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 clivorine and monocrotaline after i.p. injection in mice. Isoline was more rapidly metabolized by both microsomes than clivorine 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 inhibitor, triorthocresyl phosphate (TOCP) and phenylmethylsulfonyl fluoride, but not at all or partially by cytochrome P450 (P450) inhibitors, α-naphthoflavone and probenif (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 clivorine 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 mutagenic and mutagenic 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, clivorine, 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 P450, 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 retorosine, 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 retrocone-type PA have also been studied extensively (Huxtable, 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 $>98\%$. Acetointhiol and methanol of HPLC grade were purchased from Wako Pure Chemicals (Osaka, Japan). Proadifen (SKF 525A) was purchased from Sigma Chemicals. Triortho-crevyl phosphate (TOCP) and tetrachloro-o-benzoquinone (o-chloranil) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Phenylmethylsulfonyl fluoride (PMSF), $\beta$-NADPH, $\beta$-NADP$^-$, $\beta$-NADP$^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, the reduced form of glutathione (GSH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) test kits, 10% formalin neutral buffer solution (pH 7.4 for tissue fixation), and phenobarbital sodium (PB) were all purchased from Wako Pure Chemicals. Dimethyl sulfoxide and all other chemicals and solvents were of the purest commercially available grade. For the isolation of the two metabolites of isoline, M1 and M2, a scaled-up rat microsomal incubation with isoline as described below was used. Both isolated and synthetic metabolites were subjected to IR, UV, MS, NMR, and HPLC-UV analysis for the confirmation of their identity and purities ($>98\%$), respectively (Tang et al., 2003a,b,c).

**Instrumentation.** HPLC analysis was performed using a Tosoh HPLC system consisting of CCPM-2 dual pumps, a UV 8020 detector, an online degasser, and a Shimadzu C-R6A data processor under the same conditions as reported previously (Tang et al., 2004). TLC separation for preliminary study or for preparations purposes was performed on silica gel F$_254$ plates (0.25 mm; Merck, Darmstadt, Germany) using 1) chloroform-methanol-28% ammonia (8:2:0.1/2:8:0.1), 2) ethyl acetate-acetone-ethanol-28% ammonia (5:3:1:1), and 3) methanol-chloroform-water-triethylamine (8:0.85:1.1:0.05). Pyrrolyl metabolites were detected using Ehrlich reagent (Mattocks, 1968) sprayed directly. Parent PAs were detected using iodine or Dragendorff reagent. Pyrroles or parent PAs were also detected using iodine, acetic anhydride, and Ehrlich reagent in sequence in addition to o-chloranil in benzene, followed by Ehrlich reagent (for parent PAs). $\alpha$-Oxides of PAs were visualized by spraying with acetic anhydride, followed by Ehrlich reagent. $^1$H and $^{13}$C NMR spectra were measured using a Varian Unity Plus 500 (Varian Inc., Palo Alto, CA) spectrometer operating at 500 MHz for proton and 125 MHz for carbon in CDCl$_3$ or CD$_3$OD with trimethylsilylamine as an internal standard. Electron ionization-MS was measured using a JEOL JMS-GC-stat mass spectrometer at an ionization voltage of 70 eV (JEOL, Tokyo, Japan). High-resolution electron impact mass spectrometry and fast atom bombardment-MS were performed with a JEOL JMS-DX 300L spectrometer using glycerol as a matrix for the latter. Optical rotation was measured using a DIP-360 automatic polarimeter (Jasco, Tokyo, Japan). UV spectra were measured with a Shimadzu UV-2200 recording spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were recorded on a Jasco FT/IR-230 infrared spectrometer (Jasco). Circular dichroism spectra were measured with a Jasco J-805 spectropolarimeter (Jasco). Column chromatography was performed using silica gel BW-820MH, ODS (Fuji Siliya Co., Nagoya, Japan), and Diaion HP-20 (Mitsubishi Kasei Co., Tokyo, Japan). Preparative TLC was performed on silica gel 60 F$_{254}$ 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). Microsomes 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 pentobarbital 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 11,472 g for 30 min, and the supernatant was separated again by centrifugation at 105,000g for 90 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% glycerol and 0.1 mM EDTA disodium and then were stored in portions at $–80\ ^\circ$C. The supernatants (cytosol fraction) were also stored at $–80\ ^\circ$C. Protein content was determined using the modified method of Lowry et al. (1951).

