Inhibitory Effects of Seven Components of Danshen Extract on Catalytic Activity of Cytochrome P450 Enzyme in Human Liver Microsomes

Furong Qiu, Rong Zhang, Jianguo Sun, Jiye A, Haiping Hao, Ying Peng, Hua Ai, and Guangji Wang

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, China (F.Q., R.Z., J.S., J.A., H.H., Y.P., H.A., G.W.); and Yijishan Hospital, Wannan Medical College, Wuhu, China (F.Q.)

Received February 13, 2008; accepted April 8, 2008

ABSTRACT:
The potential for herb-drug interactions has recently received greater attention worldwide, considering the fact that the use of herbal products becomes more and more widespread. The goal of this work was to examine the potential for the metabolism-based drug interaction arising from seven active components (danshensu, protocatechuic aldehyde, protocatechuic acid, salvianolic acid B, tanshinone I, tanshinone IIA, and cryptotanshinone) of danshen extract. Probe substrates of cytochrome P450 enzymes were incubated in human liver microsomes (HLMs) with or without each component of danshen extract. IC50 and Ki values were estimated, and the types of inhibition were determined. Among the seven components of danshen extract, tanshinone I, tanshinone IIA, and cryptotanshinone were potent competitive inhibitors of CYP1A2 (Ki = 0.48, 1.0, and 0.45 μM, respectively); danshensu was a competitive inhibitor of CYP2C9 (Ki = 35 μM), and cryptotanshinone was a moderate mixed-type inhibitor of CYP2C9 (Ki = 8 μM); cryptotanshinone inhibited weakly and in mixed mode against CYP2D6 activity (Ki = 68 μM), and tanshinone I was a weak inhibitor of CYP2D6 (IC50 = 120 μM); and protocatechuic aldehyde was a weak inhibitor of CYP3A4 (IC50 = 130 and 160 μM for midazolam and testosterone, respectively). These findings provided some useful information for safe and effective use of danshen preparations in clinical practice. Our data indicated that it was necessary to study the in vivo interactions between drugs and pharmaceuticals with danshen extract.

Alternative therapies such as herbal or natural products are finding increasing use around the globe. As a consequence, herb-drug interactions do undoubtedly occur and may put individuals at risk. Recently, many metabolism-based interactions have been reported between drug and natural products. A typical example is the inhibition of CYP3A4 by grapefruit juice, which can result in an increase of bioavailability of CYP3A substrates (Bailey et al., 1998). Some furanocoumarins are the causative components in grapefruit juice for CYP3A4 inhibition (Edwards et al., 1996; Sahi et al., 2002). Schisandra extract has also a potent inhibitory effect on human liver microsomal erythromycin N-demethylation activity mediated by CYP3A4, and known components of schisandra fruit, gomisins B, C, G, and N and γ-shizandrin, have inhibitory effects on N-demethylation activity (Iwata et al., 2004). The components of herbal products may differ quantitatively and qualitatively, depending upon the cultivation areas, harvest time, and storage conditions. To predict the metabolism-based interactions between drugs and herbal products, major components of herbal products having inhibitory effects on cytochrome P450 (P450) activities should be identified, and their inhibition mechanism and inhibition kinetics should also be determined (Iwata et al., 2004).

