

Original Article

Inhibitory effect of salvianolate on human cytochrome P450 3A4 in vitro involving a noncompetitive manner

Chong-Zhen Qin^{1,2*}, Xian Ren^{3*}, Hong-Hao Zhou^{1,2}, Xiao-Yuan Mao^{1,2}, Zhao-Qian Liu^{1,2}

¹Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008, P. R. China; ²Institute of Clinical Pharmacology, Central South University; Hunan Key Laboratory of Pharmacogenetics, Changsha 410078, P. R. China; ³Shanghai Green Valley Pharmaceutical Co., Ltd., Shanghai 201203, P.R. China.
*Equal contributors.

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Abstract: Salvianolic acid B (Sal B), which is purified from Danshen, is a popular herb extract. Sal B has anti-oxidative, anti-inflammatory, anti-hypoxic, anti-arteriosclerotic and anti-apoptotic properties. This substance can also ameliorate brain injury or neurodegenerative diseases. The listed drug Salvianolate, which contains a substantial amount of Sal B, has been used for the treatment of coronary heart disease. Our present work aimed to evaluate the inhibitory effect of salvianolate on seven cytochrome P450 isoforms (CYP450), namely, CYP1A2, CYP2A6, CYP2E1, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, in human liver microsomes (HLMs) and recombinant enzymes through high-performance liquid chromatography (HPLC) assay. Salvianolate have a potent inhibitory effect on CYP3A4 activity with IC₅₀ values of 1.438 (HLMs) and 3.582 (recombinant cDNA-expressed CYP3A4) mg/L, respectively. Salvianolate strongly dose, but not time-dependently decreased CYP3A4 activity in HLMs. The typical Lineweaver-Burk plots showed that Salvianolate inhibited CYP3A4 activity noncompetitively, with a K_i value of 2.27 mg/L in HLMs. Other CYP450 isoforms are not markedly affected by Salvianolate. These findings indicate that salvianolate may be involved in potential drug interactions when co-administrated with CYP3A4 substrates.

Keywords: Salvianolate, CYP3A4, human liver microsomes, noncompetitive inhibition, drug-drug interaction

Introduction

Danshen, the dried root of *Salvia miltiorrhiza*, is widely used in the treatment of coronary heart disease, hepatitis, cerebrovascular disease, hepatocirrhosis, and chronic renal failure [1-5]. As a highly purified aqueous extract from Danshen, Salvianolate is a listed drug that primarily contains salvianolic acid B (Sal B) (≥85%), rosmarinic acid (≥10.1%), and lithospermic acid (≥1.9%) to protect microvascular reflow against ischaemia/reperfusion injury and acute coronary syndrome [6, 7]. It was previously reported that Sal B is the most abundant member of salvianolic acids [8]. Various investigations have shown that Sal B possesses various pharmacologic properties including anti-oxidative, anti-inflammatory, anti-hypoxic, anti-arteriosclerotic, anti-apoptotic properties in vivo and in vitro [9-16].

The CYP450 superfamily is an important enzyme system in humans. This enzyme is

responsible for the metabolism of several endogenous compounds and xenobiotics [17]. CYP isozymes can be inhibited by various drugs, such as ketoconazole, ritonavir, and clarithromycin. This process may lead to drug-drug interactions and subsequently exacerbate adverse clinical events [18]. Seven human hepatic CYP isoforms, namely, CYP1A2, CYP3A4, CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1, are shown to be responsible for more than 90% drug metabolism [19]. This mechanism may lead to several clinical important drug-drug interactions [20]. The blood concentration of co-administered drugs may be altered significantly and result in adverse reactions or drug withdrawal when concomitant drugs are metabolized by the same enzyme.

