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DEACETYLCLIVORINE: A GENDER-SELECTIVE METABOLITE OF CLIVORINE FORMED IN FEMALE SD RAT LIVER MICROSOMES

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Running title: Female Rat-specific metabolite of clivorine

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[1]**Abbreviations used are:** PA, pyrrolizidine alkaloid; DHR, dehydroretronecine; GSH, glutathione; 7-GSH-DHR, 7-glutathionyldehydroretronecine; PMSF, phenylmethylsulfonyl fluoride; TOCP, triorthocresyl phosphate.

Abstract:

Clivorine, a naturally occurring pyrrolizidine alkaloid, causes liver toxicity via its metabolic activation to generate toxic metabolite (pyrrolic ester). Female SD rats are reported to be less susceptible to clivorine intoxication than male SD rats. However, the biochemical mechanism causing such gender difference is largely unknown. The present study investigated hepatic microsomal metabolism of clivorine in female rats to delineate the mechanism of the gender difference. Two pathways, which directly metabolize clivorine, were observed. Firstly the metabolic activation to produce the toxic pyrrolic ester followed by formations of bound-pyrroles, DHR, 7-GSH-DHR and clivoric acid were found in female rats, and CYP3A1/2 isozymes were identified to catalyze the metabolic activation. Compared with male rats (~21%), the metabolic activation in female rats was significantly lower (~4%) possibly due to significantly lower CYP3A1/2 levels expressed in female rats. Secondly a direct hydrolysis to generate a novel female rat-specific metabolite deacetylclivorine was demonstrated as the predominant pathway (~16% clivorine metabolism) in female rat liver microsomes and was determined to be mediated by microsomal hydrolase A. Furthermore, when the metabolic activation was completely inhibited by ketoconazole, the amount of deacetylclivorine formed in 1-hour incubation significantly increased from 19.44±3.00 to 54.87±9.30 nmol/mg protein, suggesting that the two pathways compete with each other. Therefore, the lower susceptibility of female SD rats to clivorine intoxication is suggested to be due to significantly higher extent of the direct hydrolysis as well as a lower degree of the metabolic activation.

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Pyrrolizidine alkaloid (PA[1]) poisoning has drawn worldwide attention due to a wide distribution of PA-containing plants and their induced serious and diversified toxicities, especially hepatotoxicity and carcinogenicity (Buhler et al., 1990; Fu et al., 2002; 2004; Huxtable 1989; Mattocks, 1986; Mori et al., 1985) as well as pneumotoxicity (Huxtable 1990; Taylor et al, 1997), neurotoxicity (Roeder, 2000) and embryotoxicity (Tu et al, 1988). Two types of PAs, namely retronecine- and otonecine-type, are mainly responsible for the PA-induced hepatotoxicity (Buhler et al., 1990; Fu et al., 2004; Huxtable 1989; Mori et al., 1985). Clivorine, a representative toxic otonecine-type PA, is present in many *Ligularia* species, and especially exists as a predominant PA in a traditional Chinese medicinal herb *Ligularia hodgsonii* hook (Lin et al., 2000a; Xia et al., 2004). Clivorine has been reported to cause hepatotoxicity and carcinogenicity in rodents and a positive mutagenic response in the Ames test in the presence of rat liver homogenates, suggesting the importance of hepatic metabolic activation in its intoxication (Kuhara et al., 1980; Yamanaka et al., 1979; Xia et al., 2004). In our previous studies, hepatic microsomal metabolism of clivorine in male SD rats has been extensively investigated and the mechanism of metabolic activation was found to be similar to that of the other toxic type (retronecine-type) of naturally occurring PAs (Lin et al., 1998b; 2000b; Fu et al., 2004). The toxicity of clivorine was revealed to be caused by cytochrome P450 mediated biotransformation to generate a chemically reactive ‘pyrrolic ester’ (e.g. dehydroclivorine in Fig. 1), which can further react with vital cellular macromolecules such as proteins and DNA to form bound pyrroles or DNA crosslinking leading to toxicities (Fu et al., 2004; Lin et al., 2000b; Pereira et al, 1998; Xia et al., 2004; Yang et al., 2001).

Moreover, female SD rats were found to be significantly less susceptible to clivorine intoxication in our previous study. Compared with male rats, significantly lower metabolic activation rate to form toxic pyrrolic ester was observed in the hepatic microsomal metabolism in female rats, although the overall metabolic rates of clivorine in both sexes of rats were the same (Lin et al., 2003). Moreover, an additional pathway was found in female rats to directly metabolize clivorine, which might be responsible for detoxification to produce non/less toxic metabolite(s). However, identity of such a pathway, the formed metabolite and enzyme(s) involved were not investigated in our previous study (Lin et al., 2003). In the present report, the microsomal metabolic pathways of clivorine in female rats, the generated metabolites and enzymes mediating these pathways are described. The results indicate that extensive microsomal carboxylesterase mediated hydrolysis may play a key role in the detoxification of clivorine in female rats.