Danshen, the dried root of *Salvia miltiorrhiza*, has been used for hundreds of years in the treatment of numerous ailments in China. Because of its properties of improving microcirculation, causing coronary vasodilatation, suppressing the formation of thromboxane, inhibiting platelet adhesion and aggregation, and protecting against myocardial ischemia, it is widely used either alone or in combination with other herbs for patients with cardiovascular diseases, in both China and other countries, including the United States (Cheng, 2007). The clinical efficacy for danshen has been confirmed by a systematic assessment on randomized controlled trials (Wang et al., 2004). Four known hydrophilic components (danshensu, protocatechuic aldehyde, protocatechuic acid, and salvianolic acid B) and three lipophilic components (tanshinone I, tanshinone IIA, and cryptotanshinone) are thought to be the major active components of danshen extract (Fig. 1) (Zhou et al., 2006). In recent years, some studies have been reported...
on the effects of danshen extract on P450 enzymes. A clinical case was reported that danshen caused over-anticoagulation of warfarin, which is CYP2C9 substrate (Yu et al., 1997). It was reported that treatment of rats with hydrophilic components of danshen extract elevated the area under the curve of warfarin, suggesting an inhibitory effect of hydrophilic components of danshen on CYP2C9 activity (Chan et al., 1995; Chan, 2001). Kuo and Ueng (Ueng et al., 2004; Kuo et al., 2006) reported that mouse CYP1A-, CYP2C-, and CYP3A-inducing agents were present in the lipophilic components of danshen extract. Ueng also reported that tanshinone IIA decreased 7-ethoxyresorufin O-deethylation and 7-methoxyresorufin O-demethylation activities in human liver microsomes (HLMs), suggesting that tanshinone IIA inhibited the activity of CYP1A2 in HLMs, and tanshinone I, tanshinone IIA, and cryptotanshinone inhibited the activity of cDNA-expressed CYP1A2 (Ueng et al., 2003). Our previous finding suggested that orally administered danshen extract had no significant effects on CYP1A2 in healthy volunteers (Qiu et al., 2008).

In light of the widespread use of danshen and incomplete knowledge regarding the effects of multiple components of the danshen extract on P450 enzymes, a detailed study is required to determine the effects of hydrophilic and lipophilic components of the danshen extract on human P450 enzymes. In this study, we chose seven major components, not danshen extract mixture, to examine whether they had inhibitory effects on catalytic activity of CYP1A2, CYP2C9, CYP2D6, and CYP3A4, which are the isoforms involved in the majority of clinically important drug-metabolized reactions, using HLMs in accordance with well documented assay conditions (Hickman et al., 1998; Walsky and Obach, 2004) and the current regulatory guidance (http://www.fda.gov/cder/guidance/index.htm), and the mechanism, kinetics, and the type of inhibition were also determined. The purpose of this study is to provide some useful information for safe and effective use of danshen preparation in clinical practice.

**Materials and Methods**

**Chemicals and Reagents.** Lipophilic components (tanshinone I, tanshinone IIa, and cryptotanshinone), hydrophilic components (danshensu, protocatechuic acid, protocatechuic aldehyde, and salvianolic acid B), and internal standards (codeine, prednisolone, and chlorzoxazone) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of all chemicals was above 99%, and the structures of seven components of danshen extract are shown in Fig. 1. Glucose 6-phosphate, NADP⁺, and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile, methanol, and ethyl acetate were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA). perchloric acid and other chemicals and solvents were all of analytical grade. The following compounds were purchased from Sigma-Aldrich: phenacetin, acetaminophen, furafylline; diclofenac, 4-hydroxydiclofenac, sulfaphenazole; midazolam, 1-hydroxymidazolam, testosterone, 6β-hydroxytestosterone, ketocconazole; and dextromethorphan, dextrophor, and quinidine.

**Human Liver Microsomes.** HLMs used in this study were provided by the Research Institute for Liver Disease Co. (Shanghai, China). The microsomes were prepared from ten individual human donor livers. The microsomes had been previously characterized for CYP1A2, CYP2C9, CYP2D6, and CYP3A4 activities by the Research Institute for Liver Disease Co.

