Involvement of human CYP3A4 in the formation of hepatotoxic metabolites of clivorine

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Abstract: **AIM** This study was conducted to identify the human CYP isoforms responsible for the biotransformation of clivorine in human liver microsomes and the mechanism of metabolism-induced hepatotoxicity of clivorine. **METHODS** Human liver microsomes were used to investigate the metabolism of clivorine *in vitro*. Selective CYP-450 inhibitors and cDNA expressed human CYPs were used to study their effects on the formation of hepatotoxic metabolites and the metabolism of clivorine and the principal CYP-450 isoform involved in the formation of hepatotoxic metabolite. **RESULTS** Four metabolites, namely dehydroretronecine (DHR), 7-glutathionyl-dehydroretronecine (7-GSH-DHR), 7, 9-diglutathionyl-dehydroretronecine (7, 9-diGSH-DHR) and clivoric acid were found in the microsomal incubations. Chemical inhibition studies indicated that the metabolism of clivorine and the formation of pyrrolic metabolites as well as the bound pyrroles were strongly inhibited by CYP3A inhibitor ketoconazole (Ket). Whereas α-naphthoflavone (Nap), sulfaphenazole (Sulp), quinidine (Qui), diethyldithiocarbamate (DDC) have no significant effects on the metabolism of clivorine and the formation of pyrrolic metabolites in human liver microsomes. The results of metabolism of clivorine by cDNA expressed human CYPs showed that only CYP3A4 was found to be capable of catalyzing the metabolism of clivorine, while CYP1A2, CYP2C9, CYP2D6 and CYP2E1 did not play significant roles in the metabolism of clivorine and the formation of pyrrolic metabolites. **CONCLUSION** The results demonstrated that the pyrrolic metabolites were the major *in vitro* metabolites of clivorine and CYP3A4 was the major CYP isoform involved in clivorine metabolism and the formation of hepatotoxic pyrrolic metabolites in human liver microsomes. CYP3A4 plays a key role in the clivorine induced hepatotoxicity.

Key words: clivorine; liver; microsome; biotransformation

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Clivorine is one of the otonecine-type pyrrolizidine alkaloids (PAs) found in a wide variety of traditional Chinese medicinal herb and often causes acute and chronic liver damages in man through direct consumption of PA-containing herbal teas and herbal medicines[1-3]. Many of naturally occurring PAs are hepatotoxic[4]. The metabolism-induced hepatotoxicity by retronecine-type PA has been extensively investigated and it is bioactivated by hepatic cytochrome P450 to reactive pyrrolic esters, which injure liver by a rapid covalent binding with hepatic cellular macromolecules[5,6]. Previous studies[7] have found that like retronecine-type PA, a similar metabolic pathway of clivorine was observed in rat microsomal incubation and four metabolites, namely dehydroretronecine (DHR), 7-glutathionyl-dehydroretronecine (7-GSH-DHR), 7, 9-diglutathionyl-dehydroretronecine (7,9-diGSH-DHR) and clivoric acid, have been found and identified. The reactive pyrrolic metabolites also play a key role in the hepatotoxicity induced by otonecine-type PA. The aim of the work presented here is to evaluate the

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metabolic profiles of clivorine in human liver microsomes and the role of human hepatic CYP isoforms in the formation of reactive pyrrolic metabolites of clivorine.

1 MATERIALS AND METHODS

1.1 Chemicals

The reduced form of glutathione (GSH), retrorsine, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), quinidine (Qui), α-naphthoflavone (Nap), ketoconazole (Ket), sulfaphenazole (Sul), diethyldithiocarbamate (DDC), troleandomycin (TAO) were purchased from Sigma Chemical Co. Clivorine and DHR, 7-GSH-DHR, 7, 9-diGSH-DHR and clivoric acid were generous gifts from Dr. Lin in the Department of Pharmacology, the Chinese University of Hong Kong.