Materials and Methods

Chemicals. Triorthocresyl phosphate (TOCP) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Diethyldithiocarbamate, ketoconazole, monocrotaline, phenylmethylsulfonyl fluoride (PMSF), pilocarpine, retrorsine, sulfaphenazole, glutathione (GSH), and all other chemicals were purchased from Sigma Chemical Co. Clivorine was isolated from *L. hodgsonii* Hook in our laboratory by a standard extraction procedure for pyrrolizidine alkaloid (Lin et al., 2000a). The identity of the isolated clivorine was confirmed by UV, IR, NMR and MS analysis, and its purity was determined to be higher than 99% by HPLC. The authentic standards of known metabolites of clivorine were either synthesized using monocrotaline as the starting material for DHR (dehydroretronecine) and 7-GSH-DHR (7-

glutathionyldehydroretronecine) or isolated from a scaled-up male rat microsomal incubation with clivorine for clivoric acid as described previously (Lin et al., 1998b; 2000b). For the isolation of the novel metabolite, deacetylclivorine, a scaled-up female rat microsomal incubation with clivorine in the absence of the NADPH-generating system as described below was conducted. Both isolated and synthetic metabolites were subjected to MS and HPLC-UV analysis for the confirmation of their identity and purities (≥99%), respectively.

Instrumentation. The on-line HPLC-UV-MS analyses for the identification of metabolites generated from microsomal incubations were performed on an Agilent 1100 Liquid Chromatograph system connected to a photodiode-array multiple-wavelength UV detector (DAD) and a Finnigan TSQ 7000 mass spectrometer coupled with an electrospray ionization interface. Both positive and negative ion mode of MS and MS/MS analyses were carried out on the same HPLC-MS setup with direct loop infusions. Another Agilent 1100 system coupled with DAD was used for all HPLC-UV quantitative analyses.

Animals and Preparation of Liver Microsomes. Adult female Sprague-Dawley (SD) rats (body weight 220-250g) were supplied by the Laboratory Animal Services Centre at the Chinese University of Hong Kong. Female rat liver microsomes and cytosolic fractions were prepared by a standard procedure (Lin et al., 2000b, 2002; 2003). Protein content was determined using the modified method of Lowry (Lowry et al. 1951). Cytochrome P450 content was determined by a standard procedure (Omura et al., 1964; Lin et al., 2000b; 2003). The prepared sub-cellular fractions were stored in portions at –80°C until used.

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Microsomal Incubation and Treatment of Incubated Samples. Our previous reported procedure (Lin et al., 2000b, 2002; 2003) was adopted for the present study. Briefly, a typical 1 ml microsomal or cytosolic incubation mixture was consisted of potassium phosphate buffer (0.1 M, pH 7.4) containing liver microsomes or cytosolic incubation (2 mg of protein/ml), 250 μM clivorine, and 2.0 mM GSH with or without the NADPH-generating system (5 mM $MgCl_2$, 1 mM β-$NADP^+$, 10 mM glucose 6-phosphate, and 1unit/ml of glucose-6-phosphate dehydrogenase). The reaction was initiated by adding the substrate (clivorine), proceeded at 37°C for 60 min, and then terminated by chilling in an ice bath. A consistent concentration of clivorine at 250 μM was selected in all incubations as well as the enzyme inhibition studies described below. This concentration was comparable to those tested in our previous *in vitro* study of rat liver microsomal metabolism of clivorine to form DHR-derived DNA adducts, leading to hepatotoxicity (Xia et al., 2004). In addition, the same concentration was also tested in male rat live microsomal metabolism of clivorine (Lin et al., 2000b; 2003), thus the present results can be directly compared with those obtained previously from male rat studies. The incubation time was chosen for 1 hour based on our previously established methods (Lin et al., 2000b, 2002; 2003) and also the results of the initial test on the different incubation times in the present study. The formation rates of all metabolites identified were linear within 45-60 min, all reached the peak at 60 min, and then remained in plateau for at least 2 hours (data not shown). Furthermore, incubations with female rat liver S9 and male rat liver S9, microsomal and cytosolic fractions for the comparison as well as various controls, including using phosphate buffer or denatured microsomes and incubation in the absence of substrate were conducted in parallel. Incubation under each individual condition was conducted

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at least in triplicate. The resultant ice-cold incubates were immediately centrifuged at 105,000 *g* at 2°C for 20 min. Aliquots (200 μl) of the supernatant were filtered and subjected to HPLC systems for qualitative and quantitative analyses.

Enzyme Inhibition Studies. Four selective cytochrome P450 inhibitors [10 μM pilocarpine to CYP2A1 (Kimonen et al., 1995: Lewis 1998); 30 μM diethyldithiocarbamate to CYP2E1 (Eagling et al., 1998); 5 μM ketoconazole to CYP3A1/2 (Ghosal et al., 1996; Shayeganpour et al., 2006); and 100 μM sulfaphenazole to CYP2C subfamily (Eagling et al., 1998)] and two carboxylesterase inhibitors (TOCP, a nonselective microsomal carboxylesterase inhibitor, and PMSF, a selective hydrolase A inhibitor) at various concentrations were used to study their effects on the microsomal metabolism of clivorine in female rats. The concentrations of individual CYP inhibitors utilized were chosen based on the published reports as indicated above, in order to achieve significant inhibitions of the corresponding isozymes in rat liver microsomes. In the case of inhibition of the tested cytochrome P450 isozymes, 10 min preincubation with individual inhibitors in the presence of the NADPH-generating system was conducted, followed by incubations under the above mentioned condition except for the absence of GSH. Furthermore, controls in the absence of inhibitors were treated identically for the direct comparison. For the inhibition of carboxylesterase, both TOCP and PMSF were dissolved in DMSO, diluted with phosphate buffer to appropriate concentrations ranging from 2 to 50 μM, and then incubated under the similar conditions but with neither GSH nor the NADPH-generating system incorporated. A control incubation with the same volume of DMSO (less than 0.05% in the incubation mixture) as vehicle control was also performed in parallel.

