Inhibitory effect of *Aristolochia* fruit on Cytochrome P450 isozymes *in vitro* and *in vivo*

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Abstract: The mature fruits of *Aristolochia debilis*, known in China by the name, "Madouling" has been popularly prescribed in Asia, particularly in China, to treat a range of conditions including gynaecological problems, arthritis and wound healing**.** This study was aimed to evaluate the potential effect of Madouling on the cytochrome P450 (CYP) isozymes *in vitro* in microsomal fractions and *in vivo* in rats. The influence of Madouling on CYPs activity was first explored by an *in vitro* method of estimating levels of four respective metabolites in rat liver microsomes. The results were re-examined *in vivo* in rats by using a cocktail approach involving the probe drugs theophylline, tolbutamide, chlorzoxazone and dapsone. Pharmacokinetics of the four substrates was used to analyze the activities of the targeting isozymes. *In vitro* study revealed that Madouling decreased the activity of CYP1A2, 3A1 and 2E1. However, no significant influence on CYP2C6 was found. These results coincided with those of *in vivo* study to a great degree except that *in vivo* estimation the herb didn't inhibit CYP1A2 significantly. From the data obtained, Madouling is suggested as a candidate for clinically significant CYP interactions. Drug co-administrated with Madouling may need dose adjustment.

Keywords: *Aristolochia debilis*; Cytochrome P450; *in vivo*; *in vitro*; herb-drug interaction.

INTRODUCTION

The use of *Aristolochia* species in Chinese popular medicine has a long tradition. Some species have been used in the form of crude drugs as anodynes, antiphlogistics and detoxicants, especially in Mainland, China (Jiangsu New Medicine College, 1977). While *Aristolochia* species have now been disappeared from medicinal market in many countries due to its severe side effect caused by aristolochic acid (Arlt *et al*., 2002; Cosyns, 2003), they are popularly used in Asia (Ernst, 2012).The mature fruits of *Aristolochia debilis* (also called "Madouling" in Chinese) is one of them, which is still included in Chinese Pharmacopoeia (2010) and prescribed in Asia for the treatment of snakebite, tuberculosis and as antihypertensive agents (Metzger and Perry, 1980).

Cytochrome P450 (CYP450) is a large family of heme-containing monooxygenase enzymes involved in the metabolism of a variety of substances ranging from endogenous substances to xenobiotics, including carcinogens, drugs and environmental pollutants (Parke *et al*., 1991). Inhibition or induction of CYP450 can result in drug-drug interaction (Gao *et al*., 2013; Lin, 2006; Polasek *et al*., 2006). Despite its long tradition, there are few reports on the drug interactions between Madouling and other medications.

The purpose of the current study was to investigate the effect of Madouling on major CYP450: CYP1A2, CYP2C6, CYP2E1 and CYP3A1, which account for about 13%, 20%, 7% and 30% respectively of the hepatic content of CYP isoenzymes (Shimada *et al*., 1994), in microsomes and *in vivo* in rats.

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MATERIALS AND METHODS

Materials

6-hydroxychlorzoxazone, dehydronifedipine, 4-hydroxytolbutamide, NADPH tetrasodium salt and bicinchoninic acid (BCA) Protein Assay Kit were from Sigma Chemical Co. (St. Louis, MO, US). Phenacetin, theophylline, paracetamol, chlorzoxazone, tolbutamide, dapsone, nifedipine, tinidazole (internal standard), cimetidine (CIM) and diethyl dithiocarbamate (DDC) were obtained from Zhengzhou Huawen Chemical CO. LTD. (Henan, China). HPLC grade methanol, ethyl acetate and acetonitrile were purchased from Honeywell Burdick & Jackson (Muskegon, USA). Formic acid and phosphoric acid were bought from Chengdu Kelong Chemical Reagent Factory (Sichuan, China). Deionized water up to a resistivity of 18.2 MΩ was purified with an Elga water purification system (London, UK).

Madouling was purchased from local Chinese TCM shops. It was identified by Professor Wang Jun in Sun Yat-sen University. The herb was grinded into powder and sieved through a100 mesh before use.

