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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, GuangJi Wang

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University,

Nanjing, China (*Q.F.R., Z. R., S. J.G., A. J.Y., H.H.P., Y. P., H. A., W.G.J.*)

Yijishan Hospital, Wannan Medical College, Wuhu, China (*Q.F.R.*)

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Corresponding Author: GuangJi Wang

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University,

Nanjing, Jiangsu 210038, China

Phone: 0086-25-83271128

Fax: 0086-25-85306750

Email: guangjiwang@hotmail.com

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Abbreviations: CYP, cytochrome P450; AUC, area under the curve; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; IC₅₀, 50% inhibitory concentration; K_i, equilibrium dissociation constant for reversible inhibitor; V_{max}, the maximal rate of product formation; K_m, the Michaelis constant; HLMs, human liver microsomes

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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 II A and cryptotanshinone) of danshen extract. Probe substrates of CYP enzymes were incubated in HLMs with or without each component of danshen extract. IC_{50} and K_i values were estimated and the types of inhibition were determined. Among the seven components of danshen extract, tanshinone I, tanshinone II A, and cryptotanshinone were potent competitive inhibitors of CYP1A2 ($K_i=0.48\mu\text{M}$, $1.0\mu\text{M}$, $0.45\mu\text{M}$, respectively); danshensu was a competitive inhibitor of CYP2C9 ($K_i=35\mu\text{M}$), and cryptotanshinone was a moderate mixed type inhibitor of CYP2C9 ($K_i=8\mu\text{M}$); cryptotanshinone inhibited weakly and in mixed mode against CYP2D6 activity ($K_i=68\mu\text{M}$), and tanshinone I was a weak inhibitor of CYP2D6 ($IC_{50}=120\mu\text{M}$); protocatechuic aldehyde was a weak inhibitor of CYP3A4 ($IC_{50}=130\mu\text{M}$, $160\mu\text{M}$ 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.

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Introduction

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 CYP3A4 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. In order to predict the metabolism-based interactions between drugs and herbal products, major components of herbal products having inhibitory effects on cytochrome P450 (CYP) activities should be identified; 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). Known four hydrophilic components (danshensu, protocatechuic aldehyde, protocatechuic acid and salvianolic acid B) and three lipophilic components (tanshinone I, tanshinone II A and cryptotanshinone) are thought to be the major active components of danshen extract (Fig.1) (Zhou et

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al., 2006). In recent years, some studies have been reported on the effects of danshen extract on CYP enzymes. A clinical case was reported that danshen caused over-anticoagulation of warfarin, which is CYP2C9 substrate (Yu et al., 1995). It was reported that treatment of rats with hydrophilic components of danshen extract elevated the AUC of warfarin, suggesting an inhibitory effect of hydrophilic components of danshen on CYP2C9 activity (Chan, 2001; Chan et al., 1995). Kuo and Ueng reported that mouse CYP1A-, CYP2C- and CYP3A-inducing agents were present in the lipophilic components of danshen extract (Kuo et al., 2006; Ueng et al., 2004). Ueng also reported that tanshinone IIA decreased 7-ethoxyresorufin O-deethylation (EROD) and 7-methoxyresorufin O-demethylation (MROD) activities in human liver microsomes (HLMs), suggesting that tanshinone IIA inhibited the activity of CYP1A2 in HLMs, and tanshinone IIA, tanshinone I 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 CYP enzymes, a detailed study is required to determine the effects of hydrophilic and lipophilic components of the danshen extract on human CYP 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 according to 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.

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Materials and Methods

Chemicals and Reagents Lipophilic components: tanshinone I, tanshinone IIA and cryptotanshinone and hydrophilic components: danshensu, protocatechuic aldehyde, protocatechuic acid, 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 Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile, methanol and ethyl acetate were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Perchloric acid and other chemicals and solvents were all of analytical grade. The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): phenacetin, acetaminophen, furafylline; diclofenac, 4-hydroxydiclofenac, sulfaphenazole; midazolam, 1-hydroxymidazolam, testosterone, 6 β -hydroxytestosterone, ketoconazole; dextromethorphan, dextrorphan, and quinidine.

Human liver microsomes Human liver microsomes (HLMs) used in this study were provided by the Research Institute for Liver Disease Co. (RILD) (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 RILD.

