Identification of the Active Components in Shenmai Injection that Differentially Affect Cyp3a4-Mediated 1′-Hydroxylation and 4-Hydroxylation of Midazolam

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ABSTRACT

Shenmai injection (SMI) is a popular herbal preparation that is widely used for the treatment of atherosclerotic coronary heart disease and viral myocarditis. In our previous study, SMI was shown to differentially affect CYP3A4-mediated 1′-hydroxylation and 4-hydroxylation of midazolam (MDZ). The present study was conducted to identify the active components in SMI responsible for the differential effects on MDZ metabolism, using in vitro incubation systems (rat and human liver microsomes and a recombinant CYP3A4 system) to measure 1′-hydroxylation and 4-hydroxylation of MDZ. First, different fractions of SMI were obtained by gradient elution on an solid phase extraction system and individually tested for their effects on MDZ metabolism. The results demonstrated that lipid-soluble constituents were likely to be the predominant active components of SMI. Second, the possible active components were gradually separated on an high-performance liquid chromatography system under different conditions and individually tested in vitro for their effects on MDZ metabolism. Third, the active component obtained in the above experiment was collected and subjected to structural analysis, and identified as panaxytriol (PXT). Finally, it was validated that PXT had significant differential effects on 1′-hydroxylation and 4-hydroxylation of MDZ in various in vitro systems that were similar to those of SMI. We conclude that PXT is the constituent of SMI responsible for the differential effects on CYP3A4-mediated 1′-hydroxylation and 4-hydroxylation of MDZ.

Introduction

CYP3A4 is the most important member of the cytochrome P450 superfamily of enzymes and is involved in the majority of P450-mediated reactions (Guengerich, 1996). Drug interactions involving substrates, inhibitors, activators, or inducers of CYP3A4 are more prevalent and complex than those of other members of the P450 family (Kim et al., 2006; Pal and Mitra, 2006). It is becoming evident that many CYP3A4-mediated reactions proceed with atypical Michaelis-Menten kinetics, including substrate activation (Shou et al., 1999), substrate inhibition (Lin et al., 2001; Wu, 2011), and differential kinetics (Shou et al., 2001; Zhang and Lim, 2008; Xia et al., 2009). These phenomena are thought to be due to the binding of multiple substrates within the active site of the enzyme (Kenworthy et al., 2001; Galesin et al., 2003; Zhou et al., 2010).

Midazolam (MDZ) is a drug that is commonly used as a probe to estimate CYP3A4 activity (Galesin et al., 2005) and is mainly metabolized to 4-hydroxymidazolam (4-OH-MDZ) and 1′-hydroxymidazolam (1′-OH-MDZ) in reactions catalyzed by CYP3A4 (Rogers et al., 2003; Link et al., 2007). Studies have demonstrated that CYP3A4-mediated metabolism of MDZ may be altered to favor the formation of either 1′-OH-MDZ or 4-OH-MDZ. For example, Zhang and colleagues (2008) reported that cital, 6-gingerol, and D-limonene (compounds found in spices) enhance 1′-hydroxylation of MDZ but inhibit 4-hydroxylation of the drug.

Shenmai injection (SMI) is one of the most widely used herbal medicines in traditional Chinese medicine, and is made from red ginseng and Ophiopogon. It is used to treat atherosclerotic coronary heart disease and viral myocarditis and is often concomitantly administered with other prescribed medications, including certain cardiovascular drugs with a narrow therapeutic window (Janetzky and Morreale, 1997; Gurley et al., 2002; Donovan et al., 2003; Jiang et al., 2004; Yuan et al., 2004). In our previous study (Xia et al., 2009), we demonstrated that SMI could significantly enhance 1′-hydroxylation of MDZ but inhibit 4-hydroxylation of this compound in human liver microsomes (HLM), rat liver microsomes (RLM), and recombinant human CYP3A4. Furthermore, total ginsenoside, Ophiopogon total saponins, Ophiopogon total flavones, ginsenoside Rd, and ophiopogonone A (important constituents of SMI) were all found to significantly inhibit both 1′-hydroxylation and 4-hydroxylation of MDZ in HLM and RLM. Interestingly, no activation of MDZ hydroxylation was observed in the presence of these major SMI constituents, applied either alone or in combination. This contrasts with the actions of SMI, raising a question about which components of SMI are responsible for the differential effects on MDZ metabolism.