**Incubation Procedure and Enzymatic Activity Assay.** The P450 enzymatic activities were characterized based on their probe reactions: CYP1A2 (phenacetin O-deethylation), CYP2C9 (diclofenac 4-hydroxylation), CYP2D6
(dichlormethane O-demethylation), and CYP3A4 (testosterone 6β-hydroxylation; midazolam 1-hydroxylation). Incubation mixtures were prepared in a total volume of 200 μl as follows: NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mM NADP, 10 mM magnesium chloride, 1 unit of glucose 6-phosphate dehydrogenase), 100 mM phosphate buffer, pH 7.4, probe substrates of P450 enzymes, and a range of concentrations of tested compound. Midazolam previously dissolved in water, and lipophilic components were dissolved in ethanol, whose final concentration in incubation mixtures was less than 1% v/v. There was a 5-min preincubation period at 37°C before the reaction was initiated by adding the NADPH-generating system. The reactions were conducted for 25 min for CYP1A2, 10 min for CYP2C9, 6 min for CYP2D6, 10 min for CYP3A4 (testosterone 6β-hydroxylation), and 5 min for CYP3A4 (midazolam 1-hydroxylation), respectively.

For each probe drug, preliminary experiments were performed to determine whether metabolism formation rates were linear with time of incubation and microsomal protein concentrations. The percentage of metabolites conversion was less than 20% of substrate added.

Enzyme Kinetics Analysis. The marker substrates concentrations of P450 enzymes were as follows: phenacetin, 5 to 400 μM; diclofenac, 1 to 400 μM; dextromethorphan, 1 to 400 μM; testosterone, 5 to 300 μM; and midazolam, 1 to 200 μM. The Km and Vmax values were determined using seven different concentrations of P450 marker substrates by nonlinear regression analysis of the enzyme activity-substrate concentration data using the Michaelis-Menten model (DAS, version 2.0; Anhui Provincial Center for Drug Clinical Evaluation, China).

Inhibition of P450 Enzymatic Activity Assay. A typical incubation mixture contained HLMs (protein concentration, 0.2 mg/ml), NADPH-generating system, probe substrates of P450 enzymes, and different concentrations of individual tested compound, 100 mM phosphate buffer, pH 7.4. Initially, in screening experiments, triplicate samples were run to generate IC50 value by incubating P450 marker substrates at a concentration approaching Km in the presence of five concentrations of seven components (final concentrations ranging from 0.5–200.0 μM) in the incubation mixture. Hydrophilic components were dissolved in water, and lipophilic components were dissolved in dimethyl sulfoxide, whose final concentration was less than 1% v/v in the mixture. Known inhibitors were run in parallel as positive controls: furafylline for CYP1A2, sulfaphenazole for CYP2C9, quinidine for CYP2D6, and ketoconazole for CYP3A4.

For each probe drug, preliminary experiments were performed to determine whether metabolism formation rates were linear with time of incubation and microsomal protein concentrations. The percentage of metabolites conversion was less than 20% of substrate added.

Enzyme Kinetics Analysis. The marker substrates concentrations of P450 enzymes were as follows: phenacetin, 5 to 400 μM; diclofenac, 1 to 400 μM; dextromethorphan, 1 to 400 μM; testosterone, 5 to 300 μM; and midazolam, 1 to 200 μM. The Km and Vmax values were determined using seven different concentrations of P450 marker substrates by nonlinear regression analysis of the enzyme activity-substrate concentration data using the Michaelis-Menten model (DAS, version 2.0; Anhui Provincial Center for Drug Clinical Evaluation, China).

Inhibition of P450 Enzymatic Activity Assay. A typical incubation mixture contained HLMs (protein concentration, 0.2 mg/ml), NADPH-generating system, probe substrates of P450 enzymes, and different concentrations of individual tested compound, 100 mM phosphate buffer, pH 7.4. Initially, in screening experiments, triplicate samples were run to generate IC50 value by incubating P450 marker substrates at a concentration approaching Km in the presence of five concentrations of seven components (final concentrations ranging from 0.5–200.0 μM) in the incubation mixture. Hydrophilic components were dissolved in water, and lipophilic components were dissolved in dimethyl sulfoxide, whose final concentration was less than 1% v/v in the mixture. Known inhibitors were run in parallel as positive controls: furafylline for CYP1A2, sulfaphenazole for CYP2C9, quinidine for CYP2D6, and ketoconazole for CYP3A4.