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Identification of Metabolites. The supernatants of incubations were directly subjected to HPLC-UV-MS analysis for the identification of metabolites. All analytical conditions were identical to those reported previously (Lin et al., 2000b; 2003). Full scan mass spectra were obtained over the mass range of *m/z* 150-850 in a negative ion mode for the identification of glutathione conjugates such as 7-GSH-DHR and clivoric acid or in a positive ion mode for the determination of DHR, deacetylclivorine and the intact clivorine. A direct comparison of retention time, UV and mass spectra of each metabolite with that of the authentic sample was also conducted for the confirmation of the identity of each metabolite found.

Furthermore, the novel metabolite deacetylclivorine was prepared from a scaled-up microsomal incubation (100 ml) in the absence of the NADPH-generating system. The obtained incubates were lyophilized, and the residues were extracted with 80% methanol (2 × 25 ml). The combined methanol extracts were centrifuged at 10,000 *g* at 4°C for 20 min, and then concentrated under reduced pressure to dryness. The residues were reconstituted into water, filtered and subjected to preparative HPLC with a Hamilton preparative PRP-1 column (100 × 7 mm, 5 μm) coupled with a Hamilton PRP-1 guard column (50 × 4.1 mm, 5 μm). The mobile phase consisted of acetonitrile (A) and 0.01% formic acid (B) using the following gradient elution: initial 0-5 min, 100% B; 5-15 min, linear change to 60% B and then maintained for 15 min. The flow rate was kept constant at 1.0 ml/min. The eluted fractions containing deacetylclivorine were collected and concentrated under reduced pressure to remove acetonitrile and formic acid followed by lyophilization to yield colorless oil. The identity of the isolated deacetylclivorine was confirmed by mass spectrometric

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analysis with a direct loop infusion and the purity (≥99%) was determined by HPLC-UV. All spectroscopic and analytical conditions were the same as reported previously (Cui and Lin 2000; Lin et al., 1998a). For the further structural elucidation, the tandem MS/MS spectrum of deacetylclivorine was obtained under collision induced dissociation (CID). The collision energy was 50 V with argon as the collision gas setting 0.20 Pa. (Lin et al., 1998a).

Quantification of Metabolites. For the quantitative study of each metabolite in individual incubates, our previously developed HPLC-UV method was adopted by using a specific two-column setup with a Hamilton PRP-1 guard column (50 × 4.1 mm, 5 μm) and a Hamilton PRP-1 analytical column (150 × 4.1 mm, 5 μm) (Cui and Lin, 2000). The incubated blank samples spiked with standards were also analyzed under the same condition, and a direct comparison of the retention time and UV spectrum of each analyte with that of the corresponding standard was conducted. Calibration curves for clivorine and its known metabolites were prepared as described previously (Cui and Lin, 2000). Validations including the measurement of intra- and inter-day variations were conducted in the present study. In the case of the novel deacetylclivorine, the isolated pure deacetylclivorine (five concentrations over a range of 6.8 to 108 μg/ml) and retrorsine as an internal standard were spiked into the blank microsomal incubation mixtures to construct a calibration curve. A linear calibration with the concentration of deacetylclivorine as a function of peak area ratio (analyte/internal standard) was obtained. Quantities of individual analytes present in different incubates were determined from the corresponding calibration curves constructed at the detection wavelength of 230 nm. Furthermore, DHR-derived bound pyrroles was determined by adopting a previously published method using a

spectrophotometric analysis of the specific chromophore formed via reacting pyrrole functional group with Ehrlich regent (Lin et al. 2000b; 2002; 2003; Yan and Huxtable 1995).

Data Analysis. All values are expressed as mean ± standard deviation. Unpaired Student t-test was used to determine the statistical significance between individual inhibition studies and the control. A probability (*p*) value less than 0.05 was considered statistically significant.

Results

Identification of Microsomal Metabolites. Three known metabolites, namely clivoric acid, DHR, and a racemic mixture of 7-GSH-DHR (Fig. 1), were identified in the incubations of clivorine with female rat liver microsomes, by using HPLC-UV-MS analysis with a direct comparison of the retention time, UV and mass spectra of the corresponding authentic samples. In addition, the bound pyrroles were also determined. Moreover, an additional metabolite was found after female rat hepatic microsomal incubation with clivorine. Its MS spectrum showed a quasi-molecular ion ($[M+H]^+$) at *m/z* 364, which corresponded to the molecular weight of clivorine without the acetyl moiety. Further structural elucidation was conducted on this metabolite isolated from the scaled-up incubation. Using a direct loop infusion, the MS (Fig 2A) and MS/MS (Fig. 2B) spectra exhibited a quasi-molecular ion ($[M+H]^+$) as the base peak at *m/z* 364, corresponding to the deacetylated metabolite of clivorine, and diagnostic fragmentation ions at *m/z* 168, 150 and 122, respectively, which were in good agreement with the fragmentation pattern of clivorine (Lin et al., 1998a; Xia et al. 2004). Therefore, this metabolite was unequivocally identified as

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deacetylclivorine, which was produced via deacetylation (hydrolysis) of clivorine (Fig. 1).

