Full paper

Induction of CYP3A by morroniside in rats

Shan Xiong a, b, Jinglai Li b, Wenpeng Zhang b, Xiaoying Wang b, Zhenqing Zhang b, * 

a Institute of Materia Medica, Shandong Academy of Medical Sciences, Jinan, Shandong 250062, China 

b Key Laboratory of Drug Metabolism and Pharmacokinetics, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China

A B S T R A C T

Morroniside is one of the most important iridoid glycosides in the herbal drug Cornus officinalis Sieb. et Zucc. The current study was designed to investigate the ex vivo and in vivo effects of morroniside on CYP3A activity in rats after treatment with morroniside for 7 days (at 10, 30, 90 mg/kg, i.g.). Morroniside was found to induce CYP3A. According to the ex vivo experiment, the activity of CYP3A was measured by the quantification of 1-hydroxymidazolam, which was the metabolite from CYP3A probe substrate, midazolam. The concentration of 1-hydroxymidazolam was determined by using a validated liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) method. The levels of messenger RNA (mRNA) and protein of CYP3A were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting analysis, respectively. The pharmacokinetics of midazolam in rats after treatment with morroniside for 7 days (at 10, 30, 90 mg/kg, i.g.) were investigated in vivo. After treatment with morroniside, the activity, mRNA and protein expression of CYP3A were significantly induced and the absorbance and bioavailability of midazolam in rats were reduced. The results indicated that morroniside could induce the activity of CYP3A.

© 2015 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Fructus Corni, named Shan-Zhu-Yu in Chinese, is the dried sarcocarp of Cornus officinalis Sieb. et Zucc. It has been used for thousands of years as an important traditional Chinese medicine in China, which exhibits a number of biological activities, including immunological regulation, antishock, antiarrhythmia, antibiosis, hypoglycemic, etc (1, 2). Morroniside is one of the most important iridoid glycosides, which are extracted from sarcocarp of C. officinalis. Its chemical structure is shown in Fig. 1 (3). Morroniside has been proved to be effective in invigorating stomach (4), preventing diabetic angiopathies (5) and renal damage (6), inhibiting lipid metabolism and inflammation (7) and reducing bone resorption (8). In recent studies, it was also reported that morroniside had significant neuroprotective effect (9–11). Extensive studies have been carried out to investigate the pharmacokinetics of morroniside (3, 12, 13), but the direct relationship between morroniside and cytochrome P450 is unclear. The interaction between morroniside and cytochrome P450 may cause herb-drug interaction, and may potentially explain the use of herb-drug combination.

Using a probe substrate to determine the metabolic activities of cytochrome P450 is used widely in present drug–drug interaction studies. In this study, the effects of morroniside on the activity, mRNA and protein expression of CYP3A were investigated ex vivo by using rat liver microsomes. The effects of morroniside on the metabolism of midazolam, the probe substrate of CYP3A, were investigated in vivo. Combined ex and in vivo parameters might better reveal the potential of direct induced effects of morroniside on CYP3A in rats.

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats (200–240 g) were provided by Beijing Institute of Pharmacology and Toxicology (Certificate no. SCXK 2012–0004). Rats were housed in cages with 12 h day light/12 h night cycle at ambient temperature (about 23–25 °C). Animals had free access to tap water and commercially available standard...
biotechnology, Inc. (Santa Cruz, CA, USA). Acetonitrile, formic acid
bioBasic-18 column (2.1 mm, Thermo) and a mobile phase gradient (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid). An elution lasting 4.5 min was carried out. The test was accomplished by a mass spectrometer using selected reaction monitoring (SRM) in positive mode.

2.4. Preparation of rat hepatic microsomes

Rats were randomized into 5 groups (n = 3 per group) receiving control vehicle, dexamethasone at 100 mg/kg/day, for 4 consecutive days, intraperitoneally and morroniside at 10, 30 or 90 mg/kg/day by gavage for 7 consecutive days. On day 8, rats were fasted overnight with free access to water for at least 12 h. Before homogenization, the livers were perfused by ice phosphate buffer (0.1 mM, pH 7.4) through portal vein. The livers were collected and transferred to ice immediately after the surgical excision, cut into pieces, and stored at –80 °C until the microsomes were prepared. After liver samples were thawed and weighed, 3vols of ice-cold homogenization medium (50 mM Tris–HCl buffer at pH 7.4 containing 0.25M sucrose) were added. The tissue was chopped using scissors and homogenized with an automatic homogenizer at 500 rpm (IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged at 10 000 r/min for 20 min at 4 °C using a Beckman centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Then the supernatant was centrifuged at 105 000 r/min for 60 min at 4 °C using an L8–70 Beckman ultracentrifuge. The resulting microsomal pellet was resuspended with the above homogenizing buffer. And the microsomal suspensions were stored at –80 °C (14). Protein concentrations of microsomes were determined by using the Lowry method (15).