The Km values of danshen components were determined if their IC50 values were lower than 100 μM. Phenacetin (0, 37.5, 75.0, and 150.0 μM), diclofenac (0, 2.0, 5.0, and 10.0 μM), dextromethorphan (0, 2.0, 5.0, and 10.0 μM), and a range of concentrations (0.5–200.0 μM) of seven components from 55 to 4450 ng/ml. After incubation with the microsome system at 37°C, the reaction was stopped by adding 50 μl of 10% perchloric acid. The tubes were vortex mixed for 3 min and then centrifuged at 20,000 rpm for 10 min. Twenty microliters of the resulting supernatant was injected into the HPLC system (LC-2010CHT; Shimadzu, Kyoto, Japan) for analysis. The chromatographic separation was achieved using a Diamonsil C18 column (4.6 × 150 mm, 5 μm) (Dikma Technologies, Beijing, China) with mobile phase of methanol and 0.01 M phosphate buffer (18:82, v/v) at a flow rate of 1 ml/min. Acetaminophen was determined using a UV detector set at a wavelength of 275 nm.

Determination of Marker Metabolite. Acetaminophen, the marker metabolite of CYP1A2, was quantitated from 10 to 1000 ng/ml. After incubation with the microsome system at 37°C, the reaction was stopped by adding 50 μl of 10% perchloric acid. The tubes were vortex mixed for 3 min and then centrifuged at 20,000 rpm for 10 min. Twenty microliters of the resulting supernatant was injected into the HPLC system for analysis. The chromatographic separation was achieved using a Phenomenex Gemini C18 column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) and an gradient mobile phase of methanol and water at a flow rate of 1 ml/min. The interests were determined using a UV detector set at a wavelength of 245 nm.

1-Hydroxymidazolam, the marker metabolite of CYP3A4, was quantitated from 25 to 2000 ng/ml. After incubation with the microsome system at 37°C, 100 μl of acetoneitrile was added in all samples. The tubes were then centrifuged at 20,000 rpm for 10 min. Twenty microliters of the resulted supernatant was injected into the HPLC system for analysis. The chromatographic separation was achieved using a Phenomenex Gemini C18 column (4.6 × 250 mm, 5 μm; Phenomenex, Torrance, CA) and a gradient mobile phase of methanol and water at a flow rate of 1 ml/min. The peaks were determined using a UV detector set at a wavelength of 254 nm.

4-Hydroxydiclofenac, the marker CYP2C9 metabolite, was quantitated from 2.5 to 1600 ng/ml. After incubation with the microsome system at 37°C, freshly prepared samples containing 4-hydroxydiclofenac in a reaction mixture were acidified and extracted by 1 ml of ethyl acetate containing the internal standard chloroxazone. The organic layer was transferred to a new tube, evaporated to dryness, and reconstituted in 100 μl of methanol. The mixtures were centrifuged, and then an aliquot of the supernatants was analyzed by HPLC/mass spectrometry. Chromatography was performed using a C18 column (5 μm, 150 × 2.1 mm; Kromasil, Bohus, Sweden) and an HPLC system consisting of Shimadzu 10A series with a gradient mobile phase of 0.01% ammonium acetate and methanol. The flow rate was 0.2 ml/min.

### Table 1: Enzymatic kinetic parameters for P450 in human liver microsomes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay</th>
<th>Type</th>
<th>Km (μM)</th>
<th>Vmax (pmol/mg/min) pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin-O-deethylation</td>
<td>Simple</td>
<td>75</td>
<td>516</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4-hydroxylation</td>
<td>Substrate inhibition</td>
<td>6.8</td>
<td>3657</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>Simple</td>
<td>5</td>
<td>1300</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>Simple</td>
<td>75</td>
<td>6130</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam 1-hydroxylation</td>
<td>Substrate inhibition</td>
<td>3.5</td>
<td>1200</td>
</tr>
</tbody>
</table>

% activity remaining after preincubation

\[
\frac{\text{activity with inactivity}}{\text{activity with vehicle}} \times \frac{\text{NADPH}}{\text{NADPH}} \times 100
\]

% activity remaining without preincubation

\[
\frac{\text{activity with inactivity}}{\text{activity with vehicle}} \times \frac{\text{NADPH}}{\text{NADPH}} \times 100
\]
the analyte and internal standard was performed using a Shimadzu LCMS-2010A quadrupole mass spectrometer with an electrospray ionization (ESI) interface. The ESI source was set at negative ionization mode. The deprotonated ion of 4-hydroxydiclofenac at m/z 285 [M-H]− and the internal standard at m/z 294 [M-H]− were selected as monitoring ions, respectively.

