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Effects of Short-term and Long-term Pretreatment of Schisandra Lignans on Regulating Hepatic and Intestinal CYP3A in Rats

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Running title: CYP3A regulation by schisandra lignans

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Abbreviations:

SLE, Schisandra Lignans Extract; Pgp, P-glycoprotein; PXR, pregnane X receptor; MDZ, midazolam; 1-OH MDZ, 1-hydroxylation midazolam; 4-OH MDZ, 4-hydroxylation midazolam; 6-P-G, Glucose-6-phosphate; $NADP^+$, β-nicotinamide adenine dinucleotide

phosphate; PDH, glucose-6-phosphate dehydrogenase; HPLC, High-performance liquid chromatography; K_i, equilibrium dissociation constant for reversible inhibitor; $IC_{50,}$ inhibitor concentration that causes 50% inhibition; CMC-Na, carboxymethylcellulose; *i.g.*, intragastric *i.v.*, intravenous; TBS/T, Tris-buffered saline/Tween 20; HPLC/ESI/MS, High-performance liquid chromatography/electrospray ionization/mass spectrometry; HPLC/APCI/MS, High-performance liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry; S.D., standard deviation; $AUC_{0-\infty,}$ the area under the concentration-time curves from zero to infinity; C_{max}, the maximum plasma concentration; CL/F, the apparent oral total body clearance (where F represents bioavailability)

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Abstract:

This study was aimed to evaluate the effects of Schisandra Lignans Extract (SLE) with short- and long-term pretreatment on regulating rat hepatic and intestinal CYP3A, for a comprehensive evaluation of metabolism-based herb-drug interactions. Inhibitory effects of SLE and its major components on rat CYP3A were confirmed in both hepatic and intestinal microsomal incubation systems. After a single dose of SLE pretreatment, higher C_{max} and $AUC_{0-\infty}$ values were observed for the intragastrical midazolam (MDZ), whereas those for the intravenous MDZ were little changed. The mechanism-based inhibition of SLE towards CYP3A was further confirmed in *vivo*, characterized with a recovery half life of 38 h. In contrast, SLE long-term treatment enhanced both hepatic (2.5-fold) and intestinal (4.0-fold) CYP3A protein expression, and promoted the *in vivo* clearance of MDZ. When MDZ was co-administered with SLE after a consecutive long-term treatment, the $AUC_{0-\infty}$ value of MDZ was still lower than that of the control group, suggesting a much stronger inducing than inhibiting effect of SLE towards CYP3A. Furthermore, the intragastric administration of SLE exhibited a more intensive regulating effect towards intestinal than hepatic CYP3A, which could be partially explained by the relatively high exposures of lignans in the intestine. In conclusion, this study provides a comprehensive map for demonstrating the complicated effects of SLE and its components on regulating rat CYP3A. The important findings are that SLE possesses much stronger inducing than inhibiting effect on CYP3A, and more intensive regulating effect on intestinal than hepatic CYP3A.

Introduction

In recent years, the concomitant use of herbal medicines and/or natural products with synthetic drugs has gained increasing popularity in modern medical treatment regimens around the world. Due to the widespread and indiscriminate use of herbal medicines, the potential metabolic herb-drug interactions which may lead to severe adverse reactions have attracted a great attention from both pharmaceutical industries and regulating authorities. In the current literature, there are many references on the metabolic herb-drug interactions, such as typical studies on St. John's wort (*Hypericum perforatum*) (Rengelshausen *et al.*, 2005; Xie and Kim, 2005), grapefruit juice (Arayne *et al.*, 2005; Paine *et al.*, 2005) and garlic (*Allium sativum*) (Hu *et al.*, 2005; van den Bout-van den Beukel *et al.*, 2006).

Schisandra is such a herbal medicine that has been widely used not only in China for the treatments of dyspnea, cough, mouth dryness, spontaneous diaphoresis, nocturnal diaphoresis, nocturnal emission, dysentery, insomnia and amnesia, but also widely used in Japan as a component of Kampo medicines and in the United States as a dietary supplement. Recently, its lignans extract has been widely used along with routinely prescribed agents to treat viral and drug-induced hepatitis in China (Zhu *et al.*, 1999). Due to the widespread use of SLE worldwide, its potential on eliciting metabolic herb-drug interactions has become an important issue for clinical safety consideration. SLE and its components, especially gomisin C, have been identified to be potent CYP3A inhibitors in an *in vitro* study using human hepatic microsomal incubation system (Iwata *et al.*, 2004). SLE has also been found to be capable of increasing the oral bioavailability of tacrolimus, a substrate of both CYP3A and

Pgp, in healthy volunteers (Xin *et al.*, 2007). Interestingly, it was found that SLE treatment in hepatocytes induced both CYP3A and CYP2C expression through activating orphan nuclear receptor PXR (Mu *et al.*, 2006). It is thus reasonably to propose that SLE may exert a biphasic effect on regulating CYP3A expression and activity, *i.e*, short-term inhibition and long-term induction, same as that observed from St. John's wort (Rengelshausen *et al.*, 2005; Xie and Kim, 2005).

Although previous researches have shed a light on understanding the effect of SLE on regulating CYP3A, there are still several critical issues remained unresolved for predicting the clinical SLE-drug interactions mediated by CYP3A. Firstly, in view that the intestinal CYP3A contributes to a great extent to the first pass metabolism of many CYP3A substrates (Andersen *et al.*, 2002; Paine *et al.*, 2005; Hao *et al.*, 2007; Kato, 2008), it is necessary to dissect the influence of SLE on regulating intestinal and hepatic CYP3A, and thus for better understanding of its differential effect on regulating pharmacokinetic profiles of CYP3A substrates after intravenous and oral administration. Secondly, the CYP3A inducing effect of SLE with long-term treatment has not been well studied *in vivo* and it is unclear whether the long-term consumption of SLE will result in a rapid clearance of CYP3A substrates. Thirdly, since SLE possesses a biphasic effect on regulating CYP3A, it is unknown about the net effect when SLE after long-term consumptions is co-administered with CYP3A substrates. Finally, it is necessary to assess the recovery time span of CYP3A activity after SLE treatment, considering that SLE has been proven a mechanism-based inhibitor of CYP3A.