Further investigation confirmed that deacetylclivorine was formed after incubation of clivorine both in the presence and absence of the NADPH-generating system with female rat S9 and microsomes but not cytosolic fractions. On the other hand, incubations of clivorine with male rat liver S9, microsomes and cytosolic fractions as well as various controls and phosphate buffer solution did not generate this novel metabolite. The results indicated that deacetylclivorine was female rat-specific metabolite and could only be produced under the catalysis of the NADPH-independent microsomal enzymes. To the best of our knowledge, deacetylclivorine is a novel and female rat-specific metabolite characterized for the first time. Based on the results, the microsomal metabolic pathway of clivorine in female rat liver was delineated as shown in Fig. 1.

Quantification of Metabolites. All known metabolites and the intact clivorine were quantified by adopting our previously developed HPLC-UV method (Cui and Lin, 2000). The present validation studies resulted in good linearities ($R^2 \geq 0.98$) for each of individual analytes with overall intra- and inter-day variations of less than 11%. For the novel metabolite, a calibration curve of deacetylclivorine was obtained with a good linearity ($R^2 = 0.9995$). The results demonstrated that deacetylclivorine was the predominant metabolite in all female rat hepatic microsomal incubations. Furthermore, in the absence of the NADPH-generating system, amount of deacetylclivorine formed in 1-hour incubation significantly increased (54.87±9.30 vs

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19.44±3.00 nmol/mg protein, $p<0.001$) (Table 1) and was the only metabolite found in the NADPH-independent metabolism.

It is well established that the metabolic activation of clivorine is via oxidative *N*-demethylation to generate pyrrolic ester, which then undergoes adduct formation leading to hepatotoxicity (Buhler et al., 1990; Fu et al., 2002; 2004; Huxtable 1990; Lin et al., 2000b; 2002; 2003; Mori et al., 1985). Since further metabolisms of pyrrolic ester, including adduct formations, all produce clivoric acid (Fig. 1), the rate of the metabolic activation can be estimated from the clivoric acid formation. In female rat liver microsome, about 30% of clivorine was metabolized within 60 min, and approximately 16% hydrolysis and 4% metabolic activation (Fig. 3, control incubation) were observed based on the measurement of amounts of deacetylclivorine and clivoric acid formed, respectively. The results demonstrated that the hydrolysis to form deacetylclivorine was the predominant pathway in hepatic microsomal metabolism of clivorine in female rats.

Enzyme Inhibition Studies. To be simplified, the enzyme inhibition studies were conducted in the incubations without the addition of GSH, thus only formations of clivoric acid and deacetylclivorine were measured in order to estimate the extent of the metabolic activation and hydrolysis pathway respectively. Four P450 isozyme inhibitors, which inhibit specifically towards CYP3A1/2, CYP2A1, CYP2E1 isozymes, and non-specifically to all isoforms in CYP2C subfamily, were tested in the present study. CYP3A1/2 isozymes have been previously reported to catalyze the metabolic activation of various PAs, including clivorine to generate the corresponding toxic pyrrolic ester in rats of both sexes (Lin et al., 2003; Fu, et al., 2004), while

CYP2A1, CYP2E1, and CYP2C6, CYP2C7 and CYP2C12 in CYP2C subfamily are the isozymes present predominantly in female rats (Agrawal and Shapiro 2000; 2003; Bandiera and Dworschak 1992; Pampori and Shapiro 1999). Incubation with all these four inhibitors using the selected concentrations, which have been reported to produce significant inhibitions of the corresponding CYP isozymes, did not significantly affect the overall metabolism of clivorine and the deacetylclivorine formation (Table 1 and Fig. 3). However, ketoconazole, a selective CYP3A1/2 inhibitor, abolished the formation of pyrrolic ester and thus of its consequent products including DHR and clivoric acid, whereas, other inhibitors known to inhibit the constitutive female rat-predominant isoforms, such as CYP2A1, CYP2C6, CYP2C7, CYP2C12 and CYP2E1, did not affect the metabolic activation because apparently the clivoric acid formation was not inhibited. The results confirmed that similar to male rats, CYP3A1/2 isozymes were also the key enzyme catalyzing the metabolic activation of clivorine in female rats. The results are consistent with our previously reported observation (Lin et al., 2003).

On the other hand, deacetylation of clivorine to generate deacetylclivorine occurred in the absence of the NADPH-generating system, and was not affected by any of the P450 isozyme inhibitors tested (Table 1), indicating that the formation of deacetylclivorine in female rats was independent of cytochrome P450 monooxygenases. Furthermore, deacetylclivorine was generated only in female rat liver S9 and microsomal but not cytosolic incubations, thus the responsible enzymes are most likely the microsomal carboxylesterases. Both non-selective (TOCP) and selective (PMSF toward hydrolase A) carboxylesterase inhibitors were found to significantly and dose-dependently inhibit the formation of deacetylclivorine (Table

2), and significantly higher inhibitory potency of PMSF was observed i.e. IC_{50}: 6.34±0.97 μM of PMSF vs 16.43±1.18 μM of TOCP (p<0.001). Furthermore, at a high concentration of 100 μM, PMSF totally abolished the hydrolysis pathway (data not shown). The results suggested that microsomal carboxylesterases, in particular hydrolase A isoform in female rats, played a key role in catalyzing direct hydrolysis of clivorine to form the novel and gender-selective metabolite deacetylclivorine.