Animals

The study was supported by the Animal Ethics Committee of Sun Yat-sen University (Certificate No.SCXK 2011-

0029). The research was conducted according to accepted principles for laboratory animal use and care. Specific pathogen free (SPF) grade Wistar male rats weighing 180-200g were provided by Sun Yat-sen University Laboratory Animal Center. Throughout the experiment, four animals were housed per cage in an SPF environment with 12 h light/12 h night cycle and maintained at 20-26°C, 40-70% relative humidity. They were fed standard rodent diet with water *ad libitum*.

Study design

In vitro Assessment of CYPs Activity

Four groups each containing five rats was designed: control group, Madouling group (625 mg/ kg/ day), DDC group (25mg/kg/day) and CIM group (100 mg/ kg/ day). Madouling powder was suspended in 1% CMC-Na solution and orally given once a day for 7 days; control group was orally given 1% CMC-Na solution; CIM and DDC were orally given from the fourth day. All rats were fasted but with free access to water before sacrificing and removing liver samples.

Rat microsomes were prepared using a differential centrifugation method (Pan *et al*., 2009). The microsomes were suspended in Tris-HCl buffer solution (pH 7.4, contained 20% glycerol), aliquoted into several small volumes, and kept at -80 °C before analysis. Protein concentration of the prepared microsomes was determined using BCA. Microsome incubation and analysis method were according to a previous reported paper (Yao *et al*., 2012).

In vivo assessment of CYPs activity

Rats were divided into four groups each containing five: control group, Madouling low (312.5mg/kg/day), medium (625mg/kg/day) and high (937.5mg/kg/day) dose group. Madouling powder was suspended in 1% CMC-Na solution and orally given once a day for 7 days; control group received equivalent CMC-Na solution.

Pharmacokinetics of probe-drugs was carried out on the 8th day. After fasting but with free access to water for 12 h, animals were administrated orally with probe-drugs theophylline (30mg/kg), tolbutamide (10mg/kg), dapsone (20 mg/ kg) and chlorzoxazone (50mg/kg) which were suspended in 1% CMC-Na solution. Blood samples were collected into heparinized tubes at 15, 30 min, 1, 1.5, 2, 2.5, 4, 6, 8, 12, 24, 30 and 36 h. Fifty microliter plasma sample was separated and analyzed according to a validated HPLC-UV method (unpublished data).

Data analysis

Pharmacokinetic Analysis

Winnonlin (Version 5.0.1, SCI Software, Statistical Consulting, Inc., Apex, NC, USA) was used to calculate pharmacokinetic parameters using a non-compartmental pharmacokinetic (NCA) model. Peak concentrations (C_{max}) and time for C_{max} (T_{max}) were directly obtained from the experimental data. Area under the concentration-time curve ($AUC_{0-\infty}$) was calculated by the trapezoidal rule. Ke was the elimination rate constant of terminal phase and the apparent elimination half-life ($T_{1/2}$) was calculated from 0.693/ Ke.

STATISTICAL ANALYSIS

All the means were expressed with their standard deviation (mean ± SD). Statistical comparisons were performed using one-way ANOVA, followed by Dunnett test. $P<0.05$ was considered as statistically significant.

RESULTS

Constituents of madouling analyzed by HPLC

Fig. 1 showed the HPLC-UV chromatograms of 70% methanol extraction of Madouling and aristolochic acids A (peak 12). Comparing the HPLC chromatograms and PDA-UV spectra (fig. not shown) of Aristolochia fruit with literature (Wei *et al*., 2005; Zhang *et al*., 2006), we identified peak 4 as aristolochic acids C, peak 5 as 7-hydroxyaristolochic acid A, peak 6 as aristolochic acid D and peak 9 as aristolochic acid B.

In vitro assessment of CYPs activity

CYP isozyme activity was expressed as pmoL metabolite/ mg protein/ min. The effect of Madouling (625 mg/ kg) on four targeting isozymes was detailed in fig 2. It was apparent that Madouling powder didn't significantly affect the activity of CYP 2C6, but did inhibit CYP2E1, CYP3A1, CYP 1A2 in rat microsomes.