Incubation Procedure and Enzymatic activity assay The CYP enzymatic activities were characterized based on their probe reactions: CYP1A2 (phenacetin O-deethylation), CYP2C9 (diclofenac 4-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), and CYP3A4 (testosterone 6 β -hydroxylation; midazolam 1-hydroxylation). Incubation mixtures were prepared in a total volume of 200 μ l as follows: NADPH-generating system (10mM glucose 6-phosphate, 0.5mM

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NADP, 10mM magnesium chloride, 1 unit of glucose 6-phosphate dehydrogenase), 100mM phosphate buffer (pH 7.4), probe substrates of CYP enzymes and a range of concentrations of tested compound. Midazolam previously dissolved in water, and the others previously dissolved in methanol, 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 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 6 β -hydroxylation), 5 min for CYP3A4 (midazolam 1-hydroxylation), respectively.

For each probe drug, preliminary experiments were performed to determine whether metabolite 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 CYP enzymes were as follows: phenacetin 5–400 μ M, diclofenac 1–400 μ M, dextromethorphan 1–400 μ M, testosterone 5–300 μ M, and midazolam 1–200 μ M. The K_m and V_{max} values were determined using seven different concentrations of CYP-marker substrates by nonlinear regression analysis of the enzyme activity-substrate concentration data using the Michaelis-Menten model (DAS, version 2.0, Shanghai, China).

Inhibition of CYP enzymatic activity assay A typical incubation mixture contained HLMs (protein concentration: 0.2 mg/ml), NADPH-generating system, probe substrates of CYP enzymes, and different concentrations of individual tested compound, 100mM phosphate buffer (pH 7.4). Initially, in screening experiments, Triplicate samples were run to generate IC₅₀ value by incubating CYP-marker substrates at a concentration approaching K_m in the presence of five concentrations of seven components (final concentrations ranging from 0.5 μ M to 200.0 μ M) in the incubation mixture. Hydrophilic components were dissolved in water, and lipophilic components were dissolved in DMSO

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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 K_i values of danshen components were determined if their IC_{50} were lower than 100 μ M. Phenacetin (0, 37.5, 75.0 and 150.0 μ M), diclofenac (0, 2.0, 5.0 and 10.0 μ M), dextromethorphan (0, 2.0, 5.0 and 10.0 μ M) and a range of concentrations (0.5–200.0 μ M) of seven components of danshen extract were used for the construction of Dixon plots and estimation of K_i values (Liu et al., 2006). The K_i values were determined by fitting the enzyme activity-substrate concentration data at various inhibitor concentrations to the equations for competitive inhibition, noncompetitive inhibition, and mixed-type inhibition. The mode of inhibition was determined on the basis of visual inspection of the Dixon and Akaike information criterion (DAS, version 2.0, Shanghai, China).

Time-dependent inactivation screening assay Triplicate samples for time-dependent inactivation screening assay were preincubated for 15 min at 37°C with danshen components selected here with or without NADPH. The percentage of remaining activity of CYP1A2, CYP2C9, CYP2D6, and CYP3A4 was measured with their marker substrates: phenacetin, diclofenac, dextromethorphan, midazolam, and testosterone, at single concentration approximating their K_m values (Table 1). The concentrations of components selected here were all equal to their IC_{50} . Vehicle controls were run to account for any decrease in enzyme activity caused by incubation under the same conditions. The percentage of remaining activity of microsomes preincubated with NADPH was compared with that of microsomes preincubated without NADPH (Subehan et al., 2006; Obach et al, 2007):

$$\% \text{Activity remaining after preincubation} = \frac{(\text{activity with inactivity})_{+NADPH}}{(\text{activity with vehicle})_{+NADPH}} \times 100 \quad (1)$$

$$\% \text{Activity remaining without preincubation} = \frac{(\text{activity with inactivity})_{no\ NADPH}}{(\text{activity with vehicle})_{no\ NADPH}} \times 100 \quad (2)$$

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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 20000 rpm for 10 min. Twenty microliters of the resulting supernatant were injected into the HPLC system (LC-2010CHT, Shimadzu, Japan) for analysis. The chromatographic separation was achieved using a Diamosil C18 column (4.6×150 mm, 5µm) (Dikma Technologies, China) with mobile phase of methanol and 0.01M 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.