1.2 Human liver microsomes

Human liver microsomes and microsomes from human B-lymphoblastoid cell lines expressing human CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 were obtained from Gentest (Woburn, MA, USA) and stored at −70℃ until use.

1.3 *In vitro* metabolism of clivorine

A typical incubation mixture consisted of 0.1 mol·L^{-1} potassium phosphate buffer (pH 7.4), 2.0 mmol·L^{-1} GSH, NADPH generating system (5 mmol·L^{-1} $MgCl_2$ 10 mmol·L^{-1} G6P, 1 mmol·L^{-1} NADP, 1 kU·L^{-1} G6PDH), 250 mmol·L^{-1} clivorine and 1 g·L^{-1} microsomal protein, in a final volume of 1 mL. The reaction was initiated by addition of the NADPH generating system. After incubation at 37℃ for 60 min, the reaction was terminated in ice bath and retronecine (25 mg·L^{-1}) was added as an internal standard. Incubation mixture used for cDNA-expressed CYP450 was of the same composition as described above.

1.4 Chemical inhibition

Selective inhibitors of human CYP isoforms[8,9] were added to the incubation mixtures and incubated as previously decribed. Incubations with CYP form-selective inhibitors for each of the major human liver P450s at a concentration of 250 mmol·L^{-1} clivorine were performed with microsomes from human liver samples. Inhibitors and inhibitor concentrations for each of the specific P450 forms are as following: 1A2 (Nap, 10 μmol·L^{-1}), 2C9 (Sul, 100 μmol·L^{-1}), 2D6 (Qui, 10 μmol·L^{-1}), 2E1 (DDC, 100 μmol·L^{-1}), 3A4 (TAO, 200 μmol·L^{-1}). Ket was added to microsomal incubations at several concentrations ranging from 0.25 to 5.0 μmol·L^{-1}.

1.5 HPLC analysis of the metabolites of clivorine

The metabolites of clivorine in human liver microsomes were determined by HPLC[10]. The separation of the metabolites of clivorine in human liver microsomes was performed on a PRP-1 reverse-phase column (5 μm, 150 mm × 4.1 mm, Hammilton Co. Reno, NV) coupled with a PRP-1 guard column (5 μm, 50 mm × 4.1 mm, Hammilton Co. Reno, NV). The mobile phase consisted of 2% (*V*/*V*) formic acid (solvent A) and acetonitrile (solvent B). The gradient elution was as follows: at 0 – 5 min 100% A; at 5 – 35 min, linear change from 100% A to 75% A; at 35 – 40 min, linear change from 75% A to 70% A. Flow rate was kept constant at 0.8 mL·min^{-1} for the complete analysis. Peak responses were measured at 230 nm by UV detector. The resultant incubates were centrifuged at 105 000 × *g* at 2℃ for 30 min and aliquots (200 μL) of the supernatant were directly injected on to the column for quantitative analysis. The resultant pellets of the incubations were used for the determination of tissue-bound pyrroles.

1.6 Determination of bound pyrroles

It is believed that the metabolites of PAs, not the alkaloids themselves, are responsible for the toxic effects[11]. The key step in metabolic activation of PAs is the formation of unstable pyrrolic esters which may directly react with nucleophilic tissue constituents such as proteins or nucleic acid to form covalent bound adducts, called bound pyrrole, and lead to specific cytotoxic effects. Therefore in this experiment bound pyrroles were measured as an index of PAs induced hepatotoxicity.

The tissue-bound pyrrole formed after incubation was measured by a modified method reported in literature[12].

1.7 Statistical analysis

Data were expressed as $\bar{x} \pm s$ and analyzed using Student t test.

