In Vitro Metabolism of Isoline, a Pyrrolizidine Alkaloid from *Ligularia duciformis*, by Rodent Liver Microsomal Esterase and Enhanced Hepatotoxicity by Esterase Inhibitors

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**ABSTRACT:**

Isoline, a major retronecine-type pyrrolizidine alkaloid (PA) from the Chinese medicinal herb *Ligularia duciformis*, was suggested to be the most toxic known PA. Its in vitro metabolism was thus examined in rat and mouse liver microsomes, and its toxicity was compared with that of clivirone and monocrotaline after i.p. injection in mice. Isoline was more rapidly metabolized by both microsomes than clivirone and monocrotaline and converted to two polar metabolites M1 and M2, which were spectroscopically determined to be bisline (a deacetylated metabolite of isoline) and bisline lactone, respectively. Both metabolites were formed in the presence or absence of an NADPH-generating system with liver microsomes but not cytosol. Their formation was completely inhibited by the esterase inhibitors, triorthocresyl phosphate (TOCP) and phenylmethylsulfonyl fluoride, but not at all or partially inhibited by the P450 inhibitors, *H*9251-naphthoflavone and proadifen (SKF 525A), respectively. These results demonstrated that both metabolites were produced by microsomal esterase(s) but not P450 isozymes. The esterase(s) involved showed not only quite different activities but also responses to different inhibitors in rat and mouse liver microsomes, suggesting that different key isozyme(s) or combinations might be responsible for the deacetylation of isoline. Isoline injected i.p. into mice induced liver-specific toxicity that was much greater than that with either clivirone or monocrotaline, as judged by histopathology as well as serum alanine aminotransferase and aspartate aminotransferase levels. Isoline-induced hepatotoxicity was remarkably enhanced by the esterase inhibitor TOCP but was reduced by the P450 inhibitor SKF 525A, indicating that rodent hepatic esterase(s) played a principal role in the detoxification of isoline via rapid deacetylation in vivo.

Pyrrolizidine alkaloids (PAs) are a broad group of naturally occurring phytotoxins that are found in many flowering plants, such as *Senecio, Ligularia, Eupatorium* (Asteraceae), *Crotalaria* (Fabaceae), *Heliotropium*, and *Cynoglossum* (Boraginaceae) species, as well as in some insects feeding on these plants (Roeder, 2000). Many PAs are hepatotoxic (Mattocks, 1968), pneumotoxic (Huxtable, 1990), and carcinogenic (Hayes et al., 1984; Yang et al., 2001). Thus, PAs are implicated in livestock losses and can cause public health problems when ingested from foodstuffs such as traditional medicines, herbal remedies, grains, milk, and honey (Huxtable, 1989; Edgar et al., 2002).

Toxic PAs are generally retronecine and otonecine esterified PAs. The metabolism and toxicity of various PAs have been studied in vitro and in vivo (Hayes et al., 1984; Buhler et al., 1990; Lin et al., 2000; Fu et al., 2004). The hepatotoxicity of PAs largely depends on their metabolic activation by hepatic P450s to become chemically reactive pyrroles (dehydro-PAs) (Mattocks, 1968; Mattocks and Bird, 1983; Glowaz et al., 1992; Lin et al., 2000) that form covalent adducts with cellular nucleophiles including DNA (Yang et al., 2001; Xia et al., 2004). On the other hand, alternative metabolic routes, including ester hydrolysis to the subgroup retronecine by guinea pig liver carboxylesterase GPH1 (Dueker et al., 1992a, 1995; Chung and Buhler, 1995) and N-oxidation by monooxygenase-containing flavine or P450s (Williams et al., 1989; Miranda et al., 1991; Huan et al., 1998), appear to constitute a detoxification mechanism. More recently, clivirone, a representative otonecine-type PA, was found to be hydrolyzed by microsomal esterase to generate a female Sprague-Dawley rat-specific metabolite deacetylclivorine (Lin et al., 2007). Nevertheless, unlike metabolic activation by P450s, the ester hydrolysis of PAs by hepatic esterase and its physiological role in hepatotoxicity are still largely unknown.

Isoline is a major retronecine-type pyrrolizidine alkaloid found in a Chinese medicinal herb [*Ligularia duciformis* (C. Winkl.) Hand-...