In vivo assessment of CYPs activity

Changes in body weight

Animal body weight was monitored every day during the period of herb administration. Rats in control group steadily gained body weight whereas high and middle dose Madouling treated groups began to lose weight from the 4th day. The low dose group had less impact on body weight compared with high or middle dose, but the weight did not increase as much as that in control group (fig. 3).

Effect of madouling on CYP1A2 activity

The effect of Madouling at different doses on the pharmacokinetics of theophylline was shown in table 1. Compared with the control group the $T_{1/2}$ (P=0.04), T_{max} (P=0.021), $MRT_{0-\infty}$ (P=0.002) in high dose administered group were significantly increased. Therefore, the herb didn't influence the activity of CYP1A2 significantly.

Effect of madouling on CYP2C6 activity

Table 2 showed the pharmacokinetic behavior of tolbutamide in all groups. Madouling showed slight inhibitory effect on CYP2C6 (C_{max}, $AUC_{0-\infty}$), and the clearance was also slowed down despite un-obviously.

Table 1: Pharmacokinetic parameters of theophylline, in control and Madouling pretreated groups

		Control	Low (312.5 mg/ kg)	Medium (625 mg/ kg)	High (937.5 mg/ kg)
$T_{1/2}$	hr	4.60 ± 1.17	5.57± 1.42	7.08 ± 2.19	5.80 ± 2.18
T_{max}	hr	2 ± 0.55	2.08 ± 1.07	1.5 ± 0.63	1.92 ± 0.38
C_{max}	ug/mL	28.32 ± 2.09	28.69 ± 2.42	26.90 ± 2.98	36.57 ± 6.03*
C_{max}/Dose	ug/mL/mg	3.65 ± 0.35	3.61 ± 0.34	3.65 ± 0.49	5.22 ± 0.91*
$AUC_{0-\infty}$	hr*ug/mL	317.08 ± 31.92	334.02 ± 44.28	304.68 ± 61.43	353.53 ± 66.25
$AUC_{0-\infty}$/Dose	hr*ug/mL/mg	40.98 ± 5.50	42.30 ± 7.99	41.77 ± 10.94	50.69 ± 11.33
V/F	mL	161.71 ± 34.05	191.07 ± 37.63	257.12 ± 95.25	172.84 ± 80.96
Cl/F	mL/hr	24.77 ± 3.30	24.42 ± 5.05	25.74 ± 8.61	20.50 ± 4.18
$MRT_{0-\infty}$	hr	8.18 ± 1.15	8.60 ± 1.83	9.24 ± 1.58	7.24 ± 0.86

Table 2: Pharmacokinetic parameters of tolbutamide in control and Madouling treated groups

		Control	Low (312.5 mg/kg)	Medium (625 mg/kg)	High (937.5 mg/kg)
$T_{1/2}$	hr	9.16 ± 1.26	16.93 ± 10.17	11.82 ± 2.29	7.85 ± 2.97
T_{max}	hr	2.75 ± 0.99	3.4 ± 1.95	2.91 ± 1.80	4.08 ± 2.11
C_{max}	ug/mL	22.50 ± 5.79	21.12 ± 8.69	27.01 ± 7.17	32.58 ± 13.39
C_{max}/Dose	ug/mL/mg	8.65 ± 2.00	7.86 ± 3.13	11.18 ± 3.72	14.08 ± 6.19
$AUC_{0-\infty}$	hr*ug/mL	328.06 ± 66.25	418.72 ± 129.68	422.60 ± 59.87	402.61 ± 168.45
$AUC_{0-\infty}$/Dose	hr*ug/mL/mg	126.21 ± 21.89	157.79 ± 51.58	173.46 ± 37.33	174.30 ± 78.30
V/F	mL	107.91 ± 27.59	169.02 ± 110.15	101.27 ± 23.31	98.70 ± 104.59
Cl/F	mL/hr	8.10 ± 1.23	7.09 ± 3.01	6.03 ± 1.53	7.30 ± 4.64
MRT_{0-t}	hr	14.43 ± 2.58	23.88 ± 12.15*	17.37 ± 2.09	11.90 ± 1.64