Our study was thus designed to resolve such critical issues described above and to provide a systematic insight into the effects of SLE on regulating CYP3A expression and activity. The potential inhibitory activity of SLE and its major components on CYP3A was tested in both rat intestinal and hepatic microsomes systems using MDZ as the typical substrate (Ghosal *et al.*, 1996; Kotegawa *et al.*, 2002; Marathe and Rodrigues, 2006). The recovery time span of CYP3A after SLE pretreatment was determined *in vivo*. The differential effect of SLE with short- and long-term treatment on regulating intestinal and hepatic CYP3A has been determined by the pharmacokinetic monitoring of intravenous and intragastric administration of MDZ. The intestinal and hepatic expression of CYP3A was determined using western blotting assay. The concentrations of major lignans components in livers and intestines were determined for better understanding of its differential effect on modulating intestinal and hepatic CYP3A. Results obtained from this study would be helpful for the better prediction of clinical SLE-drug metabolic interactions mediated by CYP3A and thus providing useful information for safe and effective use of SLE preparation combined with prescription drugs.

Materials and methods

Chemicals and reagents. Schizandrol A, deoxyschizandrin, γ-schizandrin, gomisin C, MDZ and diazepam were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Panaxatriol was from the College of Chemistry in Jilin University. 1-OH MDZ and 4-OH MDZ were from Sigma Corporation (Shanghai, China). The purity of all chemicals above was proven exceeding 99%. SLE powder (containing 10.9 % schizandrol A, 2.4 % gomisin C, 1.9 % deoxyschizandrin and 1.8 % γ-schizandrin)

was purchased from Qing Ze Medical & Pharmaceutical Technical Development Co., Ltd (Nanjing, China). 6-P-G (purity 98-100%), $NADP^+$ (purity 97%) and PDH (200-400 units/mg protein) were purchased from Sigma Corporation (Shanghai, China). HPLC grade acetonitrile and methanol were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Radio immunoprecipitation assay lysis buffer, phenylmethanesulfonyl fluoride, BCA Assay Protein Kit and SDS-PAGE sample loading buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Rabbit anti-rat CYP3A2 polyclonal antibody was from Chemicon Corporation (U.S.A) (Binds specifically to cytochrome P450 CYP3A2 in rat hepatic microsomal fraction, http://www.millipore.com/catalogue/item/ab1276). Polyvinylidene difluoride membranes were obtained from Millipore (Shanghai, China). Horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Boster Biological Technology, Ltd (Wuhan, China). Enhanced chemiluminescence kit was purchased from Pierce Chemical Company (Rockford, USA). Ethyl acetate and other regents were all of analytical grade.

Animals. Male Sprague-Dawley rats (200-220 g) were obtained from Academy of Military Medical Sciences, China. All rats were acclimated for at least one week before all experiments and allowed water and standard chow *ad libitum.* All animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

CYP3A inhibitory assay *in vitro*. Microsomes were prepared from male rat livers and intestines through differential centrifugation (Hao *et al.*, 2007). The protein concentrations

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were determined with a commercially available kit (BCA protein assay; Pierce Chemical Co.). Rat hepatic and intestinal microsomes were stored at -80 ◦C until use.

To investigate IC_{50} values of SLE and its major components towards hepatic and intestinal microsomal MDZ 4-hydroxylation activity, a typical incubation mixture contained rat microsomal protein (1 mg/ml), NADPH-regenerating system (10 mM 6-P-G, 10 mM $MgCl_2$, 1 U/ml PDH, and 0.5 mM $NADP^+$), the typical CYP3A substrate MDZ (10 μM), SLE or lignans components (schizandrol A, deoxyschizandrin, γ-schizandrin and gomisin C) at different concentrations, and 100 mM phosphate buffer (pH 7.4) in a final volume of 200 μL. All incubations were conducted at 37 ◦C for 5 min. To determine time-dependent inactivation, rat hepatic and intestinal microsomes were preincubated with SLE for 10 min in a metabolic system as described above except without substrate MDZ. After that, 10 μL of MDZ solution was added to the mixture to reach a final volume of 200 μL. Reactions were terminated 5 min later by adding cold acetonitrile and the remained MDZ 4-hydroxylation activity was determined.

To determine K_i values, schizandrol A (0, 25, 50, and 100 μM), deoxyschizandrin (0, 6.25, 12.5, and 25 μM), γ-schizandrin (0, 3.125, 6.25, and 12.5 μM) and gomisin C (0, 0.125, 0.25, and 0.5 μM) was added to the reaction mixture containing different concentrations of MDZ (5, 10, 20, and 40 μM) in the rat hepatic microsomal incubation systems. The total volume was 200 μl. Reactions were stopped by adding cold acetonitrile. Reaction conditions and sample preparation procedures were same to that described above for the IC50 assay. All incubations

were performed in triplicate.

Pharmacokinetic studies of MDZ. Rats were fasted for 12 h with free access to water before the experiment. MDZ and SLE were suspended in 0.5% CMC-Na for *i.g.* administrations. To determine the short-term effect on CYP3A, SLE (0, 50, and 150 mg/kg) was intragastrically administered 30 min prior to the *i.v.* administration (4 mg/kg) or 5 min prior to the *i.g.* administration of MDZ (20 mg/kg). The MDZ dosing time intervals to SLE pretreatment were designed from the pharmacokinetic and tissue exposures study of lignans components to ensure MDZ at maximal exposures of lignans components. To ascertain whether SLE has a mechanism-based inhibition towards CYP3A *in vivo*, MDZ was administered intragastrically to rats (4 mg/kg) 6, 24, or 72 h after SLE administration (150 mg/kg).