**ABBREVIATIONS:** PA, pyrrolizidine alkaloid; P450, cytochrome P-450 enzyme; NMR, nuclear magnetic resonance; MS, mass spectrometry; HPLC, high-performance liquid chromatography; TOCP, triorthocresyl phosphate; PMSF, phenylmethylsulfonyl fluoride; ANF, α-naphthoflavone; GSH, glutathione; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PB, phenobarbital sodium; TLC, thin layer chromatography.
Mazz.) that has been used as an expectorant and antitussive folk remedy (Zhao et al., 1998; Tang et al., 2004). However, little is known about its metabolism and toxicity. Even though isoline might be the most toxic known PA, its lethality was compared only with that of retrorsine, which was highly hepatotoxic (Sapiro, 1953; Mattocks, 1981; Mattocks and Bird, 1983). It is also interesting to note that isoline may not exert direct hepatotoxicity at a concentration of 0.1 mM by in vitro examination on hepatocytes (Ji et al., 2004). Because the toxicity of PAs differed significantly with respect to structural features (Mattocks, 1981), the toxicity of isoline should be compared with that of other structurally similar PAs, such as clivorine and monocrotaline, which become hepatotoxic after oxidation by microsomal P450s (Lame et al., 1990; Lin et al., 2000). The metabolism and liver and lung toxicities of monocrotaline as a retrocrotine-type PA have also been studied extensively (Hustable, 1990; Glowaz et al., 1992; Fu et al., 2004). Moreover, animal resistance or susceptibility to PAs seems to be well known as a consequence of diversity in hepatic PA metabolism (Huan et al., 1998). Understanding the metabolic fate and balance of PAs in various pathways could be clinically important for prediction and prevention of emergent PA-induced threats or potent treatment of already present intoxication. Thus, we studied the in vitro metabolism of isoline by both rat and mouse liver microsomes compared with that of clivorine and monocrotaline. Isoline was most rapidly metabolized by rodent liver microsomes and was the most hepatotoxic of these three PAs in mice. Microsomal carboxylesterase(s) was the key enzyme(s) that contributed to the rapid metabolism and, consequently, the detoxification of isoline in vivo.

### Materials and Methods

**Chemicals.** Isoline and clivorine were isolated from *L. duciformis* (C. Winkl.) Hand-Mazz. and *L. hodgsonii* Hook, respectively, in our laboratory. Monocrotaline, which was originally isolated from *Crotalaria assamica* Benth. (Tang et al., 2003b) was kindly supplied by Professor Zhiben Tu (Wuhan Botanical Institute, Academia Sinica, Wuhan, China). Their structures were confirmed using UV, IR, NMR, and MS analysis, and HPLC analysis showed that the purity of all three was >98%. Acetonitrile and methanol of HPLC grade were purchased from Wako Pure Chemicals (Osaka, Japan). Preparative TLC was performed on silica gel 60 F254 plates (0.5 mm, Merck).

**Isolation of Metabolites of Isoline Using Rat Liver Microsomes.** Isoline and clivorine were isolated from *L. duciformis* (C. Winkl.) Hand-Mazz. and *L. hodgsonii* Hook, respectively, in our laboratory. Monocrotaline, which was originally isolated from *Crotalaria assamica* Benth. (Tang et al., 2003b) was kindly supplied by Professor Zhiben Tu (Wuhan Botanical Institute, Academia Sinica, Wuhan, China). Their structures were confirmed using UV, IR, NMR, and MS analysis, and HPLC analysis showed that the purity of all three was >98%. Acetonitrile and methanol of HPLC grade were purchased from Wako Pure Chemicals (Osaka, Japan). Preparative TLC was performed on silica gel 60 F254 plates (0.5 mm, Merck).

**Animals and Preparation of Liver Microsomes.** Male Sprague-Dawley rats weighing 240 to 280 g and male ddY mice weighing 30 to 37 g were purchased from Nippon SLC (Hamamatsu, Japan). Isolines were induced by injecting the rats i.p. with PB (80 mg/kg in saline) daily for 3 days (Hayes et al., 1984). Animals starved during the last 12 h were killed under pento-barbital anesthesia (50 mg/ml/kg), and then the livers were rapidly removed and weighed. Blood was removed by perfusion with cold 0.25 M sucrose, and the liver was homogenized in 4 volumes of ice-cold 0.25 M sucrose in 55-mL Wheaton tubes with a pestle. The homogenate was centrifuged at 11,472g for 30 min, and the supernatant was separated again by centrifugation at 105,000 rpm for 30 min. After washing with 0.25 M sucrose, the pellets (microsomal fraction) were suspended in ice-cold 0.1 M potassium phosphate buffer (1:2 volume/liver weight, pH 7.4) containing 20% glyc erol and 0.1 mM EDTA disodium and then were stored in portions at −80°C. The supernatants (cytosol fraction) were also stored at −80°C. Protein content was determined using the modified method of Lowry et al. (1951).