6β-Hydroxytestosterone, the marker metabolite of CYP3A4, was quantitated from 55 to 4450 ng/ml. After incubation with the microsome system at 37 °C, all samples were basified by 30 µl of 1M NaOH solution, and extracted with 1ml of ethyl acetate containing prednisolone (100ng/ml). The samples were shaken for 30 s. The organic phase was evaporated to dryness under a nitrogen stream at 40 °C. The residue was dissolved in 100µl methanol, and vortex-mixed for 3 min. The tubes were then centrifuged at 20000 rpm for 10min. Twenty microliters of the resulted supernatant were injected into the HPLC system for analysis. The chromatographic separation was achieved using a Phenomenex Gemina C18 column (4.6 ×250mm, 5µm) and a 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 245nm.

1-Hydroxymidazolam, the marker metabolite of CYP3A4, was quantitated from 25 to 2000 ng/ml. After incubation with the microsome system at 37 °C, 100µl of acetonitrile was added in all samples. The tubes were then centrifuged at 20000 rpm for 10 min. Twenty microliters of the resulting supernatant were injected onto the HPLC system for analysis. Chromatographic separation was achieved on a Diamosil C18 (150×4.6mm, I.D. 5µm) with isocratic mobile phase of 20 mM NH₄Ac-acetonitrile (60:40) at a flow rate of 1.0 ml/min. The peaks were determined using a UV detector set at a wavelength of 254 nm.

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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 ethyl acetate containing the internal standard chlorzoxazone. The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in a 100 µL methanol. The mixtures were centrifuged, and then aliquot of the supernatants was analyzed by HPLC/MS. Chromatography was performed using a C18 column (5µm, 150 × 2.1 mm, Kromasil, Sweden) and an HPLC system consisting of Shimadzu 10A series (Shimadzu, Kyoto, Japan) with a gradient mobile phase of 0.01% ammonium acetate and methanol. The flow rate was 0.2 ml/min. Detection of 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 500ng/ml. After incubation with the microsome system at 37 °C, freshly prepared samples containing dextrorphan in the mixture were extracted with 1 mL 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 mm×2.0 mm,Tokyo, Japan) and an HPLC system consisting of Finnigan Surveyor (Thermo Electron,USA) with a gradient mobile phase of 0.1% formic acid and methanol at flow rate of 0.2 ml/min. Detection of the analyte and internal standard was performed using a Finnigan TSQ Quantum Discovery Max MS/MS system (Thermo Electron,USA) in the positive ion mode using 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 39eV and 41 eV, respectively.

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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. 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 6 β -hydroxylation and midazolam 1-hydroxylation in HLMs were 75, 6.8, 5, 75, and 3.5 μ M, respectively (Table 1). The V_{max} values for phenacetin O-deethylation, diclofenac 4-hydroxylation, dextromethorphan O-demethylation, and testosterone 6 β -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 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 II A and cryptotanshinone had inhibition against CYP1A2 activities in HLMs with IC_{50} values of 0.75 μ M, 1.3 μ M and 0.75 μ M (Table 2); Danshensu and cryptotanshinone exhibited inhibition against the activity of CYP2C9 with IC_{50} values of 50 μ M and 33 μ M, respectively (Table 2). Cryptotanshinone and tanshinone I exhibited weak inhibitions against the activities of CYP2D6 with IC_{50} values of 75 μ M and 120 μ M, respectively (Table 2). Protocatechuic aldehyde was a weak inhibitor of CYP 3A4 (IC_{50} =130 μ M and 160 μ M for midazolam and testosterone, respectively) (Table 2).

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Time-dependent inactivation screening assay Five inhibitors (danshensu, salvianolic acid B, tanshinone II A, 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), tanshinone I and cryptotanshinone (inhibition of CYP2D6), and protocatechuic aldehyde (inhibition of CYP2D6). The percentage of remaining activity of CYP 2C9 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 3).

Inhibition kinetic analysis To further characterize the inhibition of CYP enzymes activity by components of danshen extract, enzyme inhibition kinetic experiments were carried out. Components of danshen extract which were found to weakly inhibit CYP enzymes ($IC_{50} > 100 \mu M$) 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 Fig3, Fig4, Fig5., Tanshinone I, tanshinone II A and cryptotanshinone exhibited competitive inhibition against CYP1A2 activities with K_i value of $0.48 \mu M$, $1 \mu M$ and $0.45 \mu M$ for CYP1A2 (Table3; Fig 3-A, Fig 3-B, Fig 3-C); danshensu exhibited competitive inhibition against the activity of CYP2C9 with K_i value of $35 \mu M$ (Table3; Fig 4-A) and cryptotanshinone exhibited mixed type inhibition against the activity of CYP2C9 in HLMs with K_i value of $8 \mu M$ (Table3; Fig 4-B), Cryptotanshinone exhibited a weak mixed type inhibition against the activity of CYP2D6 in HLMs with K_i value of $68 \mu M$ (Table3; Fig 5).