Discussion

Species and gender differences in susceptibility of retronecine-type PA intoxication are well documented (Fu et al., 2004; Huan et al., 1998; Stegelmeier et al., 1999). However, there is limited information on otonecine-type PA induced toxicity, and only our research team reported species (guinea pig and rat) and gender (male and female SD rats) differences in clivorine intoxication (Lin et al., 2002; 2003). In the case of gender difference, our previous study (Lin et al., 2003) demonstrated that male rats were more susceptible to clivorine intoxication possibly due to the predominant metabolic activation mediated by CYP3A1/2, the highly expressed isozymes in male rats, and that the metabolic activation in female rats could be significantly accelerated if their CYP3A1/2 isozymes were induced. Moreover, a total metabolic rate of clivorine was the same in both sexes of rats, and the metabolic activation was the only biotransformation pathway found to directly metabolize clivorine in male rats. The results also demonstrated that there might be other detoxification pathway(s), which might generate non/less toxic metabolite(s) and could also compete with the metabolic activation, and thus significantly reduce the formation of toxic pyrrolic ester in female rats. However, details of such

detoxification pathway(s), identity of metabolite(s) formed, and enzyme(s) involved were not investigated in the previous study.

In the present study, it is interesting to note that the constitutive female rat-predominant P450 isozymes including CYP2A1, CYP2C6, CYP2C7, CYP2C12, and CYP2E1 did not catalyze the metabolic activation of clivorine (Table 1), whereas, similar to male rats, CYP3A1/2 isozymes were also observed to mediate the metabolic activation via an oxidative *N*-demethylation to form the toxic pyrrolic ester in female rats (Fig. 1). It is well documented that lower levels of CYP3A subfamily, especially CYP3A2 were found in female rats (Agrawal et al., 2000; 2003; Bandiera and Dworschak 1992; Pampori and Shapiro 1999). Therefore, it is not surprising to observe that of the 30% metabolism of clivorine in female rats in 1 hour, the metabolic activation pathway only accounted for about 4%, which is significantly lower than the previous reported 21% in male rats (Lin et al., 2003). The lesser extent of metabolic activation of clivorine in female rat liver microsomes is probably due to the lower activity of CYP3A1/2 isozymes. In addition, a new direct hydrolysis pathway, which accounted for about 16% of clivorine metabolism, was identified, demonstrating that direct hydrolysis dominated hepatic microsomal metabolism of clivorine in female rats.

It is noted that two unknown putative metabolites were suggested in our previous report on the microsomal metabolism of clivorine in female rats (Lin et al., 2003). The present study confirmed that only deacetylclivorine was the novel and female rat-specific metabolite. The second putative metabolite, determined not to be related to clivorine, was formed after incubation of female rat liver microsomes with the

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NADPH-generating system, and decomposed within 4 hours at room temperature (data not shown). The female rat-specific metabolite deacetylclivorine was unequivocally identified for the first time. Apparently, deacetylclivorine is speculated to be more hydrophilic than clivorine, because the ester group in clivorine has been changed to a hydroxyl group in this metabolite.

The recoveries obtained for all incubations were 90─100% (Fig. 3). In addition, possible metabolites of deacetylclivorine in all incubated samples were further studied using both positive and negative ion mode of HPLC-MS/MS. Similar to the metabolic activation of clivorine, the possible metabolites generated via oxidative *N*-demethylation of deacetylclivorine to the corresponding pyrrolic ester (*m/z* 332 $[M+H]^+$) and acid (*m/z* 203 $[M-H]^-$) were carefully examined, and no such metabolites were found. The results demonstrated that apparently deacetylclivorine did not undergo further significant metabolism in female rat liver microsomes. Therefore, direct hydrolysis of clivorine to produce deacetylclivorine is considered to be not related to the metabolism-induced intoxication. Nevertheless, whether deacetylclivorine undergoes further metabolism *in vivo* and/or is directly excreted requires further investigations.

Further evaluation demonstrated that deacetylclivorine could not be formed via chemical treatment of clivorine under either acidic or basic condition, indicating that this metabolite was generated enzymatically. Moreover, this female-specific metabolite was formed in microsomal but not cytosolic incubations, suggesting a microsomal carboxylesterase mediated formation. Multiple forms of carboxylesterase are present in mammalian tissues with the highest levels in the liver located in

endoplasmic reticulum. Rat carboxylesterase consists of two isoforms: hydrolase A (ES-10) and hydrolase B (ES-4), and both isoforms hydrolyze xenobiotics containing an ester, thioester, or amide group (Lee et al., 1998; Alexson et al., 2002). Based on the nature of their responses to PMSF, hydrolase A or hydrolase B are also referred to as PMSF-sensitive or PMSF-insensitive hydrolase, respectively. Therefore, PMSF is commonly used as a selective hydrolase A inhibitor to distinguish the hydrolase A- or hydrolase B-mediated hydrolysis (Lee et al., 1998; Alexson et al., 2002). The results demonstrated that both non-selective and selective hydrolase A inhibitors significantly and dose-dependently suppressed the hydrolysis of clivorine to form deacetylclivorine. Especially, PMSF exhibited significantly higher inhibitory potency (Table 2) and totally abolished the hydrolysis pathway at a high concentration (100 μM). Therefore, hydrolase A was determined to be the key enzyme catalyzing the hydrolysis pathway. It is interesting to note that although there are no reports on gender differences in expression and activity of two rat carboxylesterase isoforms, the hydrolase A-mediated hydrolysis of clivorine could not be found in the incubations with male rat liver microsomes. The reason is unknown and is speculated to be due to a balance among various competitive pathways. Further investigations are required.