**Incubation of Pyrrolizidine Alkaloids with Microsomes and Preparation for TLC/HPLC.** Incubation mixtures (0.2 ml) consisted of a NADPH-generating system, liver microsomes (0.48 mg of protein), and 1 mM isoline in potassium phosphate buffer (0.1 M, pH 7.4) in the presence or absence of 2.0 mM GSH. The NADPH-generating system contained 5 mM MgCl2, 1 mM β-NADH, 1 mM β-NADP+, 10 mM glucose-6-phosphate, and 1.0 unit/ml glucose-6-phosphate dehydrogenase. Although the optimal pH value of the incubation system was 8.0 in the present study, the P450-mediated metabolism of PAs was usually examined at pH 7.4 (Kedzierski and Buhler, 1986). Other conditions including substrate concentration were the same as in previous reports (Kedzierski and Buhler, 1986; Williams et al., 1989; Dueker et al., 1992a; Lin et al., 2000). The reaction was initiated by adding substrate (isoline or other PA), performed at 37°C, and terminated by adding an equal volume of ice-cold methanol. Control incubations were performed without the NADPH-generating system and with or without rodent liver microsomes or substrate. The residue obtained by centrifugation at 10,000g for 30 min was immediately filtered through 0.45-µm microdiscs (Ekicrodisc 3, 3 mm; Gelman Sciences, Inc., Ann Arbor, MI), and the filtrates were analyzed by TLC and HPLC.

**Incubation with Esterase and P450 Inhibitors.** The mixtures described above were incubated at 37°C for 60 min with one of the following inhibitors: TOCP (0.01, 0.1, 1, and 2.5 mM) and PMSF (0.1, 0.5, and 1 mM) for esterase(s) or SKF 525A (0.1 and 0.5 mM) and ANF (0.1 and 0.5 mM) for P450s, each with differential selectivity. The inhibitor concentrations were defined according to the preliminary TLC studies or published data to achieve significant inhibition effects by TOCP and PMSF (Dueker et al., 1992a; Lin et al., 2007) and SKF 525A and ANF (Hayes et al., 1984; Williams et al., 1989). Because these inhibitors were dissolved in dimethyl sulfoxide (<0.5% in the incubation mixture), the control incubation also contained this solvent. Reaction inhibition is expressed as percent decrease in formed metabolites on a molar basis.

**Isolation of Metabolites of Isoline Using Rat Liver Microsomes.** The procedure described above was scaled up (400 ml) with the slight modification of a 90-min incubation. The mixture was immediately extracted with 3 volumes of methanol and stored at 4°C. The extract was centrifuged at 10,000g at...
2°C for 15 min. The supernatant was concentrated in vacuo and eluted through a Diaion HP-20 column with water, 30% methanol, 70% methanol, and 100% methanol. Each eluate was totally evaporated in vacuo to yield a residue. The 70% methanol fraction was eluted through a silica gel column with chloroform:methanol:25% ammonia (19:1.0.1 to 8:2:0.1). Fractions 2 to 4 were collected, concentrated, and purified by preparative TLC to yield a white crystalline compound, M1 (approximately 40 mg). Similarly, fractions 12 to 20 yielded an oily compound, M2 (approximately 20 mg). M1 and M2 were determined to be bisline and bisline lactone by use of UV, IR, MS, circular dichroism, and extensive use of NMR techniques, respectively, as described in our previous studies (Tang et al., 2003a, 2004).

Preparation of Authentic Samples. Retronecine was obtained from monocrotaline and isoline by both alkaline and acidic hydrolysis. Bislene was isolated from *L. duciformis* (C. Winkl.) Hand-Mazz. and identified previously in our laboratory. Dehydroretronecine and dehydroisoline were synthesized from retronecine and isoline, respectively. All detailed procedures and structure elucidations were described in our previous reports (Tang et al., 2003a,b,c).

Quantification of Metabolites. An HPLC assay method to qualitatively and quantitatively analyze M1, M2, and isoline in all incubates was developed as described previously (Tang et al., 2004).

In Vivo Animal Treatment and Dosing. Male ddY mice (6 weeks old, 30–37 g) were bred at the Experimental Animals Service Center of Toyama Medical and Pharmaceutical University on a 12-h light/dark cycle in a room with controlled temperature and humidity for 1 week before the experiments. Isoline, clivorine, and monocrotaline were dissolved in 0.9% saline containing a suitable volume of 0.2 M hydrochloric acid and neutralized with 0.2 M sodium hydroxide to give respective solutions of 12.7 (low), 25.3 (medium), and 50.6 mM (high) compounds. Each of these was administered once daily as an i.p. injection at a dose of 10 μg/g b.wt. for 1 or 3 days. Some mice were given undiluted T0CP (1 ml/kg b.wt.) via a stomach tube 4 h before administration of PAs (Mattocks, 1981; Mattocks et al., 1986; Chu et al., 1993). Others received SKF 525A at a dose of 50 mg/kg b.wt. by i.p. injection 15 min before PA administration as described by Ohhira et al. (2000). Controls were injected with saline alone. Thereafter, blood was collected via the abdominal vein under pentobarbital anesthesia, and the liver and other organs were immediately removed. Blood samples were stored at room temperature for >1 h to obtain serum for ALT and AST measurements. All animal experiments were conducted in accordance with the Guidelines of the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University approved by the Japanese Association of Laboratory Animal Care.