Discussion

We established probe substrate assay methods in HLMs conventionally applied to study inhibition toward CYP enzymes activities. Apparent K_m , V_{max} for CYP probe substrates and the IC_{50}

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of positive control CYP inhibitors (furafylline, CYP1A2; sulfaphenazole, CYP2C9; quinidine, CYP2D6; and ketoconazole, CYP3A4) obtained from the present experiments were in good 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 present study were higher than that used in literatures at high concentration.

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

Among the seven components of danshen extract, cryptotanshinone, tanshinone I and tanshinone II A, which were all lipophilic components, exhibited a strong inhibition on CYP1A2, with IC_{50} of 0.75, 1.3 and 0.75 μ M, respectively. In contrast, the inhibitory effects of the hydrophilic components on CYP1A2 were found to be weak ($IC_{50}>100\mu$ M). The inhibitory potency of these lipophilic components were comparable with that of furafylline, which is a positive inhibitor of CYP1A2 ($IC_{50}=0.63\mu$ M). Dixon plot analysis showed typical competitive inhibition mode for tanshinone I, tanshinone II A and cryptotanshinone with K_i values of 0.48 μ M, 1 μ M and 0.45 μ M, respectively. Time-dependent inactivations of cryptotanshinone, tanshinone I and tanshinone II A against CYP1A2 were not observed. However, our previous findings suggested that danshen extract tablet contained lipophilic components had little effect on in vivo CYP1A2 activity in human. This might due to the low bioavailability of lipophilic components (Qiu et al., 2008; Hao et al., 2006). 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

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interactions between the novel danshen preparations and substrates of CYP1A2 remain necessary.

Cryptotanshinone showed moderate mixed-type inhibition toward CYP2C9 activity in HLMs with K_i of $8\mu\text{M}$. Danshensu was found to inhibit competitively against CYP2C9 activity in HLMs with K_i of $35\mu\text{M}$, assumed no contribution from time-dependent inactivation to the shift in activity. Although danshensu decreased the percentage of remaining activity of CYP 2C9 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_{inact} , partitioning ratio, etc.) (Jushchyshyn et al., 2003; Atkinson et al., 2005). 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 which have narrow therapeutic index (e.g. warfarin, phenytoin) (Miners and Birkett, 1998). It was reported that treatment with oral danshen extract ($5\text{g}\cdot\text{kg}^{-1}$, 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 ($2\text{mg}\cdot\text{kg}^{-1}$) (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 $7.0\mu\text{g}/\text{ml}$ or $40\mu\text{M}$, which is above the K_i value of $35\mu\text{M}$) was observed in human plasma after oral administration of compound danshen pill at a dose of 250mg (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 overanticoagulate in vivo. However, considering the low bioavailability of cryptotanshinone, cryptotanshinone may not affect the catalytic activity of CYP2C9 at the concentration in vivo. So, warfarin should be avoided coadministering

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danshen preparation containing high quantities of danshensu.

Cryptotanshinone exhibited mixed type inhibition against the activity of CYP2D6 in HLMs with K_i of 68 μ M. CYP2D6 activity was also slightly inhibited by tanshinone I with IC_{50} of 120 μ M. 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 rather low level compared with other human CYP 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 another study showed that oral intake of danshen extract tablets for two weeks 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 pre-systemic metabolism and relatively poor oral bioavailability of CYP3A4 substrates such as felodipine, lovastatin, midazolam, aquinavir, simvastatin, triazolam (Fromm et al., 1997). In the present study testosterone 6 β -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 to that in human intestinal microsomes. From the *in vitro* data, it appeared that CYP3A4 was slightly inhibited by protocatechuic aldehyde with IC_{50} of 130 μ M (testosterone 6 β -hydroxylation) and 160 μ M (midazolam

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1-hydroxylation), respectively. As 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 II A were potent competitive inhibitors of human CYP1A2, cryptotanshinone and danshensu were moderate competitive inhibitors of human CYP2C9, tanshinone I and cryptotanshinone was 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.