2 RESULTS

2.1 Metabolism of clivorine in human liver microsomes

Metabolite profiles obtained from human liver microsomes indicated that clivorine was oxidized to four primary metabolites with the corresponding level of clivorine decreased. Using chromatography with authentic standard and HPLC mass spectrometry, all four metabolites were identified as being the previously described primary metabolites of clivorine in male rat liver microsomal incubation: DHR, 7-GSH-DHR, 7, 9-diGSH-DHR and clivoric acid respectively. In addition, bound pyrrole was also found in microsomal incubations and the total bound pyrrole in microsomal incubations was 1.026 $\mu mol \cdot L^{-1}$. The results suggests that the metabolic pattern of clivorine in human liver microsomes is similar to that in male rat liver microsomes.

2.2 Inhibition analysis

To determine the specific CYP isozyme(s) involved in the biotransformation of clivorine, the CYP-isoform selective probes were screened for inhibitory effects on the metabolism of clivorine in human liver microsomes and incubations were conducted using chemical inhibitors that are specific for various CYP isozymes. The effects of CYP-isoform selective inhibitors on the metabolism of clivorine and the formation of pyrrolic metabolites are shown in Tab 1. The results showed that Nap (CYP1A2), Sul (CYP2C9), Qui (CYP2D6), DDC (CYP2E1) had little or no significant inhibitory effects on both the metabolism of clivorine and the formation of corresponding metabolites in human liver microsomes, whereas TAO (CYP3A4) significantly inhibited the metabolism of clivorine in human liver microsomes. Ket caused a significant reduction of formation rate for DHR to 45% and for clivoric acid to 20% of control values and the levels of 7-GSH-DHR, 7, 9-diGSH-DHR was decreased to below the detect limit. Whereas intact clivorine was increased from 56.8% to 87.4%. Both the formation of the metabolites and the bound pyrrole were significantly inhibited by Ket(Fig 1 and Fig 2), even at low concentrations (0.5 $\mu mol \cdot L^{-1}$). The formation of both pyrrolic metabolites and bound pyrrole were completely inhibited by high concentration (5 $\mu mol \cdot L^{-1}$) of Ket and both of them were not detectable in the microsomal incubations, while the

Tab 1. Effect of selective CYP inhibitors on the metabolism of clivorine in human liver microsomes

Group	CYP activity/nmol·min^{-1}·g^{-1}				Clivorine content /mmol·L^{-1}
	DHR	7-GSH-DHR	7,9-diGSH-DHR	Clivoric acid	
Control	263 ± 32	86 ± 12	42 ± 0.7	674 ± 37	142 ± 13
Nap	271 ± 8	78 ± 6	45 ± 2	607 ± 61	146 ± 9
Sul	257 ± 32	83 ± 17	39 ± 2	662 ± 35	150 ± 9
Qui	265 ± 27	90 ± 10	45 ± 3	632 ± 39	150 ± 2
DDC	259 ± 22	90 ± 14	44 ± 2	633 ± 93	145 ± 5
TAO	119 ± 2**	–	–	135 ± 2**	223 ± 8*

Clivorine: 250 mmol·L^{-1}; Nap: α-naphthoflavone (10 $\mu mol \cdot L^{-1}$); Sul: sulfaphenazole (100 $\mu mol \cdot L^{-1}$); Qui: quinidine (10 $\mu mol \cdot L^{-1}$); DDC: diethyldithiocarbamate (100 $\mu mol \cdot L^{-1}$); TAO: troleandomycin (200 $\mu mol \cdot L^{-1}$); DHR: dehydroretronecine; 7-GSH-DHR: 7-glutathionyl-dehydroretronecine; 7,9-diGSH-DHR: 7,9-diglutathionyl-dehydroretronecine. (–): not detectable. $\bar{x} \pm s$, $n = 3$. * $P < 0.05$, ** $P < 0.01$, compared with control.