ABBRVATIONS: HLM, human liver microsomes; HPLC, high-performance liquid chromatography; MDZ, midazolam; 1′-OH-MDZ, 1′-hydroxymidazolam; 4-OH-MDZ, 4-hydroxymidazolam; PXT, panaxytriol; CYP3A4, recombinant CYP3A4; RILD, Research Institute for Liver Disease Co.; RLM, rat liver microsomes; SMI, Shenmai injection; SPE, solid phase extraction.
The aim of the present study was to identify the active components present in SMI that differentially affect 1'-hydroxylation and 4-hydroxylation of MDZ, using gradual separation of the constituents of SMI with a solid phase extraction (SPE) column and high-performance liquid chromatography (HPLC), combined with in vitro incubation systems (RLM, HLM, and recombinant human CYP3A4).

Materials and Methods

Chemicals. SMI was provided by Hebei Shenui Pharmaceutical Co. Ltd (Hebei, China). Each milliliter volume of SMI was comprised of the extract from 0.1 g red ginseng and 0.1 g radix ophiopogonis and contained 76.2 μg ginsenoside Rg1, 69.7 μg ginsenoside Re, 68.1 μg ginsenoside Rd, 178.4 μg ginsenoside Rb1, 15.7 μg ophiopogonone D, and 1.7 μg ophiopogonone A (Xia et al., 2009). Glucose-6-phosphate, NADP⁺, glucose-6-phosphate dehydrogenase, MDZ, 1'-OH-MDZ and 4-OH-MDZ were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were of analytical or chromatographic grade. Deionized water, obtained from a Milli-Q water purification system (Millipore, Billerica, MA), was used throughout the study.

Enzymes. Human liver microsomes (HLM) and rat liver microsomes (RLM) (CYP3A4 and CYP2D6, respectively) were prepared from livers harvested from normal animals (7–10 week old, both sexes) of specific-pathogen-free Wistar rats and Sprague-Dawley rats (Shanghai, China). The eluate was freeze-dried, and the residue was dissolved in 20% methanol.

Incubation Systems. The 1'- and 4-hydroxylations of MDZ, a specific substrate for CYP3A4, were determined using HPLC, as described previously (Xia et al., 2009). A reaction mixture (200 μl) containing MDZ (5 μM) for the HLM and CYP3A4 systems, 10 μM for the RLM system, 100 mM phosphate buffer (pH 7.4), 0.2 mg/ml liver microsomal protein or 20 nM recombinant CYP3A4, and an NADPH-generating system (0.5 mM NADP⁺, 10 mM glucose-6-phosphate, 10 mM magnesium chloride, and 1.0 unit of glucose-6-phosphate dehydrogenase) was equilibrated at 37°C for 5 minutes. Incubation with MDZ alone was used as a negative control, and incubation with 5% SMI (10 μl SMI added to a final reaction mixture volume of 200 μl) was used as a positive control. All experiments were conducted in triplicate.

Fractions of SMI Separated Using a Solid Phase Extraction System. Ten SPE C18 cartridges (Supelco Co., Bellefonte, PA) were each loaded with 1 ml SMI, and the fractions collected were separated using different concentrations of methanol. In detail, an initial eluate was collected from each cartridge as the sample (SMI) passed through the stationary phase. Then, each cartridge was washed with 1 ml water, and the eluate was collected as a water-soluble eluate. Finally, ten cartridges were, respectively, washed with 1 ml of a different concentration of methanol (10, 20, 30, 40, 50, 60, 70, 80, 90, or 100%), and the eluates were collected. These fractions were all evaporated to dryness and reconstituted in 1 ml 20% methanol. The effects of these different fractions on the metabolism of MDZ were evaluated by adding 10 μl of each reconstituted fraction to the RLM incubation system.