2.5. Measurements of CYP3A activity

The activity of CYP3A was characterized by the metabolism of the probe substrate, midazolam. The 250 μL reaction mixture contained potassium phosphate buffer (pH 7.4, final concentration = 0.1 M); microsomal protein (final concentration = 0.5 mg/mL), NADPH (final concentration = 10 mM) and midazolam (final concentration = 5 μM). The mixture was pre-incubated in a 37 °C water bath with gentle shaking for 5 min before addition of NADPH. Then the reaction was initiated by addition of 10 mM NADPH and terminated with addition of 750 μL ice-chilled acetonitrile containing 80 ng/mL 1-phenacylamine after 60 min incubation. The metabolites of the CYP3A probe substrate from all incubation were determined by previously well-validated sensitive LC-MS/MS method. The tubes were vortex-mixed for 1 min and centrifuged at 14 000 r/min for 10 min, and 15 μL of the supernatant was analysed.

2.6. Determination of mRNA levels by RT-PCR

Approximately 100–200 mg of liver tissue was homogenized and total RNA was isolated with the Trisol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined and the quality of RNA solutions was assessed by an ultraviolet light spectrophotometer. For reverse transcription, 1 μL of RNA was used in a 20 μL reaction mixture utilizing RevertAid™ M-MuLV RT (Fermentas, Hanover, MD, USA) according to the supplier’s instructions. The typical reaction mix (final volume = 20 μL) contained 10 μL of Platinum SYBR Green qPCR SuperMix-UDG, 2 μL each of the forward and reverse primers and DEPC-treated water. The reverse transcription reaction was performed for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C (16).

2.7. Western blotting analysis

Liver microsomes were prepared from rats in all groups. The proteins were subjected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Sodiumdodecyl sulphate (SDS)-PAGE was performed as described (17). Membrane was incubated overnight at 4 °C with mouse monoclonal antibody against rat CYP3A at 1:1000 dilution in TBS containing 5% milk. At the end of the incubation, the membrane was rinsed with TBS and incubated for 90 min at room temperature with the secondary anti-rabbit antibody conjugated with horseradish peroxidase. Gel image was visualized using Chem-Doc (Bid-Rad) and signal intensity of immunoreactive bands was analyzed by Quantity One software (Bid-Rad) (18–20).

2.8. Effect on the pharmacokinetics of midazolam

The pretreatment was the same as above. Midazolam was dissolved to 5 mg/mL in ethanol and then slowly diluted to 0.25 mg/L.

Fig. 1. Chemical structure of morroniside.
mL with 0.9% Sodium chloride injection. After the 7 day treatment, a single midazolam (2.5 mg/kg, intragastric administration) was given. Blood samples (0.3 mL) were collected via the jugular vein before and at 2, 5, 15, 30, 60, 120, 240 and 360 min after midazolam administration and the heparin was used as an anticoagulant. The same volume of saline was added each time after a blood sample was collected. Plasma was obtained from the samples by centrifugation at 3000 r/min for 15 min at 4 °C. Samples were prepared using the standard protein precipitation method consisted of adding 100 μL of plasma to a centrifuge vial, followed by 300 μL of acetonitrile containing 80 ng/mL L-phencyonate. The tubes were vortex-mixed for 1 min and centrifuged at 14 000 r/min for 10 min and 15 μL of the supernatant was analysed. All the data were processed by non-compartmental analysis using the DAS 2.0 package (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

2.9. Statistics

Experimental data were expressed as mean ± standard deviation (SD) of three replicates. Statistical analyses were performed with Microsoft Office Excel 2003. The significance of the difference between groups for continuous variables was evaluated with analysis of variance (ANOVA). Differences between two groups were analysed using the t-test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of morroniside treatment on rat CYP3A activity

CYP3A activity was evaluated by comparing the amount of 1'-hydroxymidazolam in control group, morroniside treatment groups and dexamethasone treatment group. Rat CYP3A activity was increased 2.57-fold by dexamethasone treatment. Compared with the control, CYP3A activity was increased 1.51-, 1.89- and 2.31-fold by morroniside treatment at 10, 30 and 90 mg/kg/day, respectively (Fig. 2).