Histopathology. The liver and other organs were fixed in buffered formalin (pH 7.4) for >24 h, embedded in paraffin, sliced into 4-μm sections, and stained with hematoxylin and eosin for light microscopic evaluation. Statistical Analysis. Serum ALT and AST activity data were analyzed using one-way analysis of variance followed by Dunnett's test. Other variables were examined using Student's *t* test. Groups were considered significantly different when *p* was < 0.05.

Results

Isolation and Characterization of Two Major Metabolites of Isoline Generated by Rat Liver Microsomes. Two major metabolites (M1 and M2) were generated by incubating isoline with rat liver microsomes in the presence of an NADPH-generating system. The amounts of the two metabolites produced almost equaled the amount by which isoline decreased on a molar basis. M1 was identified as (2S,3R,5R)-15-ethyl-12,15-dihydroxy-12,13-dimethylecene-1-ename (bisline) (Fig. 1) by comparison with an authentic sample (see Materials and Methods) and with the reported composition of bisline (Susag et al., 2000). M2 was determined as the 9-O-(δ-lactone) form of [(12S,13R,15R)-15-ethyl-15-hydroxy-12,13-dimethyl-17-oxotetrahydropyran-12,15-dicarboxylic acid] retronecine (bisline lactone) (Fig. 1) (Tang et al., 2003a). M1 is a deacetylated metabolite of isoline, and M2 is formed by the 12-membered ring-fission of M1 and subsequent ring-closure to generate a 6-membered lactone ring. By HPLC, isoline and its two metabolites could be analyzed with retention times of approximately 43.4, 27.2, and 29.7 min, respectively (Tang et al., 2004).

Metabolism of Isoline, Clivorine, and Monocrotaline by Rodent Liver Microsomes. The metabolism of isoline, clivorine, and monocrotaline (Fig. 1) was investigated using both rat and mouse liver microsomes in the presence of an NADPH-generating system. Both types of microsomes metabolized isoline much more rapidly than either clivorine or monocrotaline, and mouse liver microsomes metabolized isoline more rapidly than those from rats (Table 1). For mouse liver microsomes, higher metabolic activation with clivorine and monocrotaline could be observed. It was noteworthy that two major metabolites (M1 and M2) of isoline generated by mouse liver microsomes were confirmed to be same as those generated by rat liver microsomes by using TLC (with at least three developing systems).
Comparison of isoline, clivorine, and monocrotaline metabolism by rat and mouse liver microsomes

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Rat</th>
<th>Mouse</th>
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<tbody>
<tr>
<td></td>
<td>Isoline</td>
<td>Clivorine</td>
</tr>
<tr>
<td>Noninduced microsomes</td>
<td>0.272 ± 0.019</td>
<td>0.028 ± 0.026</td>
</tr>
<tr>
<td>PB-induced microsomes</td>
<td>0.408 ± 0.015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.145 ± 0.036&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytosolic fraction</td>
<td>—</td>
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</tbody>
</table>

—, no decrease.
<sup>a</sup> p < 0.01, by comparing mouse liver microsomes with non-induced rat ones.
<sup>b</sup> p < 0.001.
<sup>c</sup> p < 0.05, by comparing mouse liver microsomes with induced rat ones.

Enzyme Inhibition Studies. The metabolism of isoline to M1 and M2 by rat liver microsomes was dependent on the presence of an NADPH-generating system. The reaction mixture was sampled at the indicated times, and the formation of M1 and M2 was quantified.

**Fig. 2.** Time course of isoline metabolism by rat liver microsomes. Isoline (●, 1 mM) was incubated with hepatic microsomes in the presence of an NADPH-generating system. The reaction mixture was sampled at the indicated times, and isoline and metabolites (M1, ■, M2, ▲) as well as the sum of isoline and two metabolites (×) were quantified.

and HPLC analysis as well as concurrent chromatography with the authentic samples. On the other hand, rat or mouse liver cytosol did not metabolize any of the three PAs. Then the effects of PB on the microsomal metabolism of three compounds were examined by exposing rats to PB. Isoline metabolism was enhanced by approximately 1.5-fold, compared with that of clivorine (5-fold) but much lower than that of monocrotaline by a slight decrease of the parent alkaloid (Table 1).