Dextrorphan, the marker CYP2D6 metabolite, was quantitated from 10 to 500 ng/ml. After incubation with the microsome system at 37°C, freshly prepared samples containing dextrorphan in the mixture were extracted with 1 ml of acetonitrile containing codeine (internal standard), and an aliquot of the supernatants was analyzed by HPLC/tandem mass spectrometry. Chromatography was performed using Shimadzu VP-ODS (250 × 2.0 mm) and an HPLC system consisting of the Finnigan Surveyor (Thermo Electron Corporation, Waltham, MA) with a gradient mobile phase of 0.1% formic acid and methanol at a flow rate of 0.2 ml/min. Detection of the analyte and internal standard was performed using a Finnigan TSQ Quantum Discovery Max tandem mass spectrometry system (Thermo Electron Corporation) in the positive ion mode using an ESI source. Mass transitions (m/z) monitored were 258.1 → 157.03 for dextrorphan and 300.1 → 165.0 for internal standard at collision energy of 39 and 41 eV, respectively.

Statistics. The significance of the difference between the group means was assessed by one- or two-way analysis of variance and, if applicable, was followed by the Student Newman-Keuls test. A p value of 0.05 was considered to be statistically significant.

Results

Enzymatic Kinetic Parameters for P450 in HLMs. The K_m values for phenacetin O-deethylation, diclofenac 4-hydroxylation, dextromethorphan O-demethylation, testosterone 6β-hydroxylation, and midazolam 1-hydroxylation in HLMs were 75, 6.8, 5, 75, and 3.5 μM, respectively (Table 1). The V_max values for phenacetin O-deethylation, diclofenac 4-hydroxylation, dextromethorphan O-demethylation, and testosterone 6β-hydroxylation and midazolam 1-hydroxylation in human liver microsomes were 516, 3657, 1300, 6130, or 1200 pmol/min/mg protein, respectively (Table 1). The values coincided with a previous report (Yuan et al., 2002). We found a substrate inhibition phenomenon in the reaction of diclofenac 4-hydroxylation mediated by CYP2C9 and midazolam 1-hydroxylation mediated by CYP3A4 in HLMs.

Inhibition of P450 Activities by Seven Components of Danshen Extract in Human Liver Microsomes. To investigate whether these components of danshen extract affected the catalytic activity of P450, the probe reaction assays were conducted with various concentrations of danshen components. The specific inhibitors of P450 isoforms were used as positive controls. Results showed tanshinone I, tanshinone IIA, and cryptotanshinone had inhibition against CYP1A2 activities in HLMs with IC_{50} values of 0.75, 1.3, and 0.75 μM (Table 2). Danshensu and cryptotanshinone exhibited inhibition against the activity of CYP2C9 with IC_{50} values of 50 and 33 μM, respectively (Table 2). Cryptotanshinone and tanshinone I exhibited weak inhibitions against the activities of CYP2D6 with IC_{50} values of 75 and 120 μM, respectively (Table 2). Protocatechue aldehyde was a weak inhibitor of CYP3A4 (IC_{50} = 130 and 160 μM for midazolam and testosterone, respectively) (Table 2).