**Incubation of Pyrroloidine Alkaloids with Microsomes and Preparation for TLC/HPLC.** Incubation mixtures (0.2 ml) consisted of an 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 MgCl$_2$, 1 mM $\beta$-NADH, 1 mM $\beta$-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; Duerker 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 0.25 M sucrose. 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 4°C for 30 min was immediately filtered through 0.45-μm microdiscs (Ekicridose 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 (Duerker 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 mixtures), the control incubation also contained this solvent. Reaction inhibition is expressed as percent decrease in formed metabolites on incubation with $100\%$ of the control reaction. Reaction inhibition is expressed as percent decrease in formed metabolites on incubation with $100\%$ of the control reaction.

**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 4°C for 15 min.
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-dimethylsenec-1-enine (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 9-0-[(8,10,12-lactone) form of [(12S,13R,15R)-15-ethyl-15-hydroxy-12,13-dimethyl-17-oxotetrahydropran-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).

**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-dimethylsenec-1-enine (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 9-0-[(8,10,12-lactone) form of [(12S,13R,15R)-15-ethyl-15-hydroxy-12,13-dimethyl-17-oxotetrahydropran-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)
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 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).

**Transformation of Isoline to M1 by Microsomal Esterase and 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 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. 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.

**Enzyme Inhibition Studies.** The metabolism of isoline to M1 and M2 by rat liver microsomes was dose dependent and was completely inhibited by esterase inhibitors such as TOCP and PMSF (Table 2). TOCP (a carboxylesterase inhibitor) inhibited the formation of M1 and M2 by 85.9% and 80.0%, respectively, at 0.01 mM and almost completely at 0.1 mM, whereas PMSF (selective to hydrolase A) inhibited their formation by 84.2% and 81.5%, respectively, at 0.1 mM and almost completely at 0.5 mM. Moreover, among the P450 inhibitors, SKF 525A (nonspecific P450 inhibitor) considerably inhibited the formation of the metabolites, in agreement with a previous report that SKF 525A partially inhibited rat microsomal esterase (Yeh, 1982). However, ANF (relatively selective to CYP2C8/9 and 1A2) had no effect.

Mouse liver microsomes similarly tended to show an inhibitory effect of isoline metabolism, except for the absence of inhibition by SKF 525A and ANF and decreased inhibition by TOCP and PMSF, compared with rat liver microsomes (Table 2). TOCP had a dose-dependent inhibitory effect on the metabolism of isoline from 0.01 to 2.5 mM, but this inhibitory effect was much less than that of PMSF. Significant differences in formation of M1 and M2 between the control group without inhibitors and those with TOCP and PMSF were also observed (at least at \( p < 0.05 \)). These findings showed that isoline was converted to M1 by rodent liver microsomal esterase(s).

**Isoline Toxicity and Histopathology.** The in vivo toxicity of isoline and other two PAs (clivorine and monocrotaline) to mice were examined. Isoline was lethal at a dose of 0.506 mmol/kg over 2 days (Table 3). Light microscopy showed liver damage consisting of hemorrhage and coagulative necrosis around the central vein at a medium dose of 0.253 mmol/kg isoline for 3 days. The coagulative necrosis was characterized by aggregative disappearance of cell membranes. Under the high-power lens, the cells in and around the area of necrosis showed enlarged nuclei and the changes in the cytoplasm as indicated by staining. The inflammatory cells were slightly increased. At a high single dose of 0.506 mmol/kg, the liver injury was more acute, extensive, and severe. Cage observation of the mice showed that they were very sick, listless, and inert. Livers were swollen and darkened after surgical removal. Hemorrhage and lesions consisting of areas of coagulative cell necrosis were greatly increased as shown on light microscopic examination (Fig. 3). Meanwhile, apoptotic bodies and enlarged nuclei were observed in hepatocytes outside the regions of necrosis. Even in those normal areas with fewer morphologic changes (approximately 50%), the cells with enlarged nuclei and the changed cytoplasm indicated by staining could still be observed. However, isoline did not damage other mouse tissues such as the heart, lung, and kidney (data not shown).