To determine the long-term effect of SLE on CYP3A, rats were randomly divided into three groups. Rats assigned to the pretreatment group and the co-administered group were gavaged with SLE (150 mg/kg/day) for 14 consecutive days. Rats in the control group were administered with an equivalent volume of 0.5% CMC-Na vehicle. On day 15, rats in the control group and the pretreatment group received a vehicle, while those in the co-administered group were given with SLE (150 mg/kg). Five minutes later, all rats were intragastrically administered with MDZ (20 mg/kg). Blood samples were collected from the post-ocular vein at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min after *i.g.* administration , and at 0, 2, 5, 10, 20, 30, 60, 120, 180, 240, 300, and 360 min after *i.v.* administration of MDZ. All blood samples were immediately centrifuged to obtain plasma

which was immediately stored at -20◦C until analysis.

Pharmacokinetics, hepatic and intestinal distribution assays of SLE components. For assessing pharmacokinetic behaviors of the major lignans components, rats were given a single *i.g.* administration of SLE (150 mg/kg). Blood samples were collected from the post-ocular vein at 0, 0.03, 0.08, 0.17, 0.33, 0.5, 1, 2, 4, 6, 8, 12, and 24 h. For hepatic and intestinal exposures determinations, another set of rats were given a single *i.g.* administration of SLE (150 mg/kg). Tissues (livers and intestines including duodenum, jejunum, and ileum) were promptly removed at 0, 0.5, 1, and 5 h after dosing and washed with saline. Each tissue sample was diluted with 4 volumes (v/w) of saline and then homogenized.

CYP3A protein expression assay. Rats in the pharmacokinetic studies of MDZ after long-term treatment with SLE were euthanized after blood collection. Intestines and livers were immediately excised, washed with ice-cold saline, snap-frozen in liquid nitrogen, and stored at -80◦C until analysis. Tissues were homogenized (40 mg of the mucosa of small intestine, 40 mg of liver) in ice-cold radio immunoprecipitation assay lysis buffer with 0.02 mM phenylmethanesulfonyl fluoride through ultrasonication. Samples were solubilized in lysis buffer for 30 min on an ice-cold plate and then centrifuged at 9,000 g for 10 min at 4◦C. The supernatant was collected and stored at -80◦C until use. Protein concentrations were determined with a BCA Assay Protein Kit according to the manufacturer's instructions. SDS-PAGE sample loading buffer was added into the protein samples. Samples were boiled for 4 min for protein denaturation and then centrifuged at 9,000 g for 3 min at 4◦C. Protein samples equivalent to 60 μg of protein were size-fractionated by electrophoresis on an 8 %

SDS-polyacrylamide gel at 150 V for 1 h and then transferred to polyvinylidene fluoride membranes for about 2.5 h. After blocked in a buffer containing 5 % nonfat milk for 1 h, the membranes were then incubated with CYP3A2 polyclonal antibody (1:1000 for intestine samples, 1:1500 for liver samples) for 24 h at 4◦C. For reference, β-actin was detected using a polyclonal antibody (1:200). After washing three times with TBS/T buffer for 10 min each time, membranes were incubated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1500) for 1 h at 37◦C and then washed three times with TBS/T. The signals were detected by enhanced chemiluminescence kit. The CYP3A2 protein band intensity was normalized with that of β-actin.

LC/MS assays. MDZ and its metabolites were analyzed according to the previous reports (Cummins *et al.*, 2003; Arellano *et al.*, 2007) with slight modifications. Diazepam was used as the internal standard. All samples were analyzed by HPLC/ESI/MS (Shimadzu Corporation, Japan). Briefly, a Shim-Pack 250 mm × 2.0 mm column packed with 5 μm VP-ODS C_{18} from Shimadzu Company (Kyoto, Japan) was used. The column temperature was set at 40◦C. The mobile phase delivered with a flow rate of 0.2 ml/min consisted of 2 mM ammonium acetate (A) and acetonitrile (B) with a gradient program as follows: 40% B to 75%B from 0-5 min, 75%B to 40% B from 5-7 min, and holding 40% B for another 5 min. Mass spectrometry was conducted in the positive-ion mode for the detections of MDZ at m/z 326 ($[M+H]^+$), 1-OH MDZ at m/z 342 ($[M+H]^+$), 4-OH MDZ at m/z 342 ($[M+H]^+$), and diazepam at m/z 285 ($[M+H]^+$). For microsomes samples, 400 μl of acetonitrile was added after incubation and then centrifuged at 20,000 rpm for 10 min twice. Plasma samples were extracted with 0.8 ml

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of ethyl acetate after adding a saturated solution of sodium carbonate. The precipitate was removed by centrifugation at 8000 rpm for 5 min. The supernatant was transferred to an eppendorf tube and evaporated to dryness by the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was dissolved in 0.1 ml of mobile phase.

Lignans components were analyzed by HPLC/APCI/MS (Shimadzu Coporation, Japan) using panaxatriol as the internal standard. The analytical column was a Gemini 150 mm × 4.6 mm column packed with 5μm C_{18} 110A (Phenomenex Company, U.S.A). The column temperature was maintained at 40◦C. The mobile phase delivered with a flow rate of 0.75 ml/min consisted of water (A) and methanol (B) with a gradient program as follows: 70% B to 85% B from 0-5 min, holding 85% B for another 2 min, 85% B to 70% B at from7-9 min, and holding for another 3 min. Mass spectrometry was conducted in the positive-ion mode for the detections of schizandrol A at m/z 415 ($[M-H_2O+H]^+$), deoxyschizandrin at m/z 417 ($[M + H]^+$), γ-schizandrin at m/z 401 ($[M + H]^+$), gomisin C at m/z 415 ($[M-C_6H_5CO_2+H]^+$), and panaxatriol at m/z 423 ($[M-3H_2O+H]^+$). Samples (100 μl) of plasma, liver and intestine homogenates were extracted with 0.8 ml of ethyl acetate. The supernatant was transferred to an eppendorf tube and evaporated to dryness by the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was dissolved in 0.1 ml of mobile phase. Ten μl of supernatant was injected to LC/APCI/MS for analysis.