![Fig. 2. Relative activity of CYP3A after morroniside treatment in rats. Rats were pretreated with morroniside (10, 30 and 90 mg/kg/d, i.g., 7d) or dexamethasone (100 mg/kg/d, i.p., 4d) and the control group. Dexamethasone group was used as the positive group. The activity of CYP3A was evaluated by the formation rate of 1'-hydroxymidazolam. Data are mean ± SD. n = 3. *p < 0.05, **p < 0.01, compared with control (saline treatment).]

3.2. Effect of morroniside treatment on rat CYP3A mRNA expression

The effects of morroniside treatment on the expression of CYP3A were examined. Total RNA was extracted from liver samples in all groups. cDNAs were prepared by reverse transcription and the levels of CYP3A mRNA were determined by RT-PCR. The mRNA levels of CYP3A significantly increased in morroniside treatment group compared with the control group (Fig. 3). Morroniside treatment at 10, 30 and 90 mg/kg/day increased the mRNA expression of CYP3A 1.13-, 1.43- and 1.79-fold, respectively, whereas dexamethasone treatment increased the mRNA expression 2.35-fold.

![Fig. 3. Relative mRNA expression of CYP3A. Rats were pretreated with morroniside (10, 30 and 90 mg/kg/d, i.g., 7d) or dexamethasone (100 mg/kg/d, i.p., 4d) and the control group. Dexamethasone group was used as the positive group. Data are mean ± SD. n = 3. *p < 0.05, **p < 0.01, compared with control (saline treatment).]

3.3. Effect of morroniside treatment on rat CYP3A protein expression

Consistently, western blotting analysis showed that protein levels of CYP3A were significantly increased in rats treated with morroniside (Fig. 4). The CYP3A content in hepatic microsomes of rats treated with morroniside at 10, 30 and 90 mg/kg/day increased 1.23-, 1.41- and 1.44-fold, respectively, whereas dexamethasone treatment increased the protein expression 1.66-fold.

![Fig. 4. Protein expression of CYP3A. Rats were pretreated with morroniside (10, 30 and 90 mg/kg/d, i.g., 7d) or dexamethasone (100 mg/kg/d, i.p., 4d) and the control group. Dexamethasone group was used as the positive group. GAPDH was used as control for the normalization of CYP3A density.]

3.4. Effect of morroniside treatment on the pharmacokinetics of midazolam

To obtain further information of the effect of morroniside on the activity of CYP3A, an in vivo study was also carried out. CYP3A
activity was evaluated by comparing pharmacokinetic behaviours of midazolam in all groups after treatment with morroniside. Fig. 5 showed the plasma concentration–time profiles of midazolam. 7-day treatment of morroniside also decreased the AUC(0–6h) of midazolam. The AUC(0–6h) of midazolam with no morroniside treatment was 5283.76 ± 1514.82 min × ng/mL, compared with 4122.56 ± 126.29 min × ng/mL after 10 mg/kg morroniside treatment (a decrease of 21.98%), 2565.69 ± 30.93 min × ng/mL after 30 mg/kg morroniside treatment (a decrease of 51.44%), 2065.34 ± 430.46 min × ng/mL after 90 mg/kg morroniside treatment (a decrease of 60.91%) and 1980.74 ± 69.09 min × ng/mL after 100 mg/kg dexamethasone treatment (a decrease of 62.51%). Treatment with morroniside at different doses increased the clearance (CL) of midazolam. The MRT decreased by 33.39% and 25.06% after treatment with 30 mg/kg and 90 mg/kg morroniside, respectively. Listed in Table 1 and Fig. 5.

4. Discussion

Many researches had shown that cytochrome P450 enzymes could be induced or inhibited by some drugs. The changes of cytochrome P450 levels or activities could affect the drug concentration of blood, the pharmacokinetic process and biological medicinal properties (21). Europe and the United States require that drug screens and metabolic researches based on the cytochrome P450 system should contain new drugs evaluations (22). This methodology for pharmacokinetic research in new chemical drugs development was also required by the Chinese CFDA.

The concomitant treatment of herbal supplements and synthetic drugs had become popular. Chinese herbal medicines contained various biologically active ingredients. As a result, the herb–drug interactions had become a common clinical problem. Some studies reported that the mechanisms underlying the interaction between herbal medicines and conventional drugs mainly involved induction or inhibition of the activities of metabolic enzymes and drug efflux proteins. Therefore, potential herb–drug interactions involving Chinese herbal medicines were worthy to study the cytochrome P450 system (23).