### TABLE 1

**Comparison of isoline, clivornine, and monocrotaline metabolism by rat and mouse liver microsomes**

Data are expressed as micromoles of decrease in amount of respective PA per hour per milligram of protein from triplicate tests.

<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;d&lt;/sup&gt;</td>
<td>0.145 ± 0.036&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytosolic fraction</td>
<td>—</td>
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</table>

<sup>a</sup> — no decrease.
<sup>b</sup> \( p < 0.01 \), by comparing mouse liver microsomes with non-induced rat ones.
<sup>c</sup> \( p < 0.001 \).
<sup>d</sup> \( p < 0.05 \), by comparing mouse liver microsomes with induced rat ones.
<sup>e</sup> \( p < 0.01 \).
<sup>f</sup> \( p < 0.05 \), by comparing induced liver microsomes with non-induced ones.
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 (low, medium, and high doses, respectively) of each of the three PAs. Table 3 shows that isoline remarkably increased serum ALT and AST activities (ALT 1003 ± 552 IU/l and AST 1065 ± 601 IU/l) after a single high dose. The increase induced by the low and medium doses of isoline for either 1 or 3 days was also dose dependently high. In contrast, monocrotaline did not increase serum activities of either ALT or AST even after a high dose for 3 days. On the other hand, clivorine slightly affected serum activities after a single high dose and after three medium doses.

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, isoline (M1 of isoline) did not elicit any apparent toxic effects at a single medium dose (Table 3). Light microscopy showed that a single high dose of clivorine did not significantly damage the liver.

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 enzymic 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 necine 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 necine 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.,

### 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).

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.01 mM</th>
<th>0.1 mM</th>
<th>1.0 mM</th>
<th>2.5 mM</th>
<th>PMSF</th>
<th>0.1 mM</th>
<th>0.5 mM</th>
<th>1.0 mM</th>
<th>SKF 525A</th>
<th>0.1 mM</th>
<th>0.5 mM</th>
</tr>
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<tbody>
<tr>
<td>TOCP</td>
<td>85.90 ± 1.94***</td>
<td>100.0***</td>
<td>100.0***</td>
<td></td>
<td></td>
<td>84.24 ± 2.04***</td>
<td>100.0***</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANF</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td>42.04 ± 6.48**</td>
<td>32.34 ± 5.84*</td>
<td>77.41 ± 2.71***</td>
<td>76.92 ± 2.60**</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Mouse Liver Microsomesa</td>
<td>18.26 ± 3.20**</td>
<td>35.30 ± 0.19***</td>
<td>53.29 ± 4.47***</td>
<td>62.49 ± 1.07***</td>
<td>77.09 ± 2.03***</td>
<td>92.36 ± 1.67***</td>
<td>94.92 ± 0.41***</td>
<td>9.44 ± 2.50</td>
<td>21.48 ± 4.04*</td>
<td>44.08 ± 5.84***</td>
<td>55.24 ± 1.36***</td>
</tr>
</tbody>
</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.
TABLE 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT activity (U/l)</th>
<th>AST activity (U/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>10.4 ± 3.2*</td>
<td>26.8 ± 4.0</td>
</tr>
<tr>
<td>0.127 mmol/kg, single dose</td>
<td>29.3 ± 11.4#</td>
<td>34.3 ± 6.7</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>126.8 ± 51.9##</td>
<td>135.2 ± 79.9###</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose</td>
<td>1003.3 ± 552.3###</td>
<td>1065.0 ± 660.8###</td>
</tr>
<tr>
<td>0.506 mmol/kg, double doses</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Clivorine</td>
<td>7.3 ± 0.8</td>
<td>24.4 ± 3.7</td>
</tr>
<tr>
<td>0.127 mmol/kg, single dose</td>
<td>14.3 ± 6.8###</td>
<td>30.0 ± 8.6</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>9.4 ± 1.8</td>
<td>34.4 ± 3.8###</td>
</tr>
<tr>
<td>0.506 mmol/kg, single doses</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Monocrotaline</td>
<td>5.4 ± 0.5</td>
<td>22.2 ± 3.5</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>6.4 ± 2.2</td>
<td>25.8 ± 10.2</td>
</tr>
<tr>
<td>TOCP</td>
<td>11.0 ± 5.6</td>
<td>30.0 ± 11.0</td>
</tr>
<tr>
<td>1.0 ml/kg, single dose, i.p. + isoline</td>
<td>6.0 ± 0.7</td>
<td>20.6 ± 5.3</td>
</tr>
<tr>
<td>0.127 mmol/kg, single dose</td>
<td>132.5 ± 60.9###</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 ± 683.8###</td>
</tr>
<tr>
<td>0.506 mmol/kg, single dose</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>0.506 mmol/kg, single doses</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>+ clivorine</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>6.4 ± 0.9</td>
<td>16.6 ± 1.7</td>
</tr>
<tr>
<td>SKF 525A</td>
<td>6.7 ± 2.1</td>
<td>23.7 ± 2.1</td>
</tr>
<tr>
<td>50.0 mg/kg, single dose, i.p. + isoline</td>
<td>6.8 ± 2.0###</td>
<td>26.2 ± 5.3</td>
</tr>
<tr>
<td>0.253 mmol/kg, single dose</td>
<td>8.0 ± 7.9</td>
<td>24.6 ± 10.0</td>
</tr>
<tr>
<td>SKF 525A + TOCP</td>
<td>8.8 ± 3.8###</td>
<td>27.2 ± 5.4###</td>
</tr>
<tr>
<td>+ isoline</td>
<td>387.5 ± 130.8###</td>
<td>470.0 ± 318.1###</td>
</tr>
</tbody>
</table>