It is interesting to note that extensive hydrolysis of clivorine by soluble hydrolases to form guinea pig-specific metabolite clivopic acid was suggested to be the main reason responsible for guinea pigs of both sexes to be resistance to clivorine intoxication. This hydrolysis pathway competed with the metabolic activation of clivorine and thus inhibited the generation of the toxic pyrrolic ester (Lin et al., 2002). However, such a pathway could not be found in female rats cytosolic incubations. On the other hand, catalyzed by microsomal hydrolase A, extensive hydrolysis of

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clivorine to form deacetylclivorine occurred in female rats. Similarly to guinea pigs, hydrolysis of clivorine to form deacetylclivorine in female rats was also found to compete with the metabolic activation. When the metabolic activation was blocked in the absence of the NADPH-generating system, the extent of hydrolysis significantly increased from 16% (control) to 40% (Fig. 3), suggesting that the overall effects might be due to a balance among various metabolic pathways mediated by different enzymes. Thus, hydrolysis of clivorine to generate gender-selective deacetylclivorine could also be considered as a possible detoxification pathway, reducing the formation of toxic pyrrolic ester via competition with the metabolic activation in female rats.

Our previous study evidenced that male SD rats were significantly more susceptible (LD_{50}: 91±3 mg/kg, i.p.) than female rats (LD_{50}: 114±9 mg/kg, i.p., $p<0.05$) to clivorine (Lin et al., 2003). Additionally, predominance of metabolic activation and lacking of direct hydrolysis were observed in male rat liver microsomal metabolism of clivorine (Lin et al., 2000b; 2003). The present finding of the significantly lower metabolic activation (~4%) plus predominant direct hydrolysis (~16%) in female rats along with the fact of predominant bioactivation in male rats can explain the gender differences in susceptibility to clivorine-induced hepatotoxicity. Although the extent of *in vivo* metabolic activation of clivorine in rats is unknown, the gender difference in metabolism of clivorine observed in our *in vitro* studies may be extrapolated to the gender difference *in vivo*. However, further *in vivo* metabolic studies are warranted.

Together with the results of our present and previous studies, the main reasons for less susceptibility of female rats and guinea pigs of both sexes to clivorine

intoxication are most likely due to predominant detoxification hydrolysis and significantly less metabolic activation. Interestingly, the hydrolysis pathway was catalyzed by different enzymes, soluble hydrolases in guinea pigs as opposed to microsomal hydrolase A in female rats. The results suggest that the severity of PA intoxication depends upon an overall balance between the metabolic activation and detoxification pathways which vary with enzyme systems and species. On the other hand, any factors, such as enzyme induction and/or inhibition caused by diets, dietary supplements, nutraceuticals, drug-drug and herb-drug interactions, may alter the metabolic balance in the body and thus have significant impacts on PA intoxication to human health.

In conclusion, two pathways directly metabolizing clivorine were identified in female rat hepatic microsomal metabolism. The hydrolysis mediated by microsomal hydrolase A was the predominant pathway to generate novel and female rat-specific deacetylclivorine. The metabolic activation catalyzed by CYP3A1/2 to produce the toxic pyrrolic ester followed by consequent adduct formation with macromolecules possibly leading to hepatotoxicity, was a minor route in female rats. This is most likely because of the significantly lower levels of CYP3A subfamily, especially CYP3A2 isoform, expressed in female rats. Therefore, we propose that the decreased susceptibility of female rats to clivorine intoxication may be due to significantly lower rates of hepatic bioactivation along with gender specific hydrolysis to deacetylclivorine.

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Footnotes

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Figure legends:

Fig. 1. The hepatic microsomal metabolism of clivorine in female rats.

NuS: nucleophilic macromolecules. The unstable pyrrolic ester is shown in brackets.

Fig. 2. The MS (A) and MS/MS (B) spectra of deacetylclivorine obtained by a direct loop infusion with electrospray ionization. The operating conditions for MS were as follows: spray voltage 5kV, sheath gas setting 50 psi, auxiliary gas 15 units, and a heated capillary temperature of 250 °C. Full scan mass spectrum was recovered over a scan range of *m/z* 150-850 in a positive ion mode. For the tandem MS/MS analysis, the collision energy was 50 V with argon as the collision gas setting 0.20 Pa.

Fig. 3. Effects of cytochrome P450 inhibitors on the metabolic activation and hydrolysis of clivorine in 1-hour incubations with female rat liver microsomes.

**** $p<0.001$ compared with the control*

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TABLE 1

Effects of P450 isozyme inhibition on the microsomal metabolism of clivorine in 1-hour incubations with *female rat liver microsomes*

Incubation sample	Amount of metabolites formed (nmol/mg protein/h)			Intact Clivorine (nmol/ml)
	DHR	Clivoric acid	Deacetylclivorine	
<u>Female rat</u>				
Control	5.73±0.80	4.86±0.03	19.44±3.00	178.41±20.70
Cytosolic fraction[a]	---	---	---	250.40±15.70***
Without NADPH[a]	---	---	54.87±9.30***	148.73±19.57
CYP2A1 inhibition	5.41±0.45	5.86±0.85	23.70±2.46	179.62±9.69
CYP2C6/7/12 inhibition	5.37±0.28	5.01±0.78	26.01±1.14	163.70±3.69
CYP2E1 inhibition	5.63±0.52	5.29±0.34	25.49±1.16	172.63±5.21
CYP3A1/2 inhibition	---	---	22.81±1.77	181.07±8.78
<u>Male rat</u>				
Control[b]	4.82±0.12	21.90±0.21	---	152.51±5.06
Cytosolic fraction[a]	---	---	---	253.22±5.93

[a]Except indicated, all samples were incubated with liver microsomes in the presence of the NADPH-generating system (n=3).
[b]Data obtained from male rats are cited from Lin et al., 2003 and listed here for a comparison.
*** $p<0.001$ compared with the control incubations in the absence of inhibitor.
---: Not detected.