Table 3: Pharmacokinetic parameters of dapsone, a CYP3A1 substrate in control and Madouling treated groups

		Control	Low (312.5 mg/kg)	Medium (625 mg/kg)	High (937.5 mg/kg)
$T_{1/2}$	hr	9.32±4.09	14.36±4.72	15.41±4.96*	7.57±2.53
T_{max}	hr	2.08±1.07	1.30±0.45	1.33±0.41	1.08±0.58
C_{max}	ug/mL	2.11±0.81	2.38±0.74	2.91±0.40	2.09±0.42
C_{max}/Dose	ug/mL/mg	0.40±0.15	0.45±0.13	0.58±0.076*	0.45±0.09
$AUC_{0-\infty}$	hr*ug/mL	27.38±8.22	35.28±12.27	50.88±21.24*	23.82±5.35
$AUC_{0-\infty}$/Dose	hr*ug/mL/mg	5.27±1.49	6.74±2.33	10.50±5.54*	5.10±1.20
V/F	mL	2787.25±1470.85	3347.63±1267.39	2240.79±10.79	2270.83 ± 1146.84
Cl/F	mL/hr	199.80±43.91	163.11±54.61	110.21±35.79*	206.36 ± 55.56
MRT_{0-t}	hr	15.53±4.26	19.57±5.99	22.02±7.44*	12.46 ± 2.47

Table 4: Pharmacokinetic parameters of chlorzoxazone in control and Madouling treated groups

		Control	Low (312.5 mg/kg)	Medium (625 mg/kg)	High (937.5 mg/kg)
$T_{1/2}$	hr	0.55 ± 0.35	0.72 ± 0.40	0.73 ± 0.59	1.03 ± 0.95
T_{max}	hr	0.29 ± 0.10	0.58 ± 0.20*	0.33 ± 0.13	0.29 ± 0.10
C_{max}	ug/mL	8.37 ± 2.89	13.21 ± 4.25	14.68 ± 3.08*	15.079 ± 5.20*
C_{max}/Dose	ug/mL/mg	0.67 ± 0.24	0.99 ± 0.30	1.22 ± 0.37*	1.30 ± 0.49*
$AUC_{0-\infty}$	hr*ug/mL	9.44 ± 3.44	21.24 ± 10.21*	17.87 ± 6.22	22.88 ± 11.47*
$AUC_{0-\infty}$/Dose	hr*ug/mL/mg	0.73 ± 0.27	1.57 ± 0.67	1.45 ± 0.48	1.95 ± 0.94*
V/F	mL	1072.91± 68.06	660.11 ± 11.41	726.06±432.50	774.41 ± 411.95
Cl/F	mL/hr	1601.51±784.08	757.30± 60.96*	775.34 ± 324.19*	643.80 ± 344.87*
MRT_{0-t}	hr	0.99 ± 0.35	1.37 ± 0.56	1.01 ± 0.22	1.64 ± 1.14

Data expressed as mean ± SD (n =5). *$p < 0.05$ vs. control group.