**Nonenzymatic Transformation of M1 to M2.** When isoline (1 mM) was incubated with rat liver microsomes at 37°C in the presence of an NADPH-generating system, the concentration of isoline rapidly decreased and almost disappeared 120 min thereafter. Meanwhile, M1 and M2 were time-dependent inhibitors up to 60 min in almost a linear way, in which the concentration of M1 was approximately 3-fold greater than that of M2 (Fig. 2). Upon prolonged incubation for up to 180 min, M1 gradually decreased, but M2 continued to increase.

The stoichiometry of the sum of the starting material and two metabolites remained constant for 240 min, together with the time course profiles of the respective compounds, suggesting that isoline was initially converted to M1, which was subsequently changed to M2. The latter step was confirmed by incubating M1 in the presence or absence of microsomes at 37°C, in which M1 was converted to M2 at the same rates (data not shown). Moreover, the transformation of M1 to M2 was not inhibited at all by TOCP (0.1 mM), PMSF (0.5 mM), ANF (0.5 mM), or SKF 525A (0.5 mM). Isoline was also converted to M1 and M2 in the absence of the NADPH-generating system at the same rate. Similar evidence could be found for mouse liver microsomes except for slightly different contents of M1 and M2. Additionally, M2 was not transformed into any other metabolites by incubation with the liver microsomes.

**Transformation of Isoline to M1 by Microsomal Esterase and Nonenzymatic Transformation of M1 to M2.** When isoline (1 mM) was incubated with hepatic microsomes at 37°C in the presence of an NADPH-generating system, the concentration of isoline rapidly decreased and almost disappeared 120 min thereafter. Meanwhile, M1 and M2 were time-dependently increased up to 60 min in almost a linear way, in which the concentration of M1 was approximately 3-fold greater than that of M2 (Fig. 2). Upon prolonged incubation for up to 180 min, M1 gradually decreased, but M2 continued to increase.

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Monocrotaline had no effect on the liver even at a dose of 0.506 mmol/kg for 3 days. On the other hand, clivorine was lethal (Table 3), and body weight was decreased by 6.5 ± 1.1 g after the administration of 0.506 mmol/kg for 3 days, compared with increases of 0.2 to 0.5 g in the control and isoline-treated groups. However, light microscopy showed that a single high dose of clivorine did not significantly damage the liver.

**Dose-Dependent Hepatotoxicity of Isoline and Enhancement by TOCP.** The liver damage was quantified by measuring serum ALT and AST activities after administration of 0.127, 0.253, and 0.506 mmol/kg for 3 days, compared with increases of 0.2 to 0.5 g in the control and isoline-treated groups. However, light microscopy showed that a single high dose of clivorine did not significantly damage the liver.

When mice were given TOCP at a dose of 1 ml/kg 4 h beforehand, isoline was lethal at both high and medium doses and significantly increased serum ALT and AST activities even at a single low or medium dose (Table 3). Light microscopy confirmed the liver-specific damage in the TOCP-treated mice (Fig. 3). In addition, bisline (M1 of isoline) did not elicit any apparent toxic effects at a single high dose and after three medium doses. On the other hand, clivorine slightly affected serum activities after a single high dose and after three medium doses.

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**Discussion**

The toxicity of PAs is due to metabolic activation by an NADPH-dependent P450 enzymatic system, and detoxification is thought to include N-oxidation (Mattocks and Bird, 1983; Williams et al., 1989) and ester hydrolysis (Mattocks, 1970; Chung and Buhler, 1995). Understanding of the ester hydrolysis is primarily based on the formation of hydrolysis products such as retronecine, a feature of all retronecine-type PAs with various acid moieties, by purified carboxylesterase (GPH1) from guinea pig hepatic microsomes and the non-toxic and excretable property of these products (Dueker et al., 1992a, 1995).

The present study identified a distinct pathway catalyzed by rodent liver microsomal esterase(s) for PAs. Two major metabolites (M1 and M2) of isoline, a retronecine-type PA, were produced by both rat and mouse liver microsomes (either untreated or pretreated with PB) in the presence or absence of an NADPH-generating system but not in cytosolic incubations. Both structures (Fig. 1) showed hydrolysis of the acetyl group at the C-12 position of isoline. Interestingly, M2 was transformed from M1 nonenzymatically but without further hydrolysis at the C-9 position, which is generally believed to be especially susceptible to enzymatic hydrolysis (Fu et al., 2004). Further HPLC analyses demonstrated their hydrophilicity to be higher than that of isoline (Tang et al., 2004). Esterase inhibitors, such as TOCP and PMSF, significantly inhibited M1 and M2 formation, whereas the P450 inhibitors with differential selectivity, ANF and SKF 525A, were not inhibitory and partially inhibitory, respectively. All of these data indicated that microsomal esterase(s) mediated the formation of both polar metabolites.