Data and statistical analysis. IC_{50} values were determined by nonlinear regression analysis. Dixon plots were used to determine K_i values of four lignans components. Data are expressed as means ± S.D.. Pharmacokinetic parameters of MDZ, 1-OH MDZ and 4-OH MDZ were

estimated by using the computer software DAS package (version 2.0 PK software, Chinese Pharmacological Association). Comparisons between two groups were performed using Student's *t* test. For multiple comparisons, one-way analysis of variance followed by Post-Hoc test was performed. The difference was considered to be statistically significant if the probability value was less than 0.05 ($P<0.05$).

Results

CYP3A inhibitory effect of SLE and its major components *in vitro*. Microsomal MDZ 4-hydroxylation activity test was used to determine the potential inhibitory effect of SLE and its major lignans components on CYP3A. SLE showed a concentration dependent inhibitory effect on CYP3A activity with an IC_{50} value of 40±5 μg/ml in rat hepatic microsomes and of 25±3 μg/ml in intestinal microsomes, respectively. After pre-incubation with microsomes in the presence of NADPH-regenerating system, the IC_{50} values markedly reduced to 35±5 and 6.25±1.25 μg/ml, respectively, suggesting a mechanism-based inhibitory effect of SLE on CYP3A. For the components tested, deoxyschizandrin and γ-schizandrin showed a moderate inhibitory activity with an IC_{50} value of 12.5 and 6.25 μM and with a K_i value of 4.8 and 5.0 μM, respectively. Schizandrol A showed a poor inhibition activity with an IC_{50} value of 70 μM and a K_i value of 45.0 μM. Gomisin C showed a potent inhibitory effect on CYP3A with an IC_{50} value of 0.30 μM and a K_i value of 0.06 μM **(Table 1)**.

Pharmacokinetics of MDZ after short-term treatment with SLE. Pharmacokinetic interaction between SLE and MDZ *in vivo* was studied in rats. The mean plasma

concentration-time profiles of MDZ, 4-OH MDZ and 1-OH MDZ after *i.v.* (4 mg/kg) and *i.g.* (20 mg/kg) administrations of MDZ with or without SLE (0, 50, 150 mg/kg) pretreatment are shown in **Fig.1**. Pharmacokinetic parameters obtained are collected in **Table 2**. No significant differences of the pharmacokinetic parameters for the *i.v.* administration of MDZ were observed among different groups. In contrast, the $AUC_{0-\infty}$ value of MDZ with *i.g.* administration significantly increased up to 2.3- and 4.0-fold in rats pretreated with 50 and 150 mg/kg SLE, respectively. In similar, the C_{max} value of MDZ increased to 1.6- and 2.3-fold, while the CL/F value decreased to 44 % and 24 % of the control group, respectively. Consequently, the C_{max} and $AUC_{0-\infty}$ of MDZ metabolites, 4-OH and 1-OH MDZ were significantly decreased in rats pretreated with SLE, providing direct evidences on the *in vivo* inhibitory effect of SLE on CYP3A.

To determine the potential mechanism based inhibition of SLE on CYP3A, the pharmacokinetic changes of MDZ were determined 6, 24 and 72 h after SLE pretreatment. The obtained pharmacokinetic profiles of MDZ and its metabolites are shown in **Fig.2,** and the calculated pharmacokinetic parameters are summarized in **Table 3**. Accompanying with the increase of MDZ dosing time intervals to SLE pretreatment, a clear restoring trend towards the control group was observed for the pharmacokinetic profiles and parameters of both MDZ and its metabolites in rats pretreated with SLE. The CYP3A protein recovery half life was estimated at about 38 h, by plotting the net increased ratio of the $AUC_{0-\infty}$ of MDZ against MDZ dosing time intervals to SLE pretreatment (**Fig.3**).

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Pharmacokinetics, hepatic and intestinal distributions of lignans components. SLE and its major lignans components exhibited potent inhibitory effects on both hepatic and intestinal CYP3A in the microsomal incubation systems. However, pharmacokinetic studies *in vivo* showed SLE pretreatment significantly inhibited intestinal, while not hepatic CYP3A. We hypothesized that such a differential effect might be mainly resourced from the different hepatic and intestinal exposures of lignans components. To examine such a hypothesis, the pharmacokinetic profiles and hepatic and intestinal distributions of four lignans compounds (schizandrol A, deoxyschizandrin, γ-schizandrin and gomisin C) were determined. As observed from **Fig.4** and **Fig.5**, although gomisin C, the most potent inhibitor in SLE, exhibited a relatively high plasma exposure, it was failed to be detected in the liver tissues. Interestingly, schizandrol A, the poorest inhibitor in SLE, possessed the highest hepatic exposure levels among all components tested. The concentrations of all lignans components tested except the poorest inhibitor schizandrol A, in the intestine were much higher than those in the liver. Such results were in good agreement with the differential effect of SLE with intragastic administration on regulating hepatic and intestinal CYP3A.

Pharmacokinetics of MDZ after long-term treatment with SLE. The plasma profiles of MDZ, 4-OH MDZ and 1-OH MDZ obtained after *i.g.* administration of 20 mg/kg MDZ of the control, the co-administered and the pretreatment groups are shown in **Fig.6**, and the pharmacokinetic parameters are summarized in **Table 4**. Compared with the control group, rats in the co-administered and the pretreatment group showed lower MDZ $AUC_{0-\infty}$ values (63% and 34%), as well as higher CL/F values (1.6-fold and 3.1–fold). Similar changes to

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those of $AUC_{0-\infty}$ values were observed for C_{max} values of MDZ. Pharmacokinetic parameters of 1-OH MDZ in the co-administered group showed no significant differences from those in the control group, while significantly higher C_{max} and $AUC_{0-\infty}$ values were observed in the pretreatment group. For 4-OH MDZ, rats in the co-administered and the pretreatment group showed higher $AUC_{0-\infty}$ values (1.8-fold and 2.5–fold), as well as higher C_{max} values (1.4-fold and 1.8–fold), as compared with the control group.