TABLE 2

Effect of microsomal carboxylesterase inhibitors on the hydrolysis of clivorine in 1-hour incubations with female rat liver microsomes

Inhibitor (μM)	Amount of deacetylclivorine formed (nmol/mg protein/h)	Intact clivorine (nmol/ml)
Control	50.63±1.40	136.37±6.63
TOCP		
(2.0)	45.29±1.28	146.47±3.68
(5.0)	39.00±1.55	173.54±4.97
(10.0)	32.00±1.15	183.57±5.58
(50.0)	12.87±1.17	238.66±10.25
PMSF		
(2.0)	34.40±0.75	130.89±2.22
(5.0)	31.19±1.60	140.45±3.70
(20.0)	21.58±2.37	194.05±0.68
(50.0)	6.33±0.68	237.61±4.85

TOCP: triorthocresyl phosphate
PMSF: phenylmethylsulfonyl fluoride

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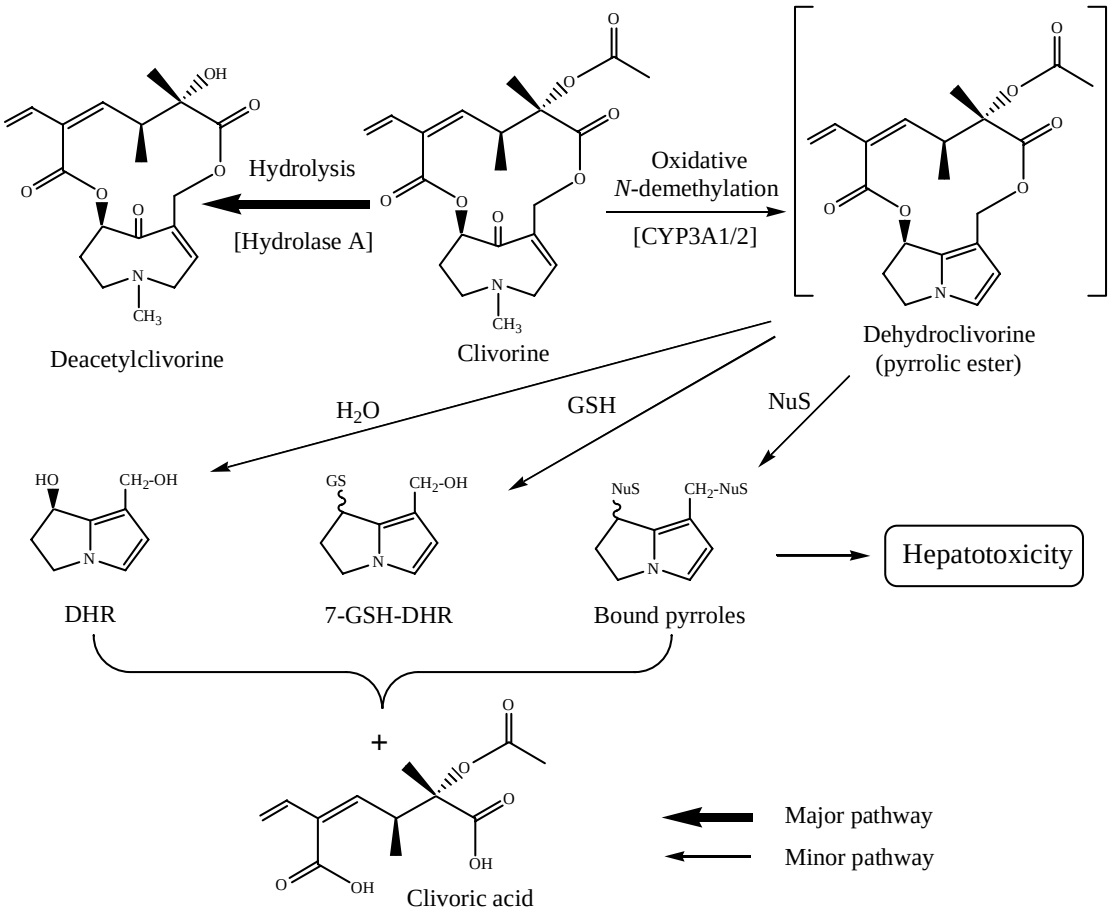