Fractions of SMI Separated Using an HPLC System. HPLC analysis was performed using a Diamonsil C18 column (150 mm × 4.6 mm, i.d. 5 μm; Dikma Technologies Inc., Beijing, China). A gradient elution, using solvent A (water) and solvent B (acetonitrile), was used as follows: 0–10 minutes, 10–20% (B); 10–30 minutes, 20–20% (B); 30–60 minutes, 20–40% (B); 60–90 minutes, 40–85% (B); 90–100 minutes, 85–100% (B); 100–110 minutes, 10–10% (B) (condition 1). The flow rate was set at 1 ml/min, and the column was run under room temperature. The eluate was monitored by the absorption at 203 nm, and fractions were collected as follows based on the HPLC chromatogram and the retention times of the peaks: SM-1 (0–15 minutes), SM-2 (15–30 minutes), SM-3 (30–45 minutes), SM-4 (45–60 minutes), SM-5 (60–72 minutes), SM-6 (72–87 minutes), and SM-7 (87–97 minutes). Then, each fraction was evaporated under a stream of nitrogen, and each residue was dissolved in 20% methanol. Finally, the effect of each residue on the metabolism of MDZ was investigated using the RLM system to determine which fraction had differential effects on the metabolism of MDZ.

The SM-5 fraction (60–72 minutes), eluted under HPLC condition 1 (described above), was further separated using different HPLC conditions. HPLC analysis was performed with a Synergi C18 column (250 mm × 4.6 mm, i.d. 4 μm; Phenomenex, Inc., Torrance, CA), using gradient elution with solvent A (water) and solvent B (acetonitrile), as follows: 0–60 minutes, 35–43% (B) (condition 2). All other HPLC conditions were the same as those described for condition 1. By using this HPLC separation, the following fractions were collected: SM-5-1 (0–10 minutes), SM-5-2 (10–24 minutes), SM-5-3 (24–32 minutes), and SM-5-4 (32–45 minutes). The effects of each of these residues on the metabolism of MDZ were also investigated. The fraction that demonstrated a differential effect on the metabolism of MDZ was defined as the active component.

Preparation and Structural Identification of the Active Component. The active component, fraction SM-5-3 (24–32 minutes), was collected by preparative liquid chromatography performed with a YMC-Pack Pro C18 column (250 mm × 10 mm, i.d. 5 μm; HanKing Instrument Equipment Co., Shanghai, China). The eluate was freeze-dried, and the residue was dissolved in a small volume of methanol and subjected to structural analysis. The 1H- and 13C-NMR spectra were measured using a Bruker AV-600 spectrometer (Bruker Corp., Billerica, MA), with the residual CHCl 3 as an internal standard. Chemical shifts were recorded as δ values. The mass spectra were obtained using an Agilent-6430 Triple Quadrupole liquid chromatography-mass spectrometry system (Agilent Technologies Inc., Santa Clara, CA). The optical rotations were measured using a JASCO DIP-4 automatic polarimeter (JASCO Corp., Billerica, MA), with the residual CHCl 3 as an internal standard. The 1H-NMR of the active component collected by preparative liquid chromatography were compared with that of a panaxytriol (PXT) standard.
Validation of the Differential Effect of PXT on MDZ Metabolism. First, the concentration of PXT in SMI was quantified using an HPLC method (this was found to be approximately 20 μg/ml). Then, the differential effects of PXT on the metabolism of MDZ in RLM, HLM, and recombinant CYP3A4 systems were assessed. The production of 1-OH-MDZ and 4-OH-MDZ was simultaneously determined in the presence of varying concentrations of isolated PXT (2.0, 1.0, and 0.5 μg/ml), standard PXT (2.0, 1.0, and 0.5 μg/ml), and SMI (10, 5, and 2.5%, v/v); PXT concentrations of 2.0, 1.0, and 0.5 μg/ml are equivalent to 10, 5, and 2.5% SMI, respectively. All experiments were conducted in triplicate.