Inducing effects of SLE on hepatic and intestinal CYP3A protein expression. After pharmacokinetic researches of MDZ and its metabolites in rats with long-term intake of SLE, the hepatic and intestinal CYP3A protein expressions were determined. After 14 days treatment, there were no significant differences in mean body weight between the control and SLE treatment groups. Compared with the control group, intestinal CYP3A protein level increased approximately by 300 % ($P<0.05$), while the hepatic CYP3A protein level increased approximately by 150 % ($P<0.05$), after 14 days pretreatment with 150 mg/kg/day SLE (**Fig.7**).

Discussion

The concomitant administration of herbal supplements and synthetic drugs has now become more and more popular. As a result, herb-drug interactions have become a common clinical problem. CYP3A, the most abundant drug-metabolizing enzyme in human liver and intestinal wall, is responsible for metabolizing a majority of drugs. Case reports, clinical trials, and *in vitro* studies have showed a number of important pharmacokinetic interaction risks of herbal

medicines with prescribed agents medicated by CYP3A (Hu *et al.*, 2005; van den Bout-van den Beukel *et al.*, 2006). Several methods have been published describing the interactions between herbal medicines and synthetic drugs based on *in vitro* data. Despite some successes, it sometimes failed to predict drug-drug interactions because of a variety of reasons (Kanamitsu *et al.*, 2000; Kanazu *et al.*, 2005). Furthermore, some herbal medicines, such as St John's wort (Rengelshausen *et al.*, 2005; Xie and Kim, 2005), showed differential effects towards CYP450 isoforms between short-term and long-term treatment. It is thus difficult to predict the herb-drug interactions in various clinical practices, especially for the herbal compounds characterized with biphasic effects on regulating drug-metabolizing enzymes. To give an example, we have designed systematic experiments to study the effects of SLE on regulating CYP3A, in order to evaluate more accurately the potentials for herb-drug interactions in different clinical practices.

In our *in vitro* study using rat hepatic and intestinal microsomes, SLE exhibited a concentration and time-dependent inhibitory effect on CYP3A. Among the lignans components tested, gomisin C was the most potent CYP3A inhibitor, while deoxyschizandrin, and γ-schizandrin showed a moderate and almost equivalent inhibitory potency. The results are generally consistent with the previous report (Iwata *et al.*, 2004), in which gomisin C was identified as a very potent mechanism based CYP3A inactivator. Considering that the mechanism based inhibitory effects of lignans *in vitro* had been previously reported (Iwata *et al.*, 2004) and the present study was mainly aimed to focus on the *in vivo* biphasic and differential effect of SLE on regulating hepatic and intestinal CYP3A, the *in vitro* inhibitory

effect determinations in our study was designed only for a confirmatory purpose, and thus the detailed mechanism based inhibition assay of SLE and its components had not been performed.

SLE with a single *i.g.* administration to rats increased both $AUC_{0-\infty}$ and C_{max} values of MDZ and consequently decreased those of its metabolites, 1-OH MDZ and 4-OH MDZ, when MDZ was *i.g.* administered 5 min after SLE pretreatment. However, pretreatment with SLE did not alter the *i.v.* pharmacokinetic behaviors of MDZ and its metabolites formation. These lines of evidences suggest that SLE with oral ingestion may exhibit a potent inhibitory effect on intestinal rather than hepatic CYP3A. Such results *in vivo* seem controversial with the *in vitro* findings. To explain such a discrepancy between *in vitro* and *in vivo*, we determined the plasma and tissue exposures of four major lignans components which showed certain inhibitory effects on CYP3A. It was found that the concentrations of most components except schizandrol A, a poor inhibitor of CYP3A, in intestines were much higher than those in livers. Gomisin C, the most potent CYP3A inhibitor among lignan components (Iwata *et al.*, 2004), was failed to be detected in liver samples, in spite of that its total plasma concentrations could reach its reported K_I value (0.399 μM). The potential high plasma protein binding of lignans components would further restrict their distribution into livers and thus compromise the inhibitory effects on hepatic enzymes. As evidenced from both *in vitro* and *in vivo* data in this study, we conclude that the oral ingestion of SLE are unlikely to induce a significant inhibitory effect on hepatic CYP3A *in vivo* due to their poor exposures in liver, which may be caused by their poor intestinal absorption and potential high plasma protein binding.

The previous report (Iwata *et al.*, 2004) and the present study *in vitro* demonstrated that SLE exhibited a mechanism-based inhibition towards CYP3A. It had been well acknowledged that the mechanism-based inhibitor caused irreversible inhibition of an enzyme until this enzyme was newly synthesized. To our knowledge, there have been no reports contributing to the mechanism-based inhibition of SLE study *in vivo*. Therefore, we have designed an experiment for determining the recovery time course of CYP3A activity in rats after a single *i.g.* dose treatment with SLE. As a result, inhibition of the enteric CYP3A activity appeared to continue for approximately 3 days, which was in consistent with the previous reports (Venkatakrishnan *et al.*, 2001; Greenblatt *et al.*, 2003) that the activity of human enteric CYP3A recovered within 3 days.

It was interesting to note that both aqueous and ethanolic extracts of *Schisandra chinensis Baill* up-regulated CYP3A expression through PXR activation (Mu *et al.*, 2006). However, the *in vivo* CYP3A inducing potency of SLE had not been previously confirmed. Results from the present study provide direct evidences on that SLE is a strong modulator of CYP3A expression in rats, as evidenced from both protein expression and activity test *in vivo*. Unlike inhibition, long-term oral pretreatment with SLE significantly up-regulated the hepatic CYP3A protein level, albeit to a lesser extent compared with that on intestinal CYP3A. Such a discrepancy is not surprising, in view that schizandrol A reported to be a potent PXR activator (Mu *et al.*, 2006) showed relatively high hepatic exposures as determined from the present study. In combinatorial considerations, we propose that the various lignans components with differential inducing and/or inhibiting potencies may underlie the biphasic

regulating effect of SLE on CYP3A. The major limitation of this study is that we have not measured the protein expression of CYP3A23/CYP3A1, which has been reported much more inducible than CYP3A2 in most cases (Huss et al., 1999). However, in view that CYP3A2 is the predominant constitutively expressed CYP3A isoform in adult male rats (Jan et al., 2006) and that it is more active than other CYP3A isoforms(Mahnke et al., 1997), we consider that the up-regulation of CYP3A2 protein levels explains to a great extent of the enhanced total CYP3A activity as measured by MDZ in the adult male rats with SLE long-term treatment in this study.