Salvianolate has been applied as a therapeutic agent in clinical practices [21]. However, there have are no reports suggesting an adverse drug-drug interaction caused by salvianolate, and no relevant publications of its effects on

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Table 1. The inhibitory effect of salvianolate on isozyme-specific cytochrome P450 activities in HLMs

CYP isozyme	Specific probe (μM)	Metabolite	Incubation time (min)	Positive control inhibitor concentration (μM)
CYP1A2	Phenacetin (10)	Acetaminophen	30	Furafylline (10)
CYP2A6	Coumarin (5)	7-hydroxycoumarin	20	Tranlycypromine (50)
CYP3A4	Midazolam (5)	1-hydroxymidazolam	30	Ketoconazole (1)
CYP2C9	Tolbutamide (100)	4-hydroxytolbutamide	30	Sulfaphenazole (10)
CYP2C19	S-Mephenytoin (100)	4'-hydroxymephenytoin	30	Ticlopidine (5)
CYP2D6	Metoprolol (7.5)	α -hydroxymetoprolol	15	Quinidine (1)
CYP2E1	Chlorzoxazone (40)	6-Hydroxychlorzoxazone	20	Diethyldithiocarbamate (50)

Table 2. HPLC conditions for the determination of CYP-dependent enzymatic activities

CYP	Mobile phase (v/v)	IS	Flow rate (mL/min)	LC-UV detection	Retention time (min) (metabolite/IS)
CYP1A2	0.1% Formic acid/ methanol (70/30)	7-hydroxycoumarin	0.7	297 nm	5.75/11.29
CYP2A6	10 mM ammonium formate /methanol (60/40)	Phenacetin	0.3	230 nm	2.56/4.97
CYP3A4	20 mM ammonium formate /methanol (35/65)	Propranolol	1	225 nm	8.5/4.0
CYP2C9	0.2% ethylic acid/ methanol (45/55)	Phenacetin	1	230 nm	4.15/5.06
CYP2C19	20 mM ammonium formate /methanol (45/55)	Phenacetin	1	204 nm	3.77/7.47
CYP2D6	20 mM ammonium formate (0.1% Formic acid)/methanol (60/40)	Phenacetin	0.5	280 nm	3.5/10.2
CYP2E1	Water/Acetonitrile (65/35)	Phenacetin	0.8	282 nm	4.94/7.3

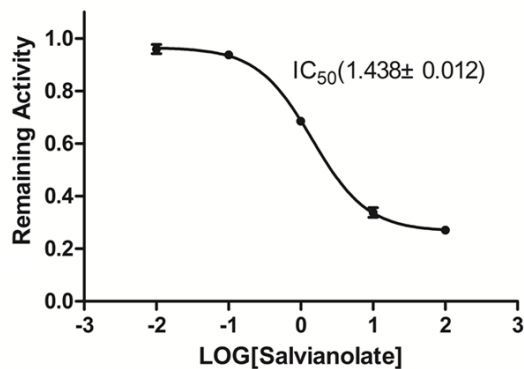


Figure 1. Inhibitory effect of salvianolate (0, 0.01, 0.1, 1, 10, and 100 mg/L) on CYP3A4 enzyme activity in HLM. The data were expressed as mean \pm SD.

CYP450s. Therefore, our present work was conducted to investigate the inhibitory effects of salvianolate on the CYP isoforms in HLMs.

Materials and methods

Chemicals

The pooled human liver microsomes (HLM) and human recombinant enzyme used in the incubation studies were purchased from BD Gentest Co. (Woburn, MA, USA). Coumarin, 7-hydroxycoumarin, tolbutamide, 4-hydroxytol-

butamide, (S)-mephenytoin, 4'-hydroxymephenytoin, metoprolol, α -hydroxymetoprolol, midazolam maleate, 1-hydroxymidazolam, phenacetin, acetaminophen, chlorzoxazone, 6-hydroxychlorzoxazone, propranolol, quinidine hydrochloride, tranlycypromine, fluconazole, ticlopidine, quinidine, ketoconazole, furafylline, and diethyldithiocarbamate were purchased from Sigma Chemicals (St. Louis, MO, USA). Formic acid, ethylic acid, ammonium formate, MgCl_2 , NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and potassium phosphate (monobase and dibase) were chromatographic-grade chemicals purchased from Sigma Chemicals (St. Louis, MO, USA). Acetonitrile and methanol were of analytical grade and purchased from Sigma Chemicals (St. Louis, MO, USA). The deionized water used in the experiments was prepared in the laboratory using a Millipore Milli-Q reverse osmosis system (Bedford, MA, USA). Salvianolate were provided by Shanghai Green Valley Pharmaceutical (Shanghai, China).