Fig. 1

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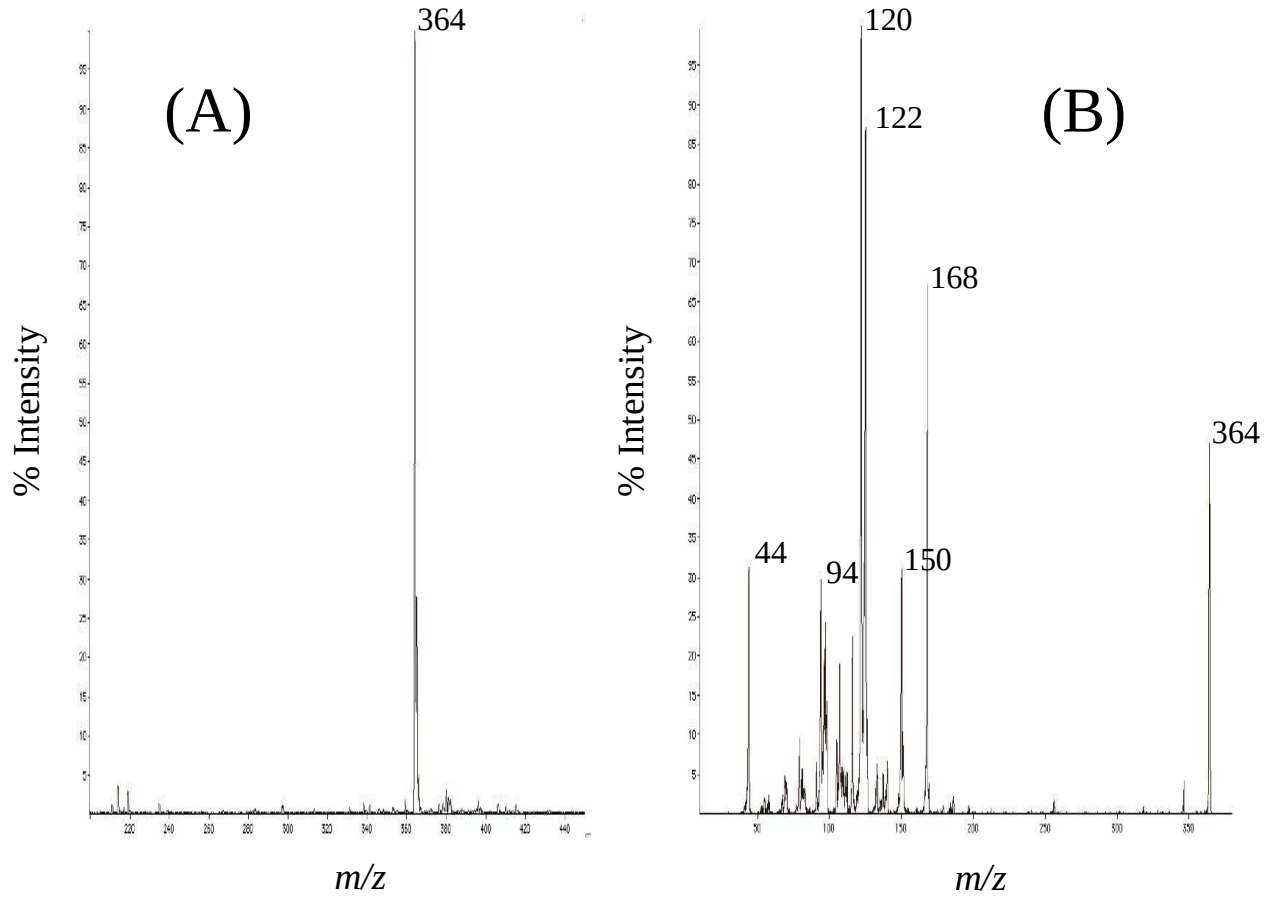

Fig. 2

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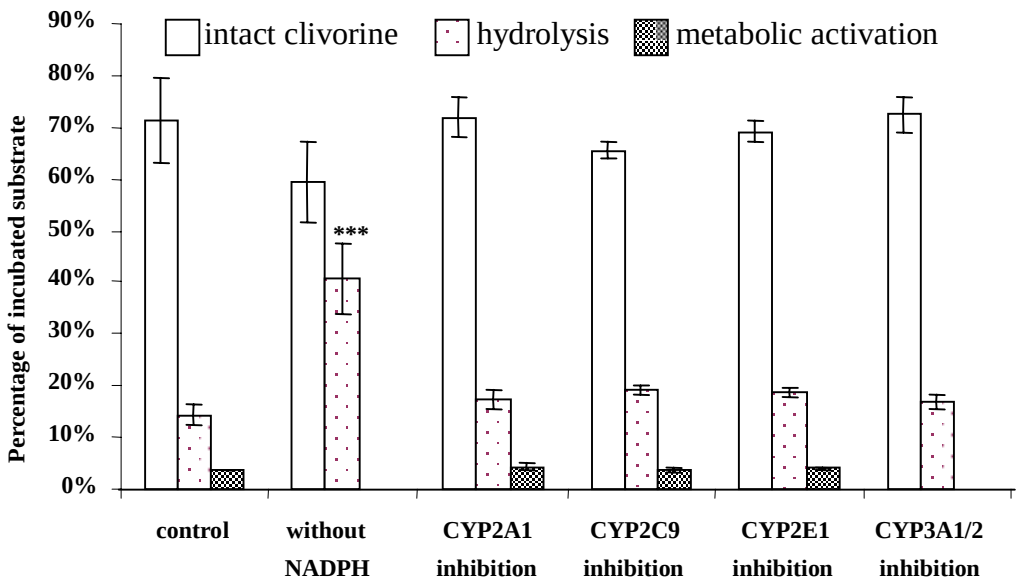

Fig. 3