Because isoline is structurally similar to clivorine in the necic acid moiety and to monocrotaline in the necic base moiety (Fig. 1), their metabolism and hepatotoxicity were compared. Rat or mouse liver microsomes metabolized the three PAs at significantly different ratios. Isoline was the most rapidly metabolized, showing the high metabolic activity of liver microsomal esterase(s), especially in mice or by PB-induced rat liver microsomes. This finding indicated not only that the same inducer of P450s also induced carboxylesterase isozymes (Satoh and Hosokawa, 1998) but also that esterase activities are probably higher in mouse liver microsomes. The rat (PB-induced) and mouse microsomal esterase(s) could also metabolize clivorine, which contains an acetyl group at the same position as the necic acid moiety, confirmed by TOCP inhibition (with 74 and 80% at concentrations of 0.01 and 0.1 mM in mice, respectively). This observation indicated that hepatic esterase(s) also played a role in clivorine metabolism by deacetylation. In contrast, monocrotaline, which contains the same necic residue (retronecine base) but no acetyl group, was unchanged under the same conditions. Although monocrotaline was a good substrate for guinea pig liver carboxylesterase GPH1 (Dueker et al.,

**TABLE 2**

Inhibitory effects of esterase and P-450 inhibitors on formation of M1 and M2 by rat and mouse liver microsomes

Data are expressed as average percent inhibition from triplicate experiments except inhibitor concentration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat Liver Microsomes</th>
<th>Mouse Liver Microsomes</th>
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<tbody>
<tr>
<td></td>
<td>M1</td>
<td>M2</td>
</tr>
<tr>
<td>TOCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 mM</td>
<td>85.90 ± 1.94***</td>
<td>79.98 ± 2.53***</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>100.0***</td>
<td>100.0***</td>
</tr>
<tr>
<td>1.0 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>0.1 mM</td>
<td>84.24 ± 2.04***</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>100.0***</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>42.04 ± 6.48**</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>77.41 ± 2.71***</td>
</tr>
<tr>
<td>ANF</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>0</td>
<td>0</td>
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<tr>
<td>0.5 mM</td>
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</table>

*The significant differences of formation of M1 and M2 were observed at *p < 0.05, **p < 0.01, and ***p < 0.001 compared with the control group in the absence of inhibitors.*
ALT and AST activities in mouse serum after i.p. administration of isoline, clivocrine, and monocrotaline and effect of TOCP or SKF 525A on hepatotoxicity

Data are expressed as means ± S.D. N = 5. Except as indicated, the regimen for PA was once daily via an i.p. injection at a dose of 10 μg/kg b.w. for 1 (single dose) or 3 doses (triple doses); TOCP and/or SKF 525A were given before any pure PA was administered.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT (IU/l)</th>
<th>AST (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>5.6 ± 1.5</td>
<td>22.8 ± 7.1</td>
</tr>
<tr>
<td>Isoline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.127 mmol/kg, triple doses</td>
<td>10.4 ± 3.2</td>
<td>26.8 ± 4.0</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>29.3 ± 11.4</td>
<td>34.3 ± 6.7</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose</td>
<td>126.8 ± 51.6</td>
<td>152.5 ± 79.4</td>
</tr>
<tr>
<td>0.506 mmol/kg, single doses</td>
<td>1003.3 ± 552.3</td>
<td>1065.0 ± 600.9</td>
</tr>
<tr>
<td>0.506 mmol/kg, double doses</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Clivocrine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.127 mmol/kg, triple doses</td>
<td>7.3 ± 0.8</td>
<td>24.4 ± 3.7</td>
</tr>
<tr>
<td>0.253 mmol/kg, triple doses</td>
<td>14.3 ± 6.8</td>
<td>30.0 ± 8.6</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose</td>
<td>9.4 ± 1.8</td>
<td>34.4 ± 3.8</td>
</tr>
<tr>
<td>0.506 mmol/kg, triple doses</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.253 mmol/kg, triple doses</td>
<td>5.4 ± 0.5</td>
<td>22.2 ± 3.5</td>
</tr>
<tr>
<td>0.506 mmol/kg, triple doses</td>
<td>6.4 ± 2.2</td>
<td>25.8 ± 10.2</td>
</tr>
<tr>
<td>M1 (bisline)</td>
<td>11.0 ± 5.6</td>
<td>30.0 ± 11.0</td>
</tr>
<tr>
<td>TOCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 ml/kg, single dose, p.o. + isoline</td>
<td>6.0 ± 0.7</td>
<td>20.6 ± 5.3</td>
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<tr>
<td>0.127 mmol/kg, single dose</td>
<td>132.5 ± 60.8</td>
<td>155.0 ± 54.5</td>
</tr>
<tr>
<td>0.253 mmol/kg, (after 18 h)</td>
<td>484.4 ± 283.7</td>
<td>977.8 ± 683.3</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose, D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose + clivocrine</td>
<td>15.8 ± 12.5</td>
<td>27.8 ± 11.2</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose</td>
<td>650.0 ± 378.0</td>
<td>1003.3 ± 518.7</td>
</tr>
<tr>
<td>+ monocrotaline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose</td>
<td>6.4 ± 0.9</td>
<td>16.6 ± 1.7</td>
</tr>
<tr>
<td>SKF 525A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50.0 mg/kg, single dose, p.o. + isoline</td>
<td>6.7 ± 2.1</td>
<td>23.7 ± 2.1</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>6.8 ± 2.0</td>
<td>26.2 ± 5.3</td>
</tr>
<tr>
<td>SKF 525A + TOCP</td>
<td>8.0 ± 7.9</td>
<td>24.6 ± 10.0</td>
</tr>
<tr>
<td>+ isoline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.127 mmol/kg, single dose</td>
<td>8.8 ± 3.8</td>
<td>27.2 ± 5.4</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>387.5 ± 130.5</td>
<td>470.0 ± 318.3</td>
</tr>
</tbody>
</table>