Since SLE possessed a biphasic effect on regulating CYP3A, we were interested in investigating the net effect when SLE was co-administered with MDZ after long-term consumptions of SLE. It has been found that the rats in both the co-administered group and the pretreatment group showed lower C_{max} and $AUC_{0-\infty}$ values of MDZ, and higher C_{max} and $AUC_{0-\infty}$ values of 4-OH MDZ compared with the control group, suggesting the net effect was of CYP3A induction. In other words, the induction capacity of SLE is much stronger than that of inhibition on CYP3A. Our studies suggest that SLE appears to exert a biphasic effect on regulating CYP3A. The initial oral dose of SLE would lead to an apparent inhibition on intestinal CYP3A both reversibly and irreversibly, while its long-term consumption is more likely to produce a significant CYP3A induction.

In summary, our results showed that SLE with *i.g.* administration inhibited the activity of intestinal but not of hepatic CYP3A. Its mechanism-based inhibition of CYP3A activity

appeared to continue for 38 h in rats. However, the long-term intake of SLE resulted in the induction of both intestinal and hepatic CYP3A protein. In addition, SLE with long-term treatment exerted a much stronger inducing than inhibiting effect on CYP3A. The present findings suggest that it is very critical to assess the potential interactions between SLE remedies and prescribed drugs as CYP3A substrates, thus avoiding clinically unwanted SLE-drug interactions.

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Footnote:

L.L. and H.P.H. contributed equally to this work.

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Legends for figures:

Fig.1 Plasma concentrations of MDZ and its metabolites *vs.* time course after *i.v.* (a-c) and *i.g.* (d-f) administrations of MDZ in rats with or without SLE pretreatment: (a, d) plasma concentrations of 4-OH MDZ; (b, e) plasma concentrations of 1-OH MDZ; (c, f) plasma concentrations of MDZ. The doses of MDZ for *i.g.* and *i.v.* administration were 20 mg/kg and 4 mg/kg, respectively. SLE was intragastrically administered with a dose of 50 and 150 mg/kg, respectively. The control rats received an equal volume of vehicle. Each data point is the average of four rats and the vertical bar represents S.D..

Fig.2 Plasma concentration-time profiles of 4-OH MDZ (a), 1-OH MDZ (b) and MDZ (c) of rats pretreated with 4 mg/kg MDZ 6, 24 and 72 h after exposure to SLE or vehicle. Each point and bar represents the mean and S.D. of 4 rats.

Fig.3 Relationship between the time post SLE exposure and the net increased ratio of the $AUC_{0-\infty}$ of MDZ over the control value. The recovery half-life time was estimated as 38 h (r^2 = 0.98). Each point represents the mean value obtained from 4 rats.

Fig.4 Plasma concentrations of four components of SLE after *i.g.* administration of SLE (150 mg/kg). (a) plasma concentrations of schizandrol A and gomisin C; (b) plasma concentrations of deoxyschizandrin and γ-schizandrin. Each data point is the average of four rats and the vertical bar represents S.D..

Fig.5 Concentrations of four compounds of SLE in the livers (a) and small intestines (b) at 0.5, 1, and 5 h after *i.g.* administration of SLE (150 mg/kg). Each data point is the average of four rats and the vertical bar represents S.D..

Fig.6 Time courses of the plasma concentrations of MDZ (a), 4-OH MDZ (b) and 1-OH MDZ (c) in rats assigned to the control, co-administered, and pretreatment group. Rats were gavaged once daily with 0.5% CMC-Na (control) or 150mg/kg/day SLE (co-administered and treatment groups) for 14 consecutive days. On day 15, rats were gavaged with vehicle (control and treatment groups) or 150 mg/kg SLE (co-administered group). After 5 min, they were gavaged with 20 mg/kg MDZ. Each data point is the average of four rats and the vertical bar represents S.D..

Fig.7 Effects of SLE on the expression of intestinal and hepatic CYP3A. (a) Data analysis of western blotting results. (b) Image of western blotting results. Rats were gavaged once daily with vehicle (control) or 150 mg/kg/day SLE for 14 consecutive days. Intestinal and hepatic tissues were collected on day 15. CYP3A2 levels were measured using the anti-rat CYP3A2 poly-antibody as described in Materials and Methods. β-Actin was used as control. Bars represent mean ± S.D. of folds relative to the control value (band densities of CYP3A2 normalized by β-Actin) (*, $P<0.05$). Each data point is the average of four rats and the vertical bar represents S.D..

Table 1 IC_{50} and K_i values of four lignans in SLE on rat hepatic microsomal MDZ 4-hydroxylation activity (n=3)

Components	IC_{50}(μM)[a]	K_i(μM)
schizandrol A	70±10	45
deoxyschizandrin	12.5±2.5	4.8
γ-schizandrin	6.25±1.5	5
Gomisin C	0.30±0.05	0.06

[a] Values are the mean ± standard deviation of three determinations (i.e., three rat hepatic microsomal preparations).