Microsomal incubations

The stock solution of salvianolate and subsequent serial dilutions were freshly prepared using deionized water and refrigerated in 4°C until use. Other stock solutions of substrates

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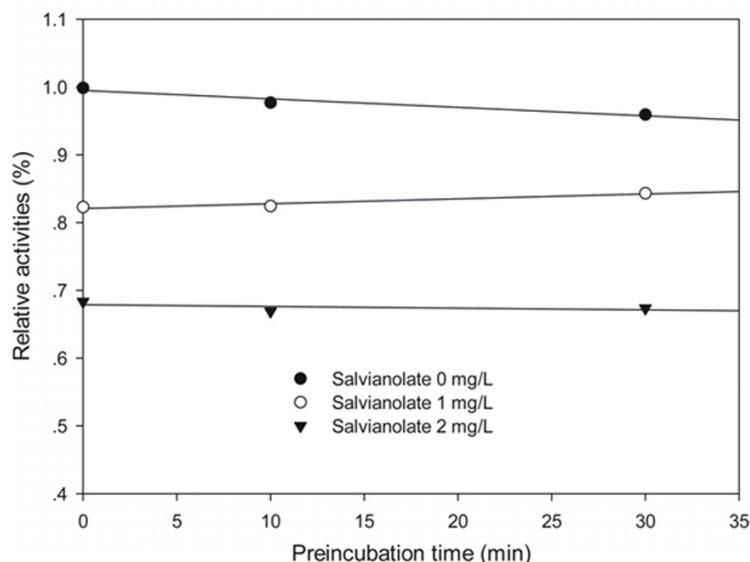


Figure 2. Time-course of CYP3A4-catalyzed midazolam 1'-hydroxylation inactivation by salvianolate in HLMs. HLMs were preincubated with salvianolate at 0 (●), 1 (○), or 2 (▼) mg/L in the presence of NADPH for 0 to 30 min. The results shown are the means of triplicate experiments.

and inhibitors were made in different ratios of acetonitrile and water. The final percentage of organic solvent in incubation mixtures was less than 1%. Incubations were conducted at 37°C in potassium phosphate buffer (100 mM KH_2PO_4 , 100 mM K_2HPO_4 , 3.3 mM MgCl_2 , pH 7.4) in the presence of 0.5 mg/mL HLM or human recombinant enzymes. Salvianolate concentrations were set from 0.01 mg/L to 100 mg/L. The incubation mixture was preincubated for 10 min prior to the initiation of metabolic reaction with NADPH system (1.3 mM NADP^+ , 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose 6-phosphate dehydrogenase), with parallel negative and positive controls in triplicate. The reactions were terminated after incubation by adding acetonitrile containing internal standard (IS). **Table 1** summarizes each enzyme that corresponds to the agents and incubation conditions for evaluating inhibitory activities of CYP450.

Inhibitory effects of salvianolate on CYP450

Compound assays were tested in triplicate using individual drug-probe substrates that were specific for each HLM-CYP or recombinant enzyme and HPLC detection. Inhibitor concentrations were set from 0.01 mg/L to 100 mg/L (5 points).

Inhibition mode of salvianolate in HLMs

To investigate the inhibition mode of salvianolate, HLMs were pre-incubated with salvianolate in potassium phosphate buffer (pH 7.4) for 0, 10, or 30 min in the presence of NADPH. Midazolam was then added and incubated for 30 min at 37°C. Midazolam was used as a probe substrate at 5, 10, or 20 μM .

cDNA-expressed CYP3A4 inactivation assay

To confirm the selective inhibition of the CYP3A4 enzyme by salvianolate, 10 pmol of human recombinant cDNA-expressed CYP3A4 was incubated with salvianolate at

0.01 to 100 mg/L, NADPH system and midazolam as a selective CYP3A4 substrate for 30 min at 37°C.

HPLC analysis

HPLC analysis was performed using the SHIMADZU LC-2010CHT with an ultraviolet detector. All chemicals were separated on Hypersil BDS C18 column (4.6×200 mm, 5 μm) (Thermo, USA) with a C18 Pre-column (4.0×3.0 mm) (Phenomenex, USA). The contents of mobile phase, flow rate, wavelength, and IS were summarized in **Table 2**. A 20 μL aliquot of each sample was subjected to HPLC analysis.

Statistic analysis

The 50% inhibitory concentration (IC_{50}) values were calculated by nonlinear regression using Graphpad Prism 5.0 (San Diego, CA, USA). Lineweaver-Burk plot was obtained from software SigmaPlot (version 10.0, Systat Software, Inc.)