For the first time, we systematically investigated the effects of morroniside on the activity of CYP1A2, CYP2B6 and CYP3A in rats. The results of the study revealed the morroniside caused a dose-dependent induction of CYP3A activity, but it had no effect on CYP1A2 and CYP2B6 (data not published). Furthermore, we had shown that the morroniside treatment increased CYP3A protein and mRNA expression in rats. In this research, the antibody we used was not specific enough to distinguish CYP3A1 and CYP3A2, which were two main CYP3A isofoms and shared an 89% sequence similarity (24).

In vivo study showed that the CYP3A activity was increased, when morroniside (10, 30 and 90 mg/kg, i.g.) was daily for seven consecutive days. Further, RT-PCR analysis showed that the induction of mRNA expression by morroniside (10, 30 and 90 mg/kg, i.g.) was 1.13-, 1.43- and 1.79-fold higher, respectively, relative to the control. And the western blot analysis showed that the induction of protein expression by morroniside was 1.23-, 1.41- and 1.79-fold higher, respectively, relative to the control. The results were well correlated with the observed CYP3A activity increase. The observed AUC(0–6h) of midazolam in morroniside treated rats also indicated that morroniside indeed enhanced the CYP3A activity. The dexamethasone treated rat model was widely used for induction of CYP3A activity (25). In this model, as plasma concentration of midazolam depended upon CYP3A activity, the observed effects of dexamethasone on the AUC of midazolam were well in accordance with the earlier report (26).

The above findings showed that morroniside treatment could induce the CYP3A activity. So, it needed to pay attention to the drug–drug interactions.

5. Conclusions

CYP3A was an important member of the cytochrome P450 superfamily. In this study, we investigated the potential influence of morroniside on CYP3A in rats after multiple doses of morroniside (at 10, 30, 90 mg/kg, i.g., 7d). based on the CYP3A activity, mRNA, protein expression and pharmacokinetic behaviours of midazolam in rats. The morroniside treatment was shown to induce CYP3A activity. The results might provide a scientific explanation for traditional Chinese medicine compatibility.

### Table 1
Pharmacokinetics of midazolam after a 7 day treatment with morroniside in rats (mean ± SD, n = 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Control</th>
<th>Low (10 mg/kg)</th>
<th>Medium (30 mg/kg)</th>
<th>High (90 mg/kg)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax</td>
<td>µg/L</td>
<td>51.58 ± 24.19</td>
<td>33.78 ± 7.02</td>
<td>25.93 ± 8.94</td>
<td>14.28 ± 4.70</td>
<td>12.19 ± 3.17</td>
</tr>
<tr>
<td>Tmax</td>
<td>min</td>
<td>16.67 ± 5.77</td>
<td>26.67 ± 5.77</td>
<td>23.33 ± 5.77</td>
<td>20.00 ± 4.00</td>
<td>23.33 ± 5.77</td>
</tr>
<tr>
<td>MRT(0–6h)</td>
<td>min</td>
<td>113.68 ± 10.88</td>
<td>106.97 ± 24.49</td>
<td>75.72 ± 16.23*</td>
<td>85.19 ± 7.19**</td>
<td>132.39 ± 5.94*</td>
</tr>
<tr>
<td>T1/2</td>
<td>min</td>
<td>115.41 ± 69.09</td>
<td>114.16 ± 30.91</td>
<td>71.43 ± 18.01</td>
<td>99.66 ± 83.09</td>
<td>138.71 ± 40.81</td>
</tr>
<tr>
<td>CL/F</td>
<td>L/min/kg</td>
<td>0.51 ± 0.17</td>
<td>0.61 ± 0.02</td>
<td>1.13 ± 0.26*</td>
<td>1.16 ± 0.22**</td>
<td>2.05 ± 0.83*</td>
</tr>
<tr>
<td>AUC(0–6h)</td>
<td>min × µg/L</td>
<td>5283.76 ± 1514.82</td>
<td>4122.56 ± 126.29</td>
<td>2565.69 ± 473.33*</td>
<td>2329.91 ± 236.61*</td>
<td>1980.74 ± 244.00*</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, compared with control (saline treatment).
Conflicts of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

The authors are grateful to the National Major Scientific and Technical Special Projects for Innovative Drug of China (No. 2012ZX09301003- 001-007).

References