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Footnote

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Legends for Figures

Fig.1 Structures of danshen components

A: Danshensu; B: Protocatechuic acid; C: Protocatechuic aldehyde; D: Salvianolic acid B; E: Cryptotanshinone; F: Tanshinone II A; G: Tanshinone I

Fig.2 Time dependent inhibition of CYP isoforms by danshen components. Comparison of the percentage of remaining activity of microsomes preincubated with NADPH and without NADPH. Asterisks (*) indicate a statistically significant difference between with NADPH and without NADPH ($p < 0.05$).

1. Inhibition of CYP1A2 by danshensu (110 μ M); 2. Inhibition of CYP1A2 by salvianolic acid B (105 μ M); 3. Inhibition of CYP1A2 by tanshinone II A (1.3 μ M); 4. Inhibition of CYP1A2 by tanshinone I (0.75 μ M); 5. Inhibition of CYP1A2 by cryptotanshinone (0.75 μ M); 6. Inhibition of CYP2C9 by danshensu (50 μ M); 7. Inhibition of CYP2C9 by cryptotanshinone (33 μ M); 8. Inhibition of CYP2D6 by tanshinone I (120 μ M); 9. Inhibition of CYP2D6 by cryptotanshinone (75 μ M); 10. Inhibition of CYP3A4 by protocatechuic aldehyde (130 μ M) (midazolam); 11. Inhibition of CYP3A4 by protocatechuic aldehyde (160 μ M) (testosterone).

Fig.3 Dixon plots of the effect of tanshinone I, tanshinone II A and cryptotanshinone on acetaminophen formation in human liver microsomes. Reactions were performed in the presence of phenacetin (0, 37.5, 75.0, 150.0 μ M), at various concentrations of tanshinone I (0, 0.25, 0.5, 1.0 μ M) (A), tanshinone II A (0, 0.25, 1.0, 2.0 μ M) (B), cryptotanshinone (0, 0.25, 0.5, 1.0 μ M) (C) in the microsomes (0.2mg/ml) and NADPH-generating system in a 100mM phosphate buffer (pH 7.4) in a final volume of 200 μ l at 37°C for 25min. Each point represents the mean of three separate experiments performed in duplicate.

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Fig.4 Dixon plots of the effect of danshensu and cryptotanshinone on 4-hydroxydiclofenac formation in human liver microsomes. Reactions were performed in the presence of diclofenac (0, 2.0, 5.0, 10.0 μ M), at various concentrations of danshensu (0, 37.5, 75, 150 μ M) (A), cryptotanshinone (0, 12.5, 50.0, 100.0 μ M) (B) in the microsomes (0.2mg/ml) and NADPH-generating system in a 100mM phosphate buffer (pH 7.4) in a final volume of 200 μ l at 37°C for 10min. Each point represents the mean of three separate experiments performed in duplicate.

Fig.5 Dixon plots of the effect of cryptotanshinone on dextrorphan formation in human liver microsomes. Reactions were performed in the presence of dextromethorphan (0, 2, 5, 10 μ M), at various concentrations of cryptotanshinone (0, 12.5, 50.0, 100.0, 200.0 μ M) in the microsomes (0.2mg/ml) and NADPH-generating system in a 100mM phosphate buffer (pH 7.4) in a final volume of 200 μ l at 37°C for 6min. Each point represents the mean of three separate experiments performed in duplicate.

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Tables

Table1 Enzymatic Kinetic Parameters for CYP in Human Liver Microsomes

Enzyme	Assay	Type	K_m (μ M)	V_{max} (pmol/min/mg pro)
CYP1A2	Phenacetin-O-deethylation	simple	75	516
CYP2C9	Diclofenac 4-hydroxylation	substrate inhibition	6.8	3657
CYP2D6	Dextromethorphan o-demthylation	simple	5	1300
CYP3A4	Testosterone 6 β -hydroxylation	simple	75	6130
CYP3A4	Midazolam 1-hydroxylation	substrate inhibition	3.5	1200

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Table2 The IC₅₀ values for the inhibition of danshen components on CYP enzymes activities