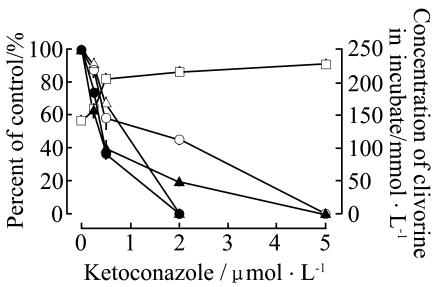

Fig 1. Effects of ketoconazole on the metabolism of clivorine and the formation of its pyrrolic metabolites. DHR(○): dehydroretronecine; 7-GSH-DHR(●): 7-glutathionyl-dehydroretronecine; 7,9-diGSH-DHR(△): 7,9-diglutathionyl-dehydroretronecine; clivoric acid(▲); clivorine(□). $\bar{x} \pm s$, $n = 3$.

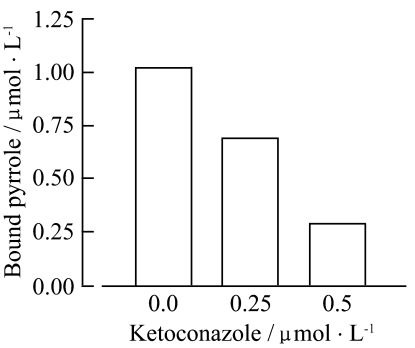

Fig 2. Effects of ketoconazole on the formation of bound pyrrole of clivorine in human liver microsomes.

intact clivorine was increased to 91%. These data indicated that CYP3A4 was responsible for about 80% of the metabolism of clivorine into pyrrolic metabolites.

2.3 Metabolism of clivorine by cDNA expressed specific P450s

The metabolism of clivorine by cDNA expressed human CYPs (CYP1A2, 2C9, 2D6, 2E1 and 3A4) was investigated. Among the five different recombinant human CYPs only CYP3A4 showed detectable catalytic activity for the metabolism of clivorine. Incubations of clivorine with cDNA-expressed CYP3A4 resulted in the formation of all four pyrrolic metabolites and bound pyrrole. But the other human CYPs (1A2, 2C9, 2D6 and 2E1) showed extremely low activity for the metabolism of clivorine and no metabolites could be measured in the incubations. The results are presented in Tab 2.

3 DISCUSSION

Previous investigation of the metabolism of retronecine-type PAs has clearly indicated that the major way of metabolism was to form hepatotoxic pyrrolic metabolites[13]. PAs naturally occur in variety of plants and traditional herbs and often cause hepatotoxicity in man through direct or indirect consumption of PA-containing plants and herbs. However, the metabolic profiles of clivorine, an otonecine-type PA, in human liver microsomes are poorly understood. Therefore such information is essential to the prevention of adverse events caused by PAs. The results of present studies showed that the major way of

Tab 2. Metabolism of clivorine by cDNA expressed human CYP isoforms

Group	CYP activity/nmol·min^{-1}·g^{-1}				Clivorine content /mmol·L^{-1}	Bound pyrrole content /μmol·L^{-1}
	DHR	7-GSH-DHR	7,9-diGSH-DHR	Clivoric acid		
CYP1A2	–	–	–	–	250 ± 20	–
CYP2C9	–	–	–	–	252 ± 20	–
CYP2D6	–	–	–	–	248 ± 16	–
CYP2E1	–	–	–	–	242 ± 10	–
CYP3A4	160 ± 18	53 ± 5	32 ± 3	327 ± 21	203 ± 4	0.288

Clivorine: 250 mmol·L^{-1}. (–): not detectable. $\bar{x} \pm s$, $n = 3$.

metabolism of clivorine was also to form hepatotoxic pyrrolic metabolites. This suggests that the major metabolic pathway of clivorine in human liver microsomes is similar to that in male rat liver microsomes.