Results

Effects of the SMI Fractions, Separated Using SPE, on the Metabolism of MDZ. To investigate which components of SMI are responsible for the differential effects on CYP3A4-mediated metabolism of MDZ, SMI was loaded onto SPE cartridges and separated using different concentrations of methanol. The effects of all collected fractions on the metabolism of MDZ are presented in Fig. 1. The initial eluate, the water-soluble eluate, and the residues eluted with 10–40% methanol did not affect the metabolism of MDZ in RLM. In contrast, after pretreatment with the residues eluted using 50–100% methanol, 1'-hydroxylation of MDZ in RLM was increased to 108.7 ± 9.8%, 149.7 ± 4.5%, 187.9 ± 12.7%, 186.8 ± 7.1%, 213.9 ± 13.4%, and 223.7 ± 8.1% compared with that of the control, whereas the formation of 4-OH MDZ was reduced to 79.6 ± 8.7%, 72.7 ± 4.4%, 76.6 ± 3.8%, 64.1 ± 12.7%, 61.6 ± 9.8%, and 70.4 ± 8.6% of that in the control. These results indicate that the possible active components responsible for the differential effects on MDZ metabolism may predominantly be the lipid-soluble constituents of SMI; this is in good agreement with our previous study (Xia et al., 2009).

Effects of the SMI Fractions, Separated by HPLC, on the Metabolism of MDZ. A representative HPLC chromatogram of SMI is shown in Fig. 2. Seven fractions were collected, according to the HPLC chromatogram and the retention times of the peaks, and the effect of each fraction on the metabolism of MDZ was investigated. Of these seven fractions, only fraction SM-5 (60–72 minutes) showed clear differential effects on the metabolism of MDZ, as shown in Fig. 3. Fraction SM-5 (60–72 minutes) was further separated using different HPLC conditions (condition 2, described in Materials and Methods). As shown in Fig. 4, several peaks were identified in fraction SM-5 (60–72 minutes). Of these, only fraction SM-5-3 (24–32 minutes), which was isolated as a single peak, demonstrated clear differential effects on the metabolism of MDZ that were similar to those of SMI (Fig. 5).

Identification of the Chemical Structure of the Active Component of SMI. The isolated compound, SM-5-3 (24-32 minute), was subjected to structural analysis using liquid chromatography-mass spectrometry, 1H-NMR, 13C-NMR and optical rotation. The physical data (Supplemental Fig. 1) obtained are shown below: [α]D -18.6° (CHCl3); liquid chromatography mass spectrometry: m/z 301(M+1)+; 1H-NMR (400 MHz, CDCl3) δ5.94 (ddd, J = 5.3, 10.1, 15.7 Hz, 1H-2), 5.47 (d, J = 17.0 Hz, 1H-1a), 5.25 (d, J = 10.1 Hz, 1H-1b), 4.92 (brs, 1H-3), 3.36 (brs, 1H-9), 3.60 (brs, J = 1H-10), 2.58 (brd, J = 3.8 Hz, 2H-8), 2.44 (brs, 3-OH-3), 2.10 (brs, 2-OH-9,10), 1.50 (m, 2H-11), 1.1–1.4 (10H-12–16), 0.87 (t, J = 6.8 Hz, 3H-17); 13C-NMR (400 MHz, CDCl3) δ117.15 (C-1), 136.04 (C-2), 63.48 (C-3), 78.11 (C-4), 70.90 (C-5), 66.51 (C-6), 74.77 (C-7), 24.98 (C-8), 73.08 (C-9), 72.13 (C-10), 31.78 (C-11), 25.55 (C-12), 29.52 (C-13), 29.19 (C-14), 35.58 (C-15), 22.62 (C-16), 14.06 (C-17). On the basis of the comparison with the known components’ stereochemistry structure in SMI, it can be found these spectral data coincide with those of PXT. In addition, the chromatographic behavior of isolated PXT was the same as that of PXT.