Danshen components	IC ₅₀ (μM)				
	CYP1A2	CYP2C9	CYP2D6	CYP3A4 ^a	CYP3A4 ^b
Hydrophilic components					
Protocatechuic acid	>200	>200	>200	>200	>200
Protocatechuic aldehyde	>200	>200	>200	130	160
Danshensu	110	50	>200	>200	>200
Salvianolic acid B	105	>200	>200	>200	>200
Lipophilic components					
Tanshinone I	0.75	>200	120	>200	>200
tanshinone II A	1.3	>200	>200	>200	>200
Cryptotanshinone	0.75	33	75	>200	>200
Positive control ^c	0.63	0.3	0.12	0.05	0.045

a Testosterone 6β-hydroxylation

b Midazolam 1-hydroxylation

c The positive controls were specific inhibitors of P450 (furaflavone for CYP1A2, sulfaphenazole for CYP2C9, quinidine for CYP2D6, and ketoconazole for CYP3A4).

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Table3 The K_i values and mode of inhibition of danshen components on CYP enzyme activities

Danshen components	CYP enzyme	K _i (μM)	Mode of inhibition
Danshensu	CYP2C9	35	Competitive
Tanshinone I	CYP1A2	0.48	Competitive
Tanshinone II A	CYP1A2	1.1	Competitive
Cryptotanshinone	CYP1A2,CYP2C9,CYP2D6	0.45,8,68	Competitive, mixed, mixed