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Table 2 Pharmacokinetic parameters of MDZ and its metabolites in rats with *i.g.* administration of MDZ after short-term administration of SLE (n = 4)

parameters	control	SLE (50 mg/kg)	SLE (150mg/kg)
4-OH MDZ			
$AUC_{0-\infty}$(μg/l*min)	99951.6±28610.3	63713.4±12439.5	44117.2±17997.5**
C_{max}(μg/l)	2436.3±302.3	977.9±105.5**	571.8±228.9**
T_{max}(min)	12.5±5.0	21.0±8.2	26.3±7.5
1-OH MDZ			
$AUC_{0-\infty}$(μg/l*min)	86071.9±27782.7	60611.5±17600.9	45954.2±19214.0*
C_{max}(μg/l)	1882.0±761.9	962.5±61.6*	607.5±262.0*
T_{max}(min)	12.5±5.0	24.0±8.2	26.3±7.5
MDZ			
$AUC_{0-\infty}$(μg/l*min)	99855.0±17681.6	229001.4±43349.9**	400312.0±29768.9**
C_{max}(μg/l)	2400.8±852.9	3799.6±711.2**	5439.7±204.5**
T_{max}(min)	10.0±5.8	15.0±0.0	15.0±0.0
CL/F(L/min/kg)	0.2062±0.0442	0.0897±0.0159*	0.0502±0.0037*

One-way ANOVA with post hoc test was conducted and statistically significant differences ($p < 0.05$ and $p < 0.01$) from control group were shown by the asterisks (* and **), respectively.

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Table 3 Pharmacokinetic parameters of MDZ and its metabolites with *i.g.* administration of MDZ at 6, 24 and 72 h after SLE short-term pretreatment (n=4)

parameters	control	6h	24h	72h
4-OH MDZ				
$AUC_{0-\infty}$(μg/l*min)	20971.4±5925.7	8612.0±731.9**	10307.1±2484.9**	13632.1±4968.2
C_{max}(μg/l)	388.9±243.5	119.6±14.4**	139.0±61.2**	252.8±19.5
T_{max}(min)	26.3±22.5	15.0±0.0	30.0±0.0	15.0±0.0
1-OH MDZ				
$AUC_{0-\infty}$(μg/l*min)	14495.8±2266.8	10897.4±109.7	8790.0±735.1**	12544.4±4051.4
C_{max}(μg/l)	244.4±101.2	123.0±9.1*	112.9±35.6**	187.4±17.8
T_{max}(min)	15.0±0.0	20.0±8.7	26.3±7.5	16.3±10.3
MDZ				
$AUC_{0-\infty}$(μg/l*min)	32699.7±1268.8	51104.4±8378.4**	45139.9±5776.5*	38262.4±8844.6
C_{max}(μg/l)	500.0±188.2	842.3±245.7	637.7±32.9*	506.2±47.1
T_{max}(min)	21.2±26.3	15.0±0.0	15.0±0.0	22.5±8.7
CL/F(l/min/kg)	0.1224±0.0047	0.0762±0.0111**	0.0897±0.0117*	0.1102±0.0328

One-way ANOVA with post hoc test was conducted and statistically significant differences ($p < 0.05$ and $p < 0.01$) from control group were shown by the asterisks (* and **), respectively

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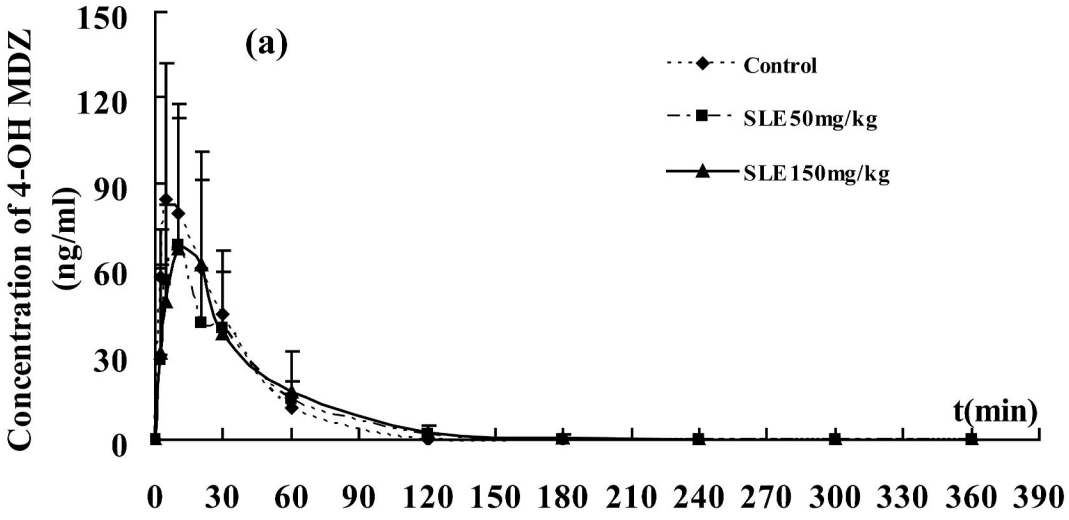
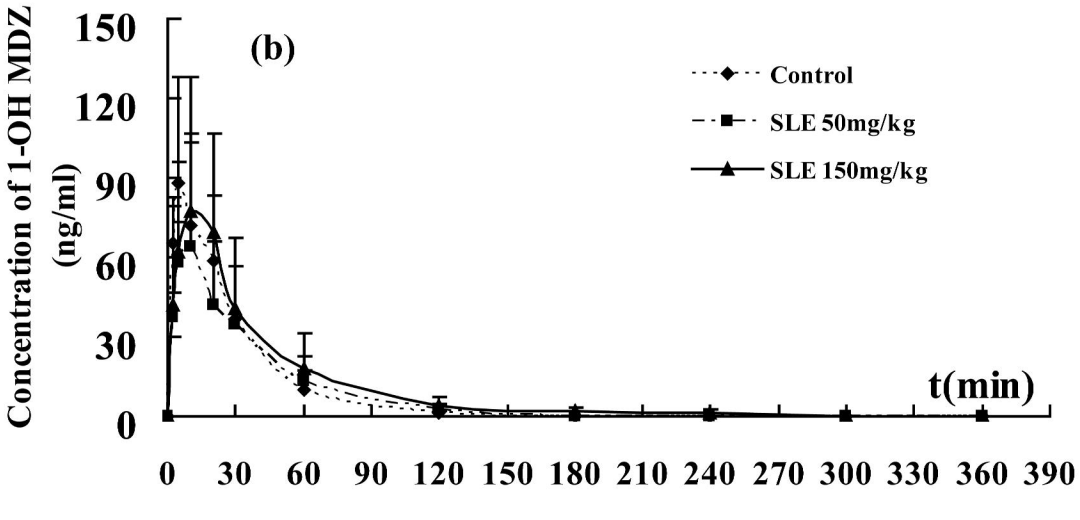
Table 4 Pharmacokinetic parameters of MDZ and its metabolites in rat plasma in control, co-administered, and pretreatment groups following *i.g.* administration of MDZ after long-term pretreatment with SLE (n=4)