Results

Inhibition of CYP450 activities by salvianolate in HLMs

The inhibitory effects of salvianolate were examined on seven typical reactions catalyzed

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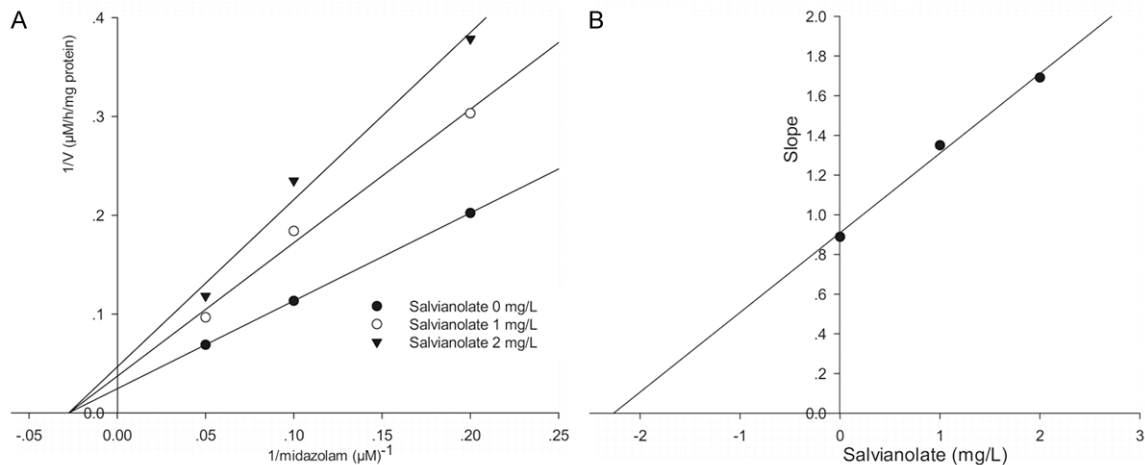


Figure 3. Lineweaver-Burk plot (A) and secondary plot (B) obtained from a kinetic study of CYP3A4-catalyzed midazolam 1'-hydroxylation following a 30 min incubation with salvianolate at 0 (●), 1 (○), or 2 (▼) mg/L. The results shown are the means of triplicate experiments.

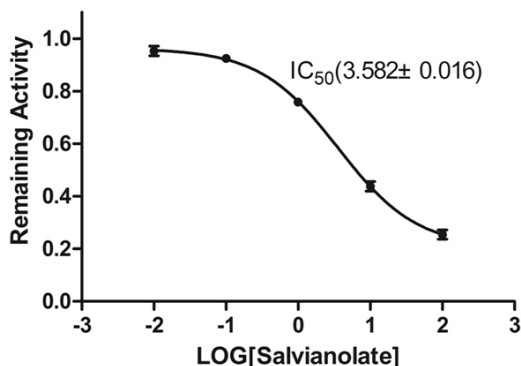


Figure 4. Inhibitory effect of salvianolate on the midazolam 1'-hydroxylation activities of human recombinant cDNA-expressed CYP3A4. The data were expressed as mean \pm SD.

by seven human CYP enzymes in HLMs. Salvianolate showed a potent inhibition of CYP3A4 activity in HLM with IC₅₀ values of 1.438 mg/L. However, salvianolate caused no significant inhibition of CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 activities (IC₅₀ >100 mg/L). **Figure 1** illustrates the inhibitory effects of salvianolate on CYP3A4 activity.

Mechanism-based inhibition of salvianolate on CYP3A4 enzyme activity

To investigate the mechanism responsible for inhibition of CYP3A4-catalyzed midazolam 1'-hydroxylation by salvianolate, CYP3A4 inhibitory activity was determined with or without pre-incubating microsomal incubation mixtures

for 0, 10, or 30 min at 37°C in HLMs. Salvianolate strongly and dose-dependently inhibited CYP3A4-catalyzed midazolam 1'-hydroxylation in HLMs, but not time-dependently (**Figure 2**). To investigate the mechanism of inhibition of salvianolate (0, 1, or 2 mg/L) on CYP3A4, concentrations of midazolam (5, 10, or 20 µM) were used. From the Lineweaver-Burk plots (**Figure 3A**) and secondary plots (**Figure 3B**), both of which linear, they indicated a noncompetitive inhibition type of salvianolate on CYP3A4. Therefore, salvianolate acts as a noncompetitive inhibitor of CYP3A4 with a K_i value of 2.27 mg/L in HLMs.