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. clivorine (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).

1992a), the macrocyclic ester of the neonic acid moiety was hydrolyzed at a different position. In the experiments described above, we did not detect retrolecine, 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 clivorine could undergo deacetylation by PMSF-sensitive microsomal hydrolase 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 isozyme(s) or combinations might be responsible for the deacetylation of isoline. The extensive metabolism to clivorine, another PA with similar acetyl structures (both S-configuration at C-12 position) (Fig. 1), further indicates that esterase(s) may have broad and overlapping substrate specificity to esters (Sato 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 isoform in mouse (MH1) could be found with homology and characteristics similar to those of rat hydrolase A (Sato 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 clivorine 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 clivorine 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, clivorine 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 toxikokinetic properties of the 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 clivorine also induced severe liver damage and sometimes death. Clivorine 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 clivorine 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
and highly reactive “pyroles” 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 pyrole (Glowaz et al., 1992) by P450s caused extrahepatic 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 responsible for the biotransformation of isoline to two deacetylated metabolites (M1 and M2) in vitro, especially in mouse and PB-induced rat microsomes. Isoline was also metabolized much more extensively than clivoring and monocrotaline but showed characteristics somewhat similar to those of clivoring, 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 of clivoring 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 microsomal 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 implications.

References

FIG. 3. Photomicrography of liver sections from mice treated with isoline, TOCP-isoline, and saline vehicle. Mice were administered with either isoline or saline vehicle, and tissues including liver, heart, lung, and kidney were removed 24 h after the last treatment and processed as described under Materials and Methods. A, liver of mouse treated with salivne vehicle for 3 days. P, portal area; C, central vein. B, liver of mouse treated with 0.253 mmol/kg isoline for 3 days. *, coagulation necrosis; P, portal area. C, liver from mouse treated with a single dose of 0.506 mmol/kg isoline; C-1, extensive hemorrhage; C-2, apoptotic body (arrows); C-3, hemorrhage and coagulation necrosis. P, portal area. D, liver of mouse pretreated with 1 ml/kg TOCP followed by one dose of 0.253 mmol/kg isoline. Extensive hemorrhage and necrosis around the central vein was dose dependently enhanced by prior exposure to TOCP. A, B, C-1, and D, bar = 200 μm; C-2 and C-3, bar = 50 μm.