Fig. 2. HPLC chromatogram of SMI obtained under condition 1.

Fig. 3. Effects of SMI fractions, separated by HPLC under condition 1, on 4-hydroxylation and 1'-hydroxylation of MDZ in RLM. *P < 0.05 versus control.
standard PXT under the same HPLC conditions (the retention time of PXT was 27.5 minutes). The co-chromatography of standard PXT spiked into isolated PXT was also in good agreement with that of standard PXT and isolated PXT alone (Fig. 6). Moreover, the spectra of MS and 1H-NMR of the isolated PXT were also consistent with that of the standard PXT. From these results, it may be concluded that the active component of SMI corresponding to SM-5-3 (24–32 minutes) was PXT, which has the structure: (3R,9R,10R)-heptadec-1-ene-9,10-isopropylidenedioxy-4,6-diyn-3-ol (Fig. 7) (Matsunaga et al., 1989; Satoh et al., 2002).

Validation of the Differential Effects of PXT on the Metabolism of MDZ. The effects of isolated PXT, standard PXT, and SMI on 19-hydroxylation and 4-hydroxylation of MDZ were investigated and compared using different incubation systems. The effects of various concentrations of isolated PXT, standard PXT, and SMI on the metabolism of MDZ are shown in Table 1. In all the incubation systems used, isolated PXT, standard PXT, and SMI all caused a concentration-dependent inhibition of 4-hydroxylation of MDZ, as well as a significant activation of 19-hydroxylation of MDZ.

Discussion

In our previous study (Xia et al., 2009), we found that SMI could significantly enhance CYP3A4-mediated 19-hydroxylation of MDZ,

Fig. 4. HPLC chromatogram of SM-5 (60–72 minutes) obtained under condition 2.

Fig. 5. Effects of the fractions, separated from SM-5 (60–72 minutes) by HPLC under condition 2, on 4-hydroxylation and 19-hydroxylation of MDZ in RLM. *P < 0.05 versus control.

Fig. 6. HPLC chromatograms of 20 μg/ml isolated PXT (A), 20 μg/ml standard PXT (B), 20 μg/ml isolated PXT + 10 μg/ml standard PXT (C) obtained under condition 2.
but inhibit its 4-hydroxylation. SMI, extracted from red ginseng and *radix ophiopogonis*, contains a large number of chemical compounds, raising the possibility that several of these components could affect the catalytic activity of CYP3A4. To explore the possible active components, various fractions of SMI obtained by gradient elution on an SPE column were individually tested for their effects on MDZ metabolism. The fraction collected initially showed no effect on the metabolism of MDZ in RLM, excluding the possibility that the effect of SMI on CYP3A4 resulted from the adjuvant used in the preparation of SMI. The water-soluble eluate and the residues eluted with methanol at low concentrations (<50%, v/v) were also without effect on the metabolism of MDZ. In contrast, the residues eluted with higher concentrations of methanol (≥60%, v/v) showed significant differential effects on 1'-hydroxylation and 4-hydroxylation of MDZ in RLM, indicating that the lipid-soluble components of SMI may predominantly underlie these differential actions on MDZ metabolism.

Further investigation of the components in SMI that may be responsible for the differential effects on the metabolism of MDZ, were therefore carried out.

To further explore the possible active constituents, an HPLC system was used to separate the complex components in SMI. Seven fractions were obtained, according to the HPLC chromatogram and the retention times of the peaks, and the effects of each fraction were investigated on the metabolism of MDZ. It was found that only fraction SM-5 (60–72 minutes) showed obvious differential effects on 1'-hydroxylation and 4-hydroxylation of MDZ. As a result, the SM-5 (60–72 minutes) fraction was further separated using different HPLC conditions, until the main components were isolated as individual peaks on the HPLC chromatogram. Fraction SM-5-3 (24–32 minutes), which displayed the main components were isolated as individual peaks on the HPLC chromatogram. Fraction SM-5-3 (24–32 minutes) exhibited the same chromatographic behavior and spectra of mass spectrometry (MS) and 1H-NMR as a PXT standard.