Time-Dependent Inactivation Screening Assay. Five inhibitors (danshensu, salvianolic acid B, tanshinone IIA, tanshinone I, or cryptotanshinone) reduced the CYP1A2 activities by the same amount when preincubations were performed in the presence and absence of NADPH, indicating reversible but not time-dependent inhibition. The same was true for cryptotanshinone (inhibition of CYP2C9), tanshine I and cryptotanshinone (inhibition of CYP2D6), and protocatechuic aldehyde (inhibition of CYP2D6). The percentage of remaining activity of CYP2C9 in HLMs was 49% when preincubated with danshensu and NADPH and 32% without NADPH. Our data implied that danshensu inhibited against CYP2C9 with 17% decrease in activity when preincubation for 10 min with microsomes in the time-dependent inhibition screening assay (Fig. 2).

Inhibition Kinetic Analysis. To further characterize the inhibition of P450 enzymes activity by components of danshen extract, enzyme inhibition kinetic experiments were carried out. Components of danshen extract that were found to weakly inhibit P450 enzymes (IC_{50} > 100 μM) in human liver microsomes were not estimated for their K_i values. Based on the analysis of nonlinear regression of inhibition data and Dixon plots presented in Figs. 3 to 5, tanshinone I, tanshinone IIA, and cryptotanshinone exhibited competitive inhibition against CYP1A2 activities with K_i values of 0.48, 1, and 0.45 μM for CYP1A2 (Table 3; Fig. 3, A–C), danshensu exhibited competitive inhibition against the activity of CYP2C9 with a K_i value of 35 μM (Table 3; Fig. 4A), and cryptotanshinone exhibited mixed-type inhibition against the activity of CYP2C9 in HLMs with a K_i value of 8 μM (Table 3; Fig. 4B). Cryptotanshinone exhibited a weak mixed-type inhibition against the activity of CYP2D6 in HLMs with a K_i value of 68 μM (Table 3; Fig. 5).

Discussion

We established probe substrate assay methods in HLMs conventionally applied to study inhibition toward P450 enzymes activities. Apparent K_m, V_max for P450 probe substrates, and the IC_{50} of positive control P450 inhibitors (furafylline, CYP1A2; sulfaphenazole, CYP2C9; quinidine, CYP2D6; and ketoconazole, CYP3A4) obtained from the present experiments were in good

---

**TABLE 2**

The IC_{50} values for the inhibition of danshen components on CYP enzymes activities

<table>
<thead>
<tr>
<th>Danshen Components</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50}</td>
<td>IC_{50}</td>
<td>IC_{50}</td>
<td>IC_{50}</td>
<td>IC_{50}</td>
</tr>
<tr>
<td>Hydrophilic components</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protocatechue acid</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Protocatechue aldehyde</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Danshensu</td>
<td>110</td>
<td>50</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Salvianolic acid B</td>
<td>105</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Lipophilic components</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanshinone I</td>
<td>0.75</td>
<td>&gt;200</td>
<td>120</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Tanshinone IIA</td>
<td>1.3</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Cryptotanshinone</td>
<td>0.75</td>
<td>33</td>
<td>75</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.63</td>
<td>0.3</td>
<td>0.12</td>
<td>0.05</td>
<td>0.045</td>
</tr>
</tbody>
</table>

*IC_{50} values for midazolam 1-hydroxylation, 3-demethylation, and testosterone 6\-hydroxylation.*

---

The significance of the difference between the group means was assessed by one- or two-way analysis of variance and, if applicable, was followed by the Student Newman-Keuls test. A p value of 0.05 was considered to be statistically significant.
agreement with values previously reported in the literatures (Yuan et al., 2002; Yao et al., 2007). It was interesting to find that substrate inhibition existed in both the diclofenac 4-hydroxylation catalyzed by CYP2C9 and midazolam 1-hydroxylation catalyzed by CYP3A4 in HLMs. These might contribute to that the substrate concentrations used in the present study were higher than those used in the literature at high concentration.

In the present study, the effects of seven components of danshen extract on activities of P450 enzymes were characterized by examining the activities of marker reactions in HLMs. Their time-dependent inactivations toward P450 enzymes were also assayed. It was the first detailed study that was conducted to investigate the P450 enzyme inhibition potential of the seven components of danshen extract in HLMs.