Potent inhibition of CYP3A4 by salvianolate

To evaluate the selectivity of the inhibitory effect of salvianolate on CYP3A4, salvianolate was incubated with human recombinant cDNA-expressed CYP3A4. Salvianolate showed a potent inhibition of CYP3A4 activity with IC₅₀ values of 3.582 mg/L (**Figure 4**).

Discussion

Our present investigation firstly illustrated the inhibitory effects of salvianolate on seven CYP450 enzymes activities in HLMs, as well as to identify the inhibition in recombinant enzymes. Salvianolate significantly inhibited CYP3A4 enzyme activity in HLMs.

CYP3A4 is responsible for the metabolism of a variety of drugs and endogenous compounds in humans. In general, it contains up to 60% CYP isoforms in liver. Previous reports revealed that

over 50% of the marketed drugs were metabolized by CYP3A4 [22, 23]. At present, coronary heart disease could be treated by numerous drugs, such as statins [24], antiplatelet drugs [25], anticoagulant drugs [26], ACEI, and β -receptor antagonists [27]. Many of these drugs are related to CYP3A4 metabolism. Salvianolate, a new water-soluble phenolic compound that is one of the most bioactive compounds, has been widely used in treatment of various cardiovascular diseases, especially coronary heart disease [6, 7]. Multi-drug therapy for patients with multiple complications was likely to cause interactions due to uncertain alterations in CYP metabolism, which led to serious or even fatal adverse drug reactions (ADRs) [28]. Drug-drug interaction between salvianolate and other drugs mentioned may decrease the safety and increase toxicity. When these drugs are co-administered with salvianolate, the drugs metabolized by CYP3A4 should be taken carefully to avoid severe adverse reactions.

Salvianolate, a series of aqueous extract from Danshen, contained mainly Sal B ($\geq 85\%$), rosmarinic acid ($\geq 10.1\%$) and lithospermic acid ($\geq 1.9\%$) [6, 7]. Sal B accounts for the most part of aqueous extract from Danshen. However, Qiu et al. reported that Sal B did not significantly affect the activity of CYP3A4 enzyme in HLM [29]. Hence, other aqueous extracts, such as rosmarinic acid and lithospermic acid, may inhibit the activity of CYP3A4 enzyme, but this requires further investigation to investigate how salvianolate affects activities of CYP450 enzymes.

Moreover, salvianolate showed a potent inhibition of CYP3A4 activity in HLM with IC₅₀ values of 1.438 mg/L. However, salvianolate caused no significant inhibition on CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 activities (IC₅₀ > 100 μ M). As deep into study, salvianolate may be a potent noncompetitive inhibitor of CYP3A4 enzyme activity with Ki value of 2.27 mg/L. The data enriched the CYP3A4 enzyme kinetic research of salvianolate in HLMs.

The in vitro experiment about salvianolate provide medicine co-administration advice to a certain extent. However, animal experiment and clinical trials are still needed to determine whether the salvianolate can favorably influ-

ence the treatment of other candidate drugs and the adverse effect that may caused in the condition of co-administration.

Conclusions

In conclusion, this study demonstrates that salvianolate selectively inhibited CYP3A4-catalyzed midazolam 1'-hydroxylation in HLMs and suggested that it might cause drug-drug interactions when co-administrated with CYP3A4 substrates. However, animal experiment and clinical trials are still needed to determine pharmacokinetic effects of salvianolate with respect to CYP3A4 to confirm these results.

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Disclosure conflict of interest

None.

Address correspondence to: Zhao-Qian Liu and Dr. Xiao-Yuan Mao, Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008, P. R. China; Institute of Clinical Pharmacology, Hunan Key Laboratory of Pharmacogenetics, Central South University, Changsha 410078, Hunan, P. R. China. Tel: +86 731 84805380; Fax: +86 731 82354476; E-mail: liuzhaoqian63@126.com (ZQL); maoxiaoyuan2011@163.com (XYM)

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