by monitoring LDH released to the media. An approximately 2-fold increase in LDH activity was observed in the media where the microdissected airway tissues were
exposed to dauricine at a concentration of 100 μM for 24 h (Fig. 2). Unlike dauricine, 4-ipomeanol at the same molar concentration only produced a mild increase in LDH activity in the media relative to the control group (Fig. 2).

**Cell Culture.** Human lung WI-38 cells were exposed to dauricine at concentrations of 5, 10, 20, and 40 μM, and cell viability was examined 24 h after the exposure. Approximately 40% cell death was observed after exposure to dauricine at 20 μM, and exposure to 40 μM dauricine caused more than 60% cell death (Fig. 3A). The same studies were performed in BEAS-2B and A549 cell lines, and similar observed toxicity was obtained (Supplemental Fig. 1).

**Protective Effect of Ketoconazole.** Dauricine was administered in mice pretreated with ketoconazole or vehicle, followed by examining LDH activity in BAL fluids 24 h after administration. As expected, the exposure of dauricine produced elevated LDH activity in BAL fluids (Fig. 4). However, the pretreatment with ketoconazole significantly reduced LDH activity in BAL fluids induced by dauricine (Fig. 4). Histopathological evaluation study also revealed the similar protective effect of ketoconazole on dauricine-induced pulmonary toxicity. As shown in Fig. 1C, the pretreatment of ketoconazole substantially reduced alveolar edema and hemorrhage induced by dauricine.

**Bioactivation of Dauricine in Mouse Lung Microsomes.** In our earlier metabolism studies, quinone methide metabolite 2 (Scheme 1) was trapped by glutathione in human liver microsomal incubations. Glutathione conjugate 3 (Scheme 1) was characterized by mass spectrometry and NMR spectroscopy (Wang et al., 2009). A similar metabolism study was performed to ensure that the same quinone methide metabolite is formed from dauricine in the incubations with mouse lung microsomes. Dauricine was incubated with lung microsomes in the presence or absence of NADPH, and the resulting electrophilic metabolites were trapped with glutathione, followed by LC/MS.

### Table 1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Rates of Product Formation (pmol/20 min/10⁶ cells)</th>
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</thead>
<tbody>
<tr>
<td>BEAS-2B cells</td>
<td>0.345 ± 0.0017</td>
</tr>
<tr>
<td>WI-38 cells</td>
<td>0.128 ± 0.0004</td>
</tr>
<tr>
<td>A549 cells</td>
<td>0.069 ± 0.0002</td>
</tr>
<tr>
<td>Mouse lung primary cells</td>
<td>0.197 ± 0.001</td>
</tr>
</tbody>
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**Fig. 2.** Toxic effects of dauricine on the lung airways. Changes in LDH released to media in cultured microdissected lung airways after exposure to 4-ipomeanol (IPO, 100 μM) and dauricine (DAU, 100 μM). Data represent three independent experiments (means ± S.E.M.). *, P < 0.05 versus IPO.

**Fig. 3.** Cytotoxicity of dauricine in human lung cell line WI-38. Concentration- (A) and time-dependent (B) cytotoxicity of dauricine in WI-38. Cells were incubated with dauricine at the indicated concentrations for 24 h. Data represent the values of triplicates (means ± S.E.M.). *, P < 0.05 versus control; ***, P < 0.01.

**Fig. 4.** Protective effect of ketoconazole. Changes in LDH activity in BAL fluids. Mice were given with vehicle (vehicle) and dauricine (DAU). Mice pretreated intraperitoneally with ketoconazole (KTC) at 50 mg/kg for 1.5 h were administered with dauricine (KTC + DAU). Data represent the values of six replicates (means ± S.E.M.). ***, P < 0.01 versus control; ##, P < 0.01 versus DAU.)
analysis. As expected, a peak monitored at \( m/z \) 930 responsible for GSH conjugate 3 was observed in samples from the incubations in the presence of NADPH (Fig. 5B), whereas no such peak was detected in the samples from the NADPH-absent incubations (Fig. 5A). The retention time (10.4 min) of the peak was the same as that of the synthetic GSH conjugate 3 (Fig. 5D). The effect of ketoconazole on the bioactivation of dauricine was examined, and the presence of ketoconazole (10 \( \mu M \)) in lung microsomal incubations caused substantial suppression (42.7% based on peak areas) of quinone methide metabolite (2) formation as shown in Fig. 5C. Figure 6 shows MS/MS spectra of authentic GSH conjugate 3 and the conjugate detected in the microsomal incubations. As expected, the multiple-stage mass spectra of GSH conjugate 3 detected in the microsomal incubations were found to be identical with authentic GSH conjugate 3 that we chemically synthesized (Fig. 6).

**Bioactivation of Dauricine in Human Recombinant P450 Enzymes.** We tested the activity of human recombinant P450s 1A1, 2B6, and 3A5 in biotransformation of dauricine to quinone methide 2, and CYP3A5 was the only P450 found to catalyze the formation of the quinone methide trapped by GSH. The apparent \( K_m \) and \( V_{max} \) values for the formation of dauricine-derived quinone methide in recombinant CYP3A5 incubations were determined, and they were 17.0 \( \mu M \) and 8.03 pmol/min/nmol CYP3A5, respectively. Table 2 lists the values of parameters \( K_m \), \( V_{max} \), and \( V_{max}/K_m \) obtained from the enzyme kinetic studies. The corresponding biochemical parameters for CYP3A4 are adopted from our previous work (Wang et al., 2009).