To further validate these data, the differential effects of isolated PXT, standard PXT, and SMI on the metabolism of MDZ were compared in RLM, HLM, and recombinant CYP3A4 systems, with the synthesis of 1'-OH-MDZ and 4-OH-MDZ simultaneously determined in the presence of varying concentrations of PXT and SMI. It was found that isolated PXT and standard PXT behaved in a similar manner to SMI in all these incubation systems, significantly enhancing MDZ 1'-hydroxylation and inhibiting MDZ 4-hydroxylation.

In many cases, CYP3A4 exhibits non-Michaelis-Menten kinetics, as well as homo- and heterotropic activation for numerous substrates. Recent advances have demonstrated that CYP3A4 cooperativity involves the binding of multiple (at least 2) substrate molecules to the binding pocket and represents a case of true allostery characterized by effector-induced conformational transitions (Davydov and Halpert, 2008). On the basis of the above theory and our results, the differential effects of PXT on the metabolism of MDZ, similar to the effectors of citral, 6-gingerol, and α-limonene (Zhang and Lim, 2008), can be explained by a two-site model (Korzekwa et al., 1998; Domanski et al., 2001) or a multi-site model (Kenworthy et al., 2001; Manoj et al., 2010; Smirnow et al., 2011) of CYP3A4. It can be hypothesized that PXT can bind to one defined site that is also occupied by MDZ in the position required for 4-hydroxylation (or 1'-hydroxylation) and that both substrates can displace each other. The binding of PXT to one site causes an allosteric effect on the other MDZ-binding site that is required for its 1'-hydroxylation (or 4-hydroxylation). Alternatively, it can be hypothesized that, in addition to substrate binding sites, an activator binding site also exists that is closely related to the substrate binding sites. PXT can bind to the activator binding site, leading to conformational transitions in CYP3A4 and ultimately to differential effects on the substrate binding sites.

In summary, we successfully isolated the active component of SMI that is responsible for the differential effects on CYP3A4-mediated 1'-hydroxylation and 4-hydroxylation of MDZ and identified this constituent as PXT. SMI and PXT both had the potential to inhibit MDZ 4-hydroxylation but activate its 1'-hydroxylation. MDZ is mainly metabolized to 4-OH MDZ and 1'-OH MDZ mediated by CYP3A, but the preferential metabolic pathway of MDZ is 1'-hydroxylation in humans and 4-hydroxylation in rats (Rogers et al., 2003). After pretreatment with SMI or PXT, therefore, a predominant inhibitory effect on MDZ metabolism in rats would be observed (Xia et al., 2009). But it is possible to obtain an activation effect of SMI or PXT on MDZ metabolism in humans. The potential interactions

![Chemical structure of PXT.](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>4-OH MDZ</th>
<th>1'-OH MDZ</th>
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<tbody>
<tr>
<td></td>
<td>RLM</td>
<td>HLM</td>
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<tr>
<td>Control (%)</td>
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<td>100</td>
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<td>10% SMI</td>
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<td>36.9</td>
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<td>2.0 µg/ml isolated PXT</td>
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<tr>
<td>0.5 µg/ml standard PXT</td>
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<td>71.2</td>
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RLM, human liver microsome; MDZ, midazolam; HLM, rat liver microsome; PXT, panaxtriol; SMI, Shenmai injection.
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Authorship Contributions

Participated in research design: Xia, Xiong.
Conducted experiments: Zeng, He, Zhang.
Performed data analysis: Zeng, He, Xia.
Wrote or contributed to the writing of the manuscript: Zeng, Xia.

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