D, died.

*p < 0.01.

*p < 0.05 vs. control (saline alone).

*p < 0.05 vs. isoline (0.253 mmol/kg, single dose).

*p < 0.01.

*p < 0.05 vs. isoline (0.127 mmol/kg, three doses).

*p < 0.001 vs. control (saline alone).

*p < 0.001 vs. isoline (0.253 mmol/kg, single dose).

*p < 0.05 vs. clivocrine (0.506 mmol/kg, single dose).

*p < 0.05 vs. TOCP + isoline (0.127 mmol/kg, single dose).

*p < 0.01.

*p < 0.05 vs. SKF 525A + isoline (0.253 mmol/kg, single dose).

TABLE 3

1992a), the macrocyclic ester of the neic acid moiety was hydrolyzed at a different position. In the experiments described above, we did not detect retorinec, a common PA hydrolysis product generated by GPH1 (Dueker et al., 1992a,b, 1995). This finding implies that the esterase(s) present in our microsomal incubation system might be different isoforms specially targeted for the acetyl substituent.

Similarly, a recent study (Lin et al., 2007) showed that clivocrine could undergo deacetylation by PMSF-sensitive microsomal hydro-lase A, suggesting a possible detoxification pathway in female rats. In our study, M1 and M2 were deacetylated metabolites of isoline, with formation inhibited dose dependently in mice by either TOCP or PMSF. PMSF almost completely abolished the hydrolysis in mice at a concentration of 1 mM. Additionally mouse liver microsomes were more resistant to TOCP inhibition than PMSF, showing more PMSF sensitivity than rat liver microsomes (Table 2). This observation indicates that different key isoyme(s) or combinations might be responsible for the deacetylation of isoline. The extensive metabolism to clivocrine, another PA with similar acetyl structures (both S-config-uration at C-12 position) (Fig. 1), further indicates that esterase(s) may have broad and overlapping substrate specificity to esters (Satoh and Hosokawa, 1998). It is well established that the liver microsomal fraction contains the highest activity of esterase(s), but there are large species differences, especially among small laboratory animals. Although the corresponding isom in form (MH1) could be found with homology and characteristics similar to those of rat hydrolylase A (Satoh and Hosokawa, 1998; Lin et al., 2007), further research to verify its identity and functions is needed.

To better understand the toxicity of isoline among the known and possible detoxification feature of the esterase(s), the toxic effects of isoline and clivocrine and monocrotaline on mice were examined. Isoline was the most hepatotoxic of the three, judging from both histopathological findings and serum ALT and AST values (Table 3; Fig. 3). Isoline induced a marked elevation in the serum ALT level in mice and caused liver lesions at a single medium dose (0.253 mmol/kg). At a single high dose (0.506 mmol/kg) or a medium dose for 3 days, isoline produced even more significant severe damage (Fig. 3). On the basis of the serum ALT and AST activities, the toxicity was increased in a dose-dependent manner from low to high doses. On the other hand, the high doses of clivocrine and monocrotaline were not significantly hepatotoxic after 1 and 3 days, respectively. Moreover, at an equivalent molar dose (0.253 mmol/kg) for 3 days, clivocrine and monocrotaline showed much lower toxicity than isoline. Furthermore, M1 (bisline), a metabolite of isoline produced by microsomal esterase, administered at a single medium dose of 0.253 mmol/kg, was not significantly toxic to the mouse liver. Together with in vitro incubations and the altered chemical and probably toxicokinetic properties of two metabolites, we suggested that the high toxicity of parent PA (isoline) may not be derived from its secondary metabolism. However, further in vivo studies using multiple dosing of M1 or M2 are warranted.