Among the seven components of danshen extract, cryptotanshinone, tanshinone I, and tanshinone IIA, which were all lipophilic components, exhibited a strong inhibition on CYP1A2, with IC50 of 0.75, 1.3, and 0.75 μM, respectively. In contrast, the inhibitory effects of the hydrophilic components on CYP1A2 were found to be weak (IC50 > 100 μM). The inhibitory potency of these lipophilic components was comparable with that of furafylline, which is a positive inhibitor of CYP1A2 (IC50 = 0.63 μM). Dixon plot analysis showed typical competitive inhibition mode for tanshinone I, tanshinone IIA, and cryptotanshinone, with Ki values of 0.48, 1, and 0.45 μM, respectively. Time-dependent inactivations of cryptotanshinone, tanshinone I, and tanshinone IIA against CYP1A2 were not observed. However, our previous findings suggested that danshen extract tablet that contained lipophilic components had little effect on in vivo CYP1A2 activity in human. This might due to the low bioavailability of lipophilic components (Hao et al., 2006; Qiu et al., 2008). Since new pharmaceutical strategies such as micronization have been developed for promoting the absorption of tanshinone IIA into blood (Cai et al., 2006), further studies of in vivo drug-drug interactions between the novel danshen preparations and substrates of CYP1A2 remain necessary.

Cryptotanshinone showed moderate mixed-type inhibition toward CYP2C9 activity in HLMs with Ki of 8 μM. Danshensu was found to inhibit competitively against CYP2C9 activity in HLMs with Ki of 35 μM and assumed no contribution from time-dependent inactivation to the shift in activity. Although danshensu decreased the percentage of
remaining activity of CYP2C9 in HLMs preincubated for 10 min with NADPH approximately 17% compared with that without NADPH, further studies are required to confirm whether danshensu had time-dependent inhibitory effect on CYP2C9 and estimate the parameters of time-dependent inhibition ($K_{i,k\text{incat}}$, portioning ratio, etc.) (Jushchysyn et al., 2003; Atkinson et al., 2005). The other five components of danshen extract were not found to inhibit CYP2C9 activity.

As we know, CYP2C9 plays an important role in the metabolism of many clinically used drugs, including the anticoagulant drug warfarin and a number of nonsteroidal anti-inflammatory drugs. The inhibition of CYP2C9 is involved in the increases of the plasma concentration and toxicity of concomitant substrate drugs of CYP2C9, especially those which have narrow therapeutic index (e.g., warfarin, phenytoin) (Miners and Birkett, 1998). It was reported that treatment with oral danshen extract (5g/kg, twice daily) for 3 days increased the plasma concentrations of both R- and S-warfarin and prolonged the prothrombin time after a single oral dose of racemic warfarin (2 mg/kg) (Chan et al., 1995). A case was reported that over-anticoagulation had happened in a patient after coadministering danshen and warfarin for about 1 month (Yu et al., 1997). High concentration of danshensu (about 7.0 g/ml or 40 μM, which is above the $K_i$ value of 35 μM) was observed in human plasma after oral administration of compound danshen pill at a dose of 250 mg (Pei et al., 2004). Furthermore, the concentration level of danshensu will be much higher than oral administration if danshen injection preparation is used, which contains large amounts of danshensu. Our result offered the in vitro evidence that danshen may cause warfarin to over-anticoagulate in vivo. However, considering the low bioavailability of cryptotanshinone, cryptotanshinone may not affect the catalytic activity of CYP2C9 at the concentration in vivo. Therefore, warfarin should be avoided when coadministering a danshen preparation containing high quantities of danshensu.