FIGURE 1

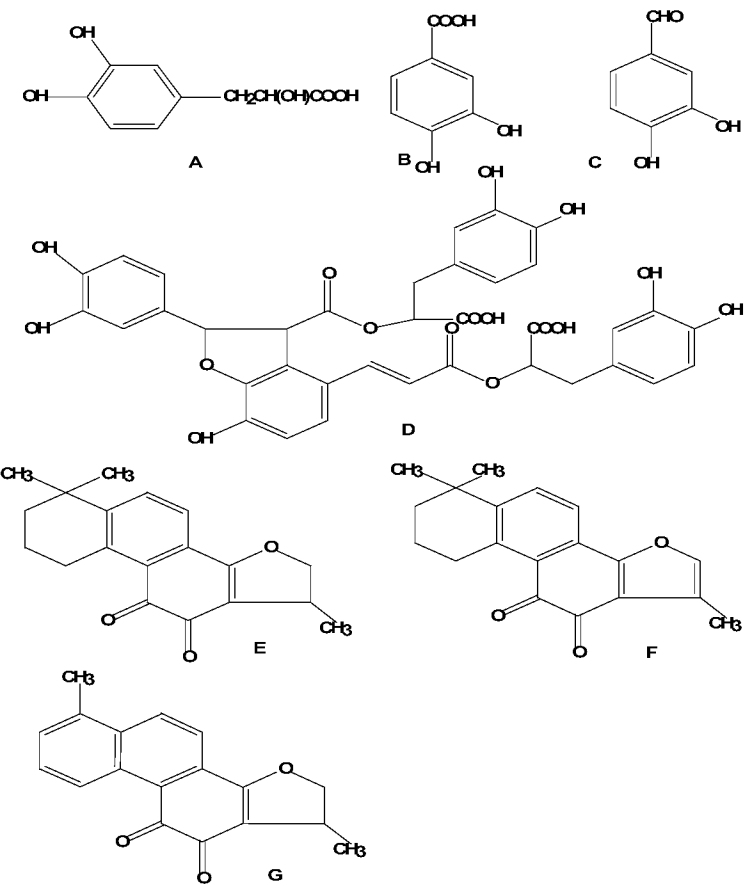


FIGURE 2

%Activity remaining

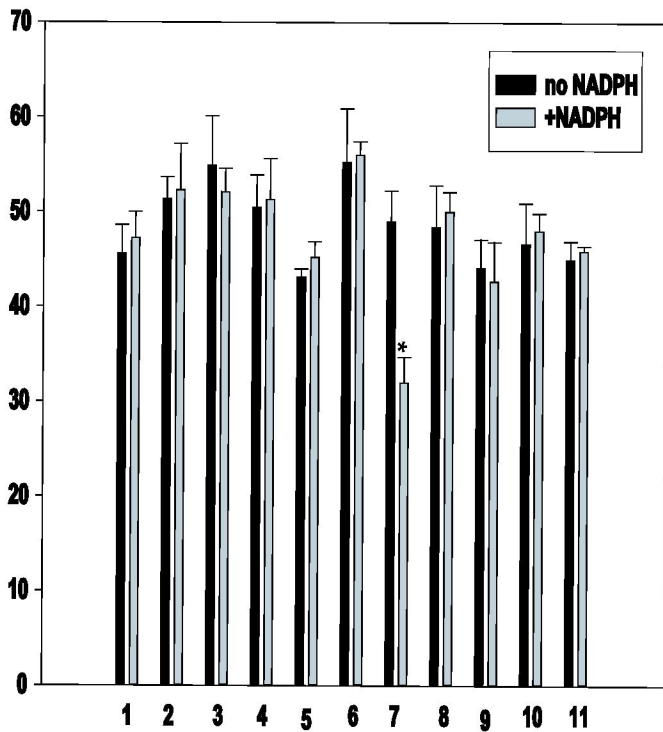


FIGURE 3-A

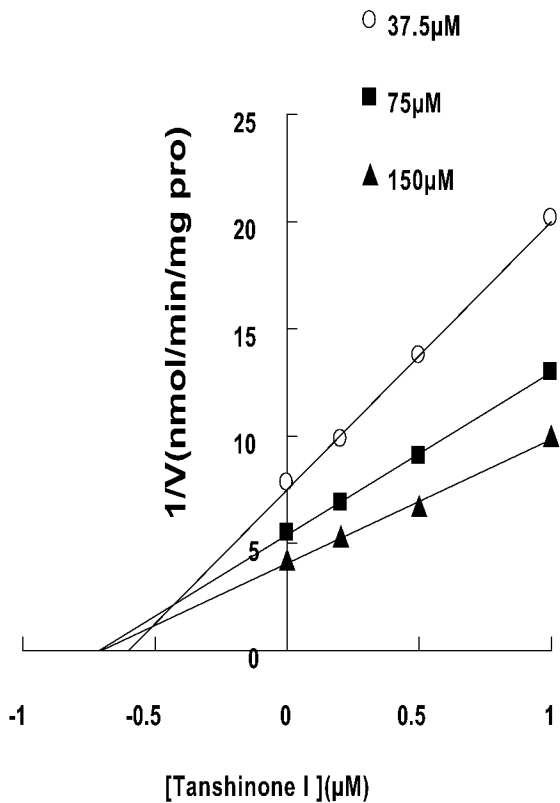


FIGURE 3-B