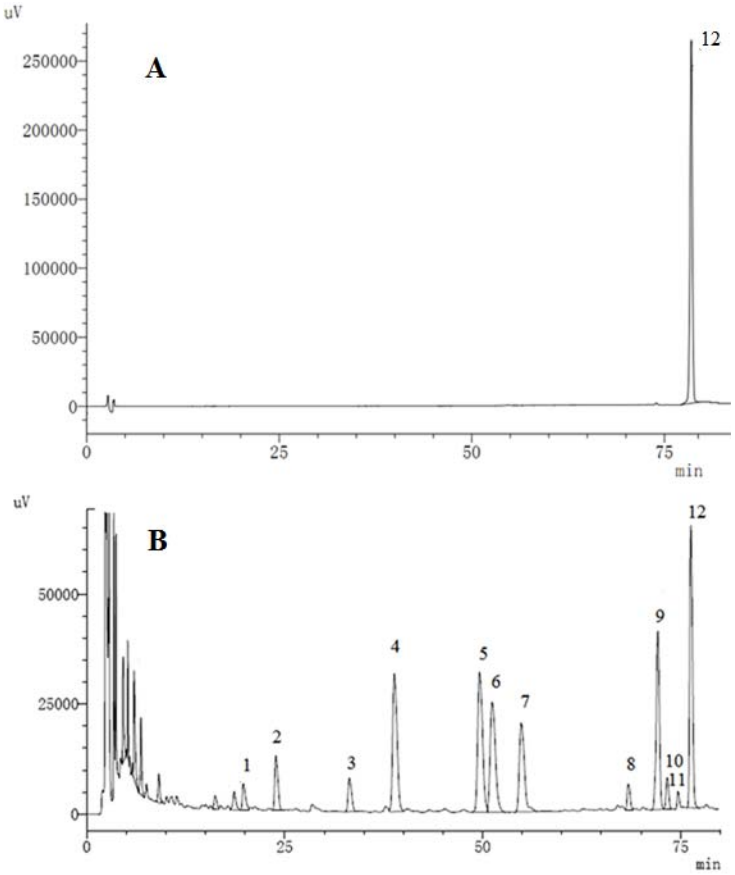

Fig. 1: Constituent analysis of Madouling by HPLC-UV. Conditions: mobile phase, 1% Acetic acid buffer–acetonitrile; flow rate, 1 mL/min; detection wavelength, 260 nm; column temperature, 45°C; injection volume, 10 µL. Peak 4 as aristolochic acids C, 5 as 7-hydroxyaristolochic acid A, 6 as aristolochic acid D, 9 as aristolochic acid B, 12 as aristolochic acids A.

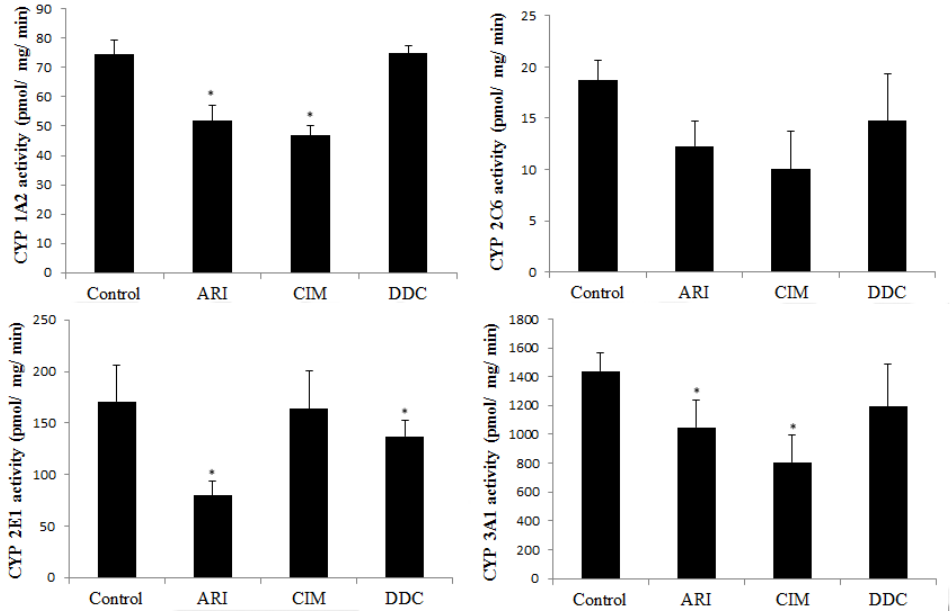

Fig. 2: Influence of Madouling (625 mg/ kg) on the activities of CYP1A2, CYP2C6, CYP2E1 and CYP3A1 in rat microsomes. n = 5, * $p < 0.05$ vs. control group. CIM: an inhibitor of CYP1A2 and CYP3A1; DDC: an inhibitor of CYP2E1. ARI: Fruit of *Aristolochia* (Madouling)