**Tissue Distribution of Dauricine.** We monitored the distribution of the parent alkaloid in the liver, lung, and kidney in mice given dauricine. Dauricine was found in all organ tissues examined, but no huge difference in its distribution abundance was observed among the liver, lung, and kidney. The alkaloid reached the liver faster than the lung and kidney, whereas it stayed longer in the lung and kidney than in the liver (Fig. 7).

**Discussion**

This current study reports that acute exposure of bisbenzylisoquinoline dauricine produced pulmonary toxicity in CD-1 mice. In previous studies, we found that dauricine was biotransformed to quinone methide metabolite 2 in human liver microsomal incubations (Wang et al., 2009). The quinone methide metabolite was trapped by glutathione and characterized by mass spectrometry and NMR. Quinone methides are known electrophilic species, and it has been speculated that quinone methides have great potential to induce a variety of toxicities. The present toxicity study was started by our previous finding of quinone methide metabolite 2 along with the knowledge about toxic significance of quinone methides. As an initial step,
we examined toxic effects of dauricine on liver, kidney, and lung in CD-1 mice. Hepatotoxicity was assessed by monitoring serum AST and ALT activity, nephrotoxicity was monitored by determining serum BUN, and pneumotoxicity was evaluated by examining LDH activity in BAL fluids. No increase in serum AST/ALT activity and BUN level was observed 24 h after the treatment at all doses (data not shown). However, the administration of dauricine was found to produce an elevation of LDH activity in BAL fluids, and the elevated LDH activity in BAL fluids was dose-dependent (Fig. 1A).

We further examined the histopathological changes of lung tissues obtained from mice treated with dauricine. As expected, significant pulmonary lesions were observed, including intra-alveolar edema and hemorrhage (Fig. 1C). Furthermore, we imaged the respiratory injury at a tissue/cell level by use of a staining technique that is based on the assessment of cell membrane integrity by ethidium homodimer-III permeability and fluorescence microscopy. The ethidium homodimer-based cell permeability evaluation provided us a semiquantitative assay to assess pulmonary cell lesions. The dye successfully stained the lung cells that lost integrity of cell membrane in animals given dauricine. The injured cells permeable to ethidium homodimer was proportional to the doses of dauricine administered in animals (Fig. 1B). This provides quantitative histopathological evidence for the toxic effect of dauricine on the pulmonary system.

4-Ipomeanol is a prototype for potent selective pulmonary toxin. Boyd and co-workers reported that 4-ipomeanol caused morphological damage in lungs at dose of 30 mg (0.20 mmol/kg; Michael, 1977), and we found that dauricine produced detectable lung injury at 0.16 mmol (100 mg/kg). We also compared the pulmonary toxicity induced by 4-ipomeanol and dauricine by incubation of microdissected mouse airways with the individual toxins. At concentration of 100 μM, 4-ipomeanol caused mild elevation of LDH activity in the media, whereas at the same concentration, dauricine caused 2-fold increases relative to the vehicle-treated group. This indicates that dauricine is more toxic than 4-ipomeanol, a model agent of pulmonary toxin.

In previous study, we identified quinone methide metabolite 2 in human liver microsomal incubations with dauricine (Wang et al., 2009). We speculated possible involvement of the quinone methide metabolite of dauricine in the lung injury induced by dauricine. Similar study was performed to seek quinone methide metabolites of dauricine using mouse lung microsomes. As expected, quinone methide 2 was trapped by coincubated glutathione, and the resulting GSH conjugate 3 (Scheme 1) was detected by LC/MS (Figs. 5 and 6), implicating that dauricine can be bioactivated not only in the liver but also in lung tissues. The observed requirement of NADPH for the formation of the quinone methide metabolite provided the evidence for the metabolism dependence of biochemical activation of dauricine.

In previous studies, we tested the catalytic role of human liver P450 enzymes, including CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, in the formation of quinone methide metabolites of dauricine, and we found only CYP3A4 is the enzyme responsible for the metabolic activation of dauricine (Wang et al., 2009). The composition of human lung P450 enzymes is a little different from that in human liver, and the major P450 enzymes in human lung include CYPs1A1, 2A6, 2B6, 2E1, and 3A4/3A5 (Zhang et al., 2006). We examined the catalytic activity of human recombinant CYPs1A1, 2B6, and 3A5, in addition to CYPs2A6, 2E1, and 3A4 that we had tested previously (Wang et al., 2009), to transform dauricine to quinone methide metabolite 2. Among the three P450 enzymes tested, only CYP3A5 showed catalysis for the formation of the quinone methide. As expected, quinone methide 2, existing as GSH conjugate 3, was detected by LC/MS.

The CYP3A subfamily comprises the major P450 proteins in human lung (Zhang et al., 2006). Anttila et al. (1997)
reported that CYP3A5 is the dominant CYP3A protein in human pulmonary system relative to CYP3A4. In our previous and current studies, we examined the catalytic activity of a total of 10 human recombinant P450 enzymes, including CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, and CYP3A4 to better understand the susceptibility of lung tissues to dauricine toxicity. In summary, our present study showed that dauricine selectively causes acute pulmonary toxicity in mice. The alkaloid was also found to induce cytotoxicity in representative human lung cell lines. Pretreatment with ketoconazole protected mice from dauricine-induced pulmonary injury. Dauricine was metabolized to the corresponding quinone methide metabolite that reacts with GSH. The CYP3A subfamily is responsible for the formation of the electrophilic quinone methide metabolite. However, whether pulmonary toxicity induced by dauricine correlates with the quinone methide metabolites in dopamine needs to be further investigated.

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**References**


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