TOCP is a well-known carboxylesterase inhibitor in vivo (Mattocks et al., 1986) and has very low toxicity. Prior exposure to TOCP significantly enhanced liver damage at all tested doses of isoline (Fig. 3; Table 3). TOCP followed by a single high dose of clivocrine also induced severe liver damage and sometimes death. Clivocrine also possesses an acetyl group and was not significantly toxic alone. On the contrary, monocrotaline does not possess an acetyl group, and this compound did not elicit any effect, even at a single high dose, regardless of prior TOCP exposure. These results demonstrated the different responses of mice to the three PAs and in particular much less susceptibility to clivocrine than in another report (Lin et al., 2007), corresponding with large species differences in vitro. These in vivo toxicity tests associated with in vitro metabolic findings indicated that the hydrolysis of the acetyl group by esterase was a detoxification process and suggested that an acetyl group attached to the macro ring of PAs constituted a vital part of the hepatotoxicity.

On the other hand, the real reason for isoline hepatotoxicity may depend on the bioactivation pathway largely mediated by P450 isozymes (Mattocks, 1968; Buhler et al., 1990). To confirm this point, the effects of P450 inhibition by SKF 525A on isoline-induced hepatotoxicity were studied. SKF 525A completely abolished the induction of hepatotoxicity by a single subsequent medium dose of isoline or a single low dose of isoline after pretreatment with TOCP and significantly repressed the severe hepatotoxicity induced by a single medium dose of isoline with prior dosing of TOCP (Table 3). Accordingly, the high in vivo toxicity of isoline might be related to the bioactivation pathway by P450s, which metabolized PAs into unstable
Mal esterase isozymes played a key role in the detoxification of isoline 525A, a P450 inhibitor. All of these data demonstrated that microsomal isoline-induced hepatotoxicity could be remarkably decreased by SKF with clivorine but not with monocrotaline. On the other hand, the isoline was enhanced by esterase inhibition by TOCP as in the case PA among the three and also was liver-specific. The hepatotoxicity of in vivo experimental results indicated that isoline was the most toxic what similar to those of clivorine, with the same acetyl structure. The than clivorine and monocrotaline but showed characteristics some-

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metabolic pathway involving P450s. Therefore, further investigation

P450s. Further studies will be conducted to identify the metabolic fate of isoline by P450s and microsomal esterases, particularly their intrinsic role in vivo. The underlying regulatory mechanisms could have potential clinical implications.

and highly reactive “pyrroles” These compounds efficiently reacted

with many nucleophiles such as GSH to spontaneously decompose or

polymerize in seconds (Mattocks et al., 1989; Tang et al., 2003b,c) or

attack macromolecules such as DNA (Kim et al., 1999; Yang et al.,

2001). However, monocrotaline that was probably metabolized to the

pyrrole (Glowaz et al., 1992) by P450s caused extrahaepatic diseases

with liver damage. Thus, the acute and severe hepatotoxicity induced

by isoline or other PAs containing an acetyl group in the necic acid

moiety might be due to their rapid metabolism predominately by the

metabolic pathway involving P450s. Therefore, further investigation

on P450 isozyme-mediated metabolic activation of isoline would be

our next subject.

In summary, the rodent hepatic microsomal esterases were respon-

sible for the biotransformation of isoline to two deacetylated metab-

olites (M1 and M2) in vitro, especially in mouse and PB-induced rat

microsomes. Isoline was also metabolized much more extensively than clivorylne and monocrotaline but showed characteristics somewhat similar to those of clivorylne, with the same acetyl structure. The in vivo experimental results indicated that isoline was the most toxic PA among the three and also was liver-specific. The hepatotoxicity of isoline was enhanced by esterase inhibition by TOCP as in the case with clivorylne but not with monocrotaline. On the other hand, the isoline-induced hepatotoxicity could be remarkably decreased by SKF 525A, a P450 inhibitor. All of these data demonstrated that microso-

mal esterase isozymes played a key role in the detoxification of isoline

associated with metabolic activation initiated by P450s. Further studies

will be conducted to identify the metabolic fate of isoline by P450s

and microsomal esterases, particularly their intrinsic role in vivo. The

underlying regulatory mechanisms could have potential clinical im-

References

Buhler DR, Miranda CL, Kedzierski B, and Reed RL (1990) Mechanisms for pyrrolizidine alkaloid activa-


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