The results of chemical inhibition showed that both Ket and TAO, selective human CYP3A inhibitor, significantly inhibited the formation of hepatotoxic pyrrolic metabolites and bound pyrrole and lowered the rate of metabolism for clivorine. Therefore the formation of pyrrolic metabolites was the major metabolic pathway in human liver microsomes. The inhibitory effect of Ket showed concentration-related and the formations of pyrrolic metabolites and bound pyrroles generated from clivorine were abolished by high level of Ket. Whereas other CYP inhibitors, *i. e.* Nap (1A2), Sulp (2C9), Qin (2D6) and DDC (2E1) had little or no significant inhibitory effect on the metabolism and the formation of pyrrolic metabolites. Furthermore cDNA expressed CYP3A4 catalyzed the metabolism of clivorine and while incubations with other cDNA expressed CYP1A2, CYP2C9, CYP2D6 and CYP2E1 did not produce detectable levels of metabolites. These data strongly suggest that human CYP3A4 is the primary isoform involved in the metabolism of clivorine and the formation of reactive metabolites and this is the major *in vitro* metabolic pathway of clivorine in human liver microsomes. CYP3A4 is known to be the major human CYP isoforms involved in the oxidative metabolism of many clinically used drugs as well as xenobiotics. In addition, CYP3A4 is inducible and inhibitable by a lots of xenobiotics[14,15]. Pharmacokinetic processes are regarded as the most important factors that affect the levels of drugs in the body. The findings obtained in this study suggests that there is a possibility that the clivorine-induced hepatotoxicity could be decreased by CYP3A inhibitors.

In conclusion, the *in vitro* metabolic pathway of clivorine in human liver microsomes is to generate hepatotoxic pyrrolic metabolites and bind with hepatic tissues that leads to hepatotoxicity and this is the major *in vitro* metabolic pathway of clivorine in human liver microsomes. Human CYP3A is the primary CYP isoforms involved in the metabolism of clivorine and formation of hepatotoxic pyrrolic metabolites and plays a key role in the clivorine induced hepatotoxicity.

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人肝 CYP3A 参与了山冈囊吾碱的代谢及其肝毒性代谢物的形成

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摘要:目的 在体外研究山冈囊吾碱在人肝微粒体内的代谢及参与其代谢的主要的 CYP450 酶,探讨其代谢致毒机理。**方法** 采用人肝微粒体研究山冈囊吾碱的主要代谢方式和代谢物。在体外运用 CYP450 酶的选择性抑制剂和 cDNA 表达的人肝 CYP450 酶,探讨其对山冈囊吾碱的代谢及肝毒性的吡咯代谢物形成的影响及参与山冈囊吾碱代谢的主要的 CYP450 酶。**结果** 山冈囊吾碱在人肝微粒体内 的主要代谢物为肝毒性的吡咯代谢物:去氢倒千里光裂碱, 7-谷胱甘肽基-去氢倒千里光裂碱,7,9-二谷胱甘肽基去氢倒千里光裂碱和山冈囊吾酸。CYP450 特异性抑制剂 α-萘黄酮(抑制 CYP1A2)、黄胺苯吡唑(抑制 CYP2C)、奎尼丁(抑制 CYP2D6)和二乙基二硫代氨基甲酸钠(抑制 CYP2E1)对山冈囊吾碱的代谢无明显的影响。但 CYP3A 的特异性抑制剂酮康唑和三乙酰竹桃霉素可以显著地抑制山冈囊吾碱的代谢及其吡咯代谢物和结合型吡咯物的形成。此外,在 cDNA 表达的人肝 CYP3A4 的温孵液中,山冈囊吾碱被代谢成相应的吡咯代谢物,而山冈囊吾碱在 cDNA 表达的人肝 CYP1A2、CYP2C9、CYP2D6 和 CYP2E1 温孵液中无代谢。**结论** 山冈囊吾碱在人肝微粒体内的主要代谢方式是形成肝毒性吡咯代谢物,CYP3A 作为主要的 CYP450 酶参与了山冈囊吾碱的代谢及其肝毒性吡咯代谢物的形成。CYP3A 在山冈囊吾碱所致的肝毒性中发挥了重要的作用。

关键词:山冈囊吾碱; 肝脏; 微粒体; 生物转化

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