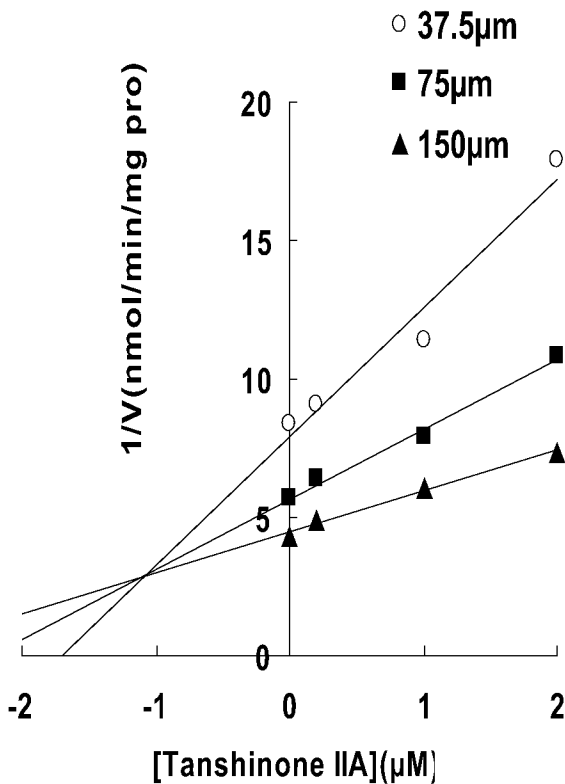


FIGURE 3-C

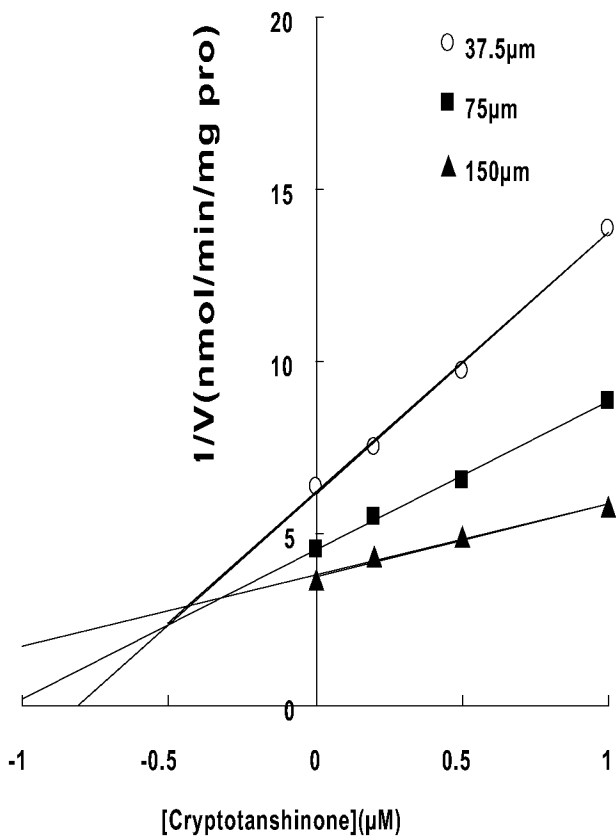


FIGURE 4-A

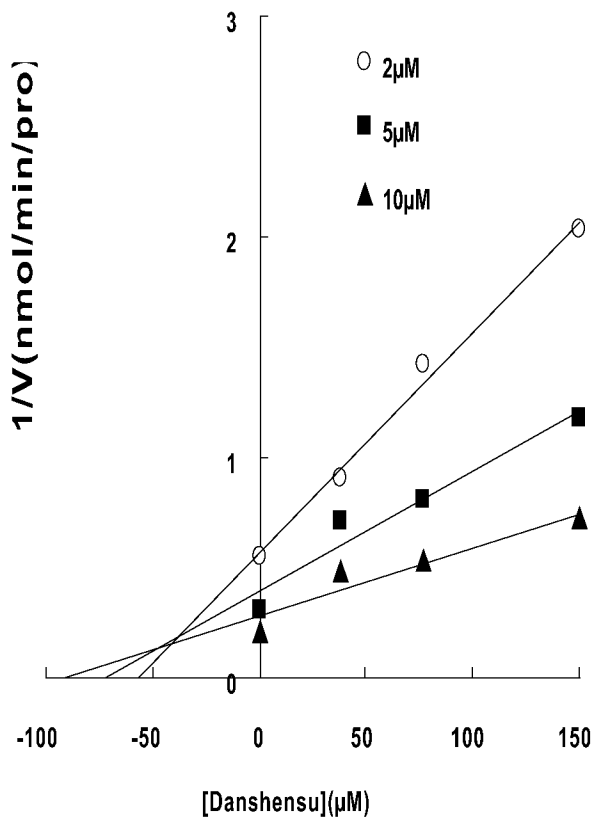


FIGURE 4-B

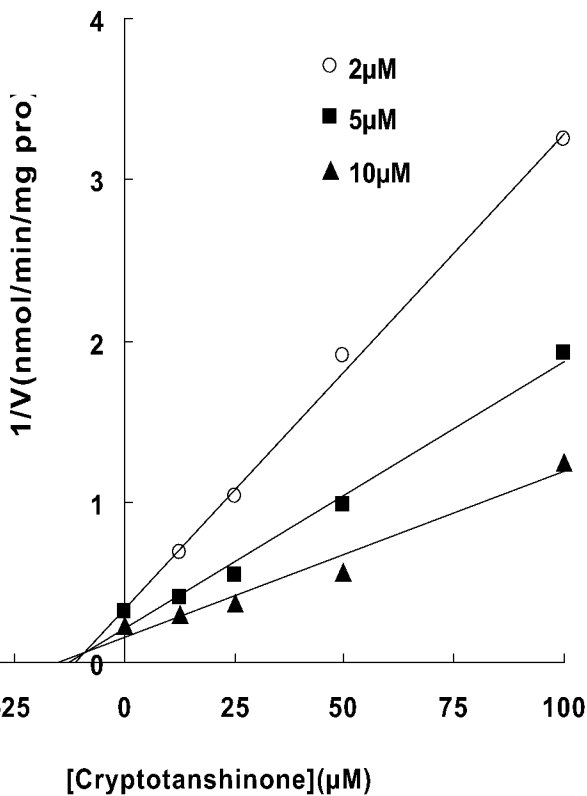


FIGURE 5