Effect of madouling on CYP3A1 activity

CYP3A1 activity was evaluated by comparing the pharmacokinetic behaviors of dapsone in control group and Madouling treated groups. One-way ANOVA indicates that middle dose treatment markedly influence the enzyme. As shown in table 3, the level of T1/2 (P= 0.048), Cmax/Dose (P=0.038), AUC0-∞ (P=0.014) and AUC0-∞/Dose (P=0.021) increased compared to non-Madouling treatment group, whereas the Cl/F (P=0.009) decreased dramatically. Therefore, Madouling inhibited CYP3A1.

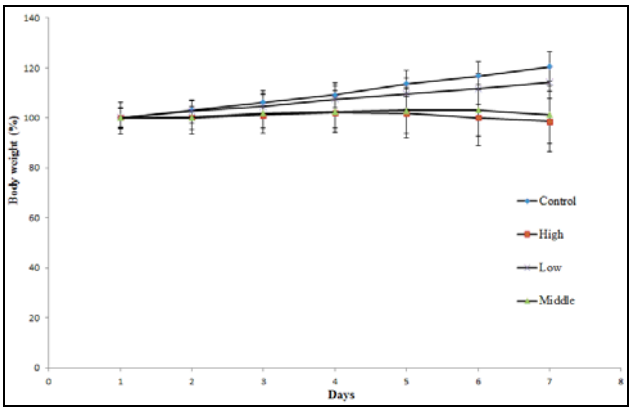

Fig. 3: Changes in body weight of rats in four experimental groups. Date expressed as mean ± stand deviation (n=5).

Effect of madouling on CYP2E1 activity

Pharmacokinetic profiles of chlorzoxazone after Madouling treatments were used to describe the activity of CYP2E1. Pharmacokinetic parameters in table 4 further showed that high dose (937.5mg/kg/day) of Madouling tends to increase the level of C_{max} (*P*= 0.024), C_{max}/Dose (*P*=0.016), $AUC_{0-\infty}$ (*P*=0.027) and $AUC_{0-\infty}$/Dose (*P*= 0.008) but reduce the oral clearance (*P*= 0.007). The low dose (625mg/kg/day or 312.5mg/kg/day) improves the ability of absorption, whereas decreases the clearance. Overall, Madouling significantly inhibited the CYP2E1 activity.

DISCUSSION

In the *in vivo* study, we applied a cocktail approach to estimate the effect of *Aristolochia contorta* on the four CPY isozymes in rats, ie, CYP1A2, CYP2C6, CYP2E1 and CYP3A1, based on the pharmacokinetic behaviors of the four probe substrates theophylline, tolbutamide, chlorzoxazone, and dapsone respectively. From the data analysis, the middle dose of *Aristolochia contorta* powder (625 mg/kg) might inhibit the CYP3A1 activity significantly, as indicated by increase in the area under curve ($AUC_{0-\infty}$) and decrease in the clearance (CL) of dapsone. The CYP2E1 enzyme was inactivated by *Aristolochia contorta* powder of the tested dose. But *Aristolochia contorta* powder has less significant effect on CYP1A2 and CYP2C6.

Based on the in vivo experiment, we choose to investigate the middle dose of *Aristolochia contorta* (625 mg/ kg) in the in vitro study.

To the best of our knowledge, it is the first report covering the evaluation of the inhibitory effect of *Aristolochia* species on CYPs. From the present study, it is obvious that Madouling can influence the activity of CYPs.

CONCLUSIONS

In conclusion, Madouling is a potent inhibitor of CYP3A1 and CYP2E1 in rats and it is possible that CYP1A2 is inhibited by Madouling in liver. Hence, drugs or herbal products co-administrated with Madouling may need dose adjustment to avoid the complications due to the increase bioavailability.

ACKNOWLEDGEMENTS

The study was supported by the innovative training program of college students in Guangdong province (No. 1055812312).

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