parameters	control	co-administered	treatment
4-OH MDZ			
$AUC_{0-\infty}$(μg/l*min)	38001±3613	69105±8276**	93161±6524**
C_{max}(μg/l)	614.3±57.2	841.3±10.5**	1111.0±76.9**
T_{max}(min)	15.0±5.8	15.0±5.8	20.0±0.0
1-OH MDZ			
$AUC_{0-\infty}$(μg/l*min)	22916±3203	28538±4216	38349±11636**
C_{max}(μg/l)	410.8±40.9	384.4±22.0	668.2±137.5**
T_{max}(min)	12.5±5.0	20.0±0.0	17.5±9.6
MDZ			
$AUC_{0-\infty}$(μg/l*min)	99410±13185	62494±4307**	33744±6999**
C_{max}(μg/l)	1822.6±234.1	1263.4±47.4*	786.5±241.0**
T_{max}(min)	15.0±5.8	12.5±5.0	17.5±5.0
CL/F(l/min/kg)	0.20±0.03	0.32±0.02	0.61±0.14**

One-way ANOVA with post hoc test was conducted and statistically significant differences ($p < 0.05$ and $p < 0.01$) from control group were shown by the asterisks (* and **), respectively

Figure 1 (a-c)


(a)
Concentration of 4-OH MDZ (ng/ml)
150
120
90
60
30
0
Control
SLE 50mg/kg
SLE 150mg/kg
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390


(b)
Concentration of 1-OH MDZ (ng/ml)
150
120
90
60
30
0
Control
SLE 50mg/kg
SLE 150mg/kg
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

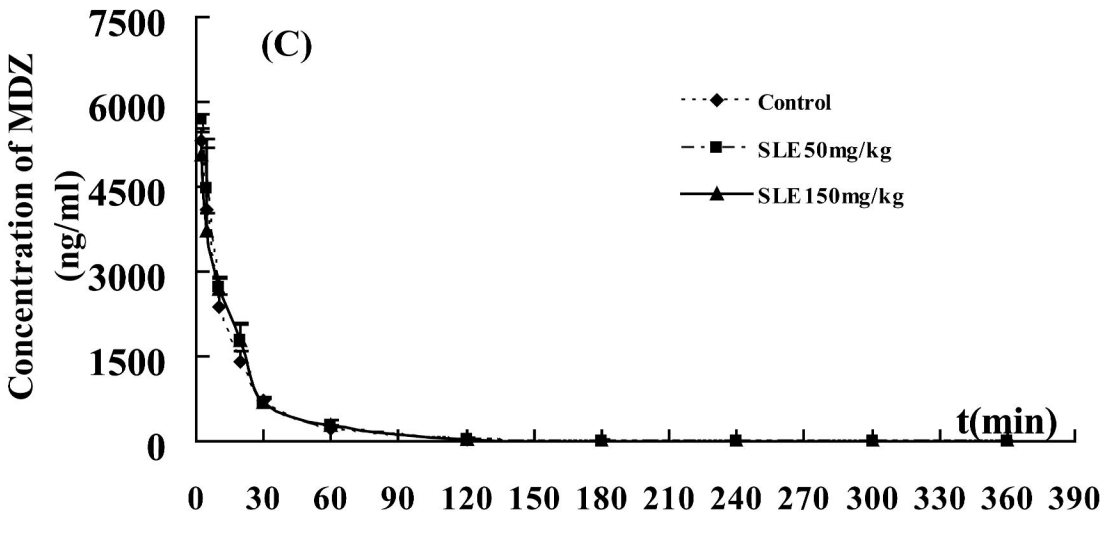
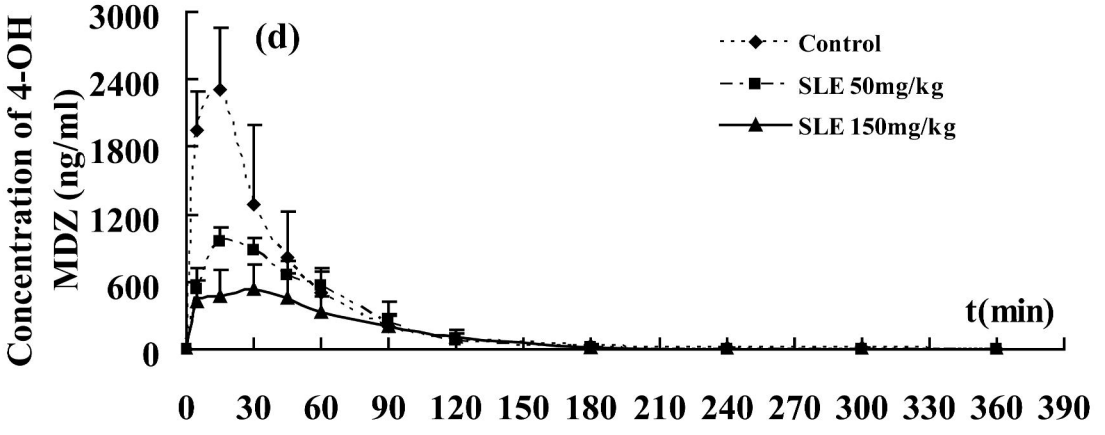