Cryptotanshinone exhibited mixed-type inhibition against the activity of CYP2D6 in HLMs with $K_i$ of 68 μM. CYP2D6 activity was also slightly inhibited by tanshinone I with $IC_{50}$ of 120 μM. Other five danshen components were not found to inhibit CYP2D6 activity in HLMs. Also, no time-dependent inactivation of seven danshen components toward CYP2D6 was observed. Although CYP2D6 is expressed at a rather low level compared with other human P450 enzymes in human, it plays an important role in oxidative metabolism of a variety of psychoactive and cardiovascular drugs with narrow therapeutic index (Subehan et al., 2006). Our other study showed that oral intake of danshen extract tablets for 2 weeks to reduce clearance of dextromethorphan, probe substrate of CYP2D6, from the body in 12 healthy subjects, suggesting danshen extract inhibited in vivo CYP2D6 activity. However, very low levels of cryptotanshinone and...
tanshinone I were detected in human blood (data not shown). The reason for the discrepancy between in vitro and in vivo is not clear. It might be due to some unknown compounds with potential inhibition ability in the danshen extract were absorbed into bloodstream. CYP3A4, the most abundant isoenzyme in the human liver and the intestinal wall, is responsible for the metabolism of a majority of drugs. High quantities of CYP3A4 expressed in the intestinal wall lead to extensive presystemic metabolism and relatively poor oral bioavailability of CYP3A4 substrates such as felodipine, lovastatin, midazolam, aminopyrine, simvastatin, and triazolam (Fromm et al., 1997). In the present study, testosterone 6β-hydroxylation and midazolam 1-hydroxylation were selected as indicators for the activity of CYP3A4 according to the current regulatory guidance (http://www.fda.gov/cder/guidance/index.htm). Although hepatic and intestinal CYP3A4 are different in tissue sources, their functions are the same (Zhang et al., 1999). The inhibition assay in human liver microsomes can be representative of that in human intestinal microsomes. From the in vitro data, it appeared that CYP3A4 was slightly inhibited by protocatechuic aldehyde with IC_{50} values of 130 (testosterone 6β-hydroxylation) and 160 (midazolam 1-hydroxylation) μM, respectively. Because the gut metabolism, solubility, and efflux transport can reduce the absorption of components of danshen, the hepatic concentration may be lower than the IC_{50}; however, the gut concentration of protocatechuic aldehyde may reach the IC_{50} value after orally administering danshen preparation. At the same time, we found that the lipophilic components of danshen extract could activate CYP3A4 activity (data not shown). Thus, complex interactions between danshen and substrates of CYP3A4 can be caused, and some further studies should be needed.

In conclusion, our data indicated that cryptotanshinone, tanshinone I, and tanshinone IIA were potent competitive inhibitors of human CYP1A2, cryptotanshinone and danshen were moderate competitive inhibitors of human CYP2C9, tanshinone I and cryptotanshinone were weak inhibitors of human CYP2D6, and danshen multiple components had complicated effects on CYP3A4. This finding provided some useful information for safe and effective use of danshen preparations in clinical practice. Our data suggested that it is necessary to study the interactions between drugs and pharmaceuticals with danshen extract.

Acknowledgments. We thank Fenzhi Sun and Haizhi Bu for thorough reviews of the manuscript and ZhiXiang Shi for providing editorial assistance.

References


TABLE 3

<table>
<thead>
<tr>
<th>Danshen Components</th>
<th>P450 Enzyme</th>
<th>( K_i )</th>
<th>Mode of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danshensu</td>
<td>CYP2C9</td>
<td>35</td>
<td>Competitive</td>
</tr>
<tr>
<td>Tanshinone I</td>
<td>CYP1A2</td>
<td>0.48</td>
<td>Competitive</td>
</tr>
<tr>
<td>Tanshinone IIA</td>
<td>CYP1A2</td>
<td>1.1</td>
<td>Competitive</td>
</tr>
<tr>
<td>Cryptotanshinone</td>
<td>CYP1A2, CYP2C9, CYP2D6</td>
<td>0.45, 8, 68</td>
<td>Competitive, mixed, mixed</td>
</tr>
</tbody>
</table>


Address correspondence to: Guangji Wang, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, Jiangsu 210038, China. E-mail: guangjiwang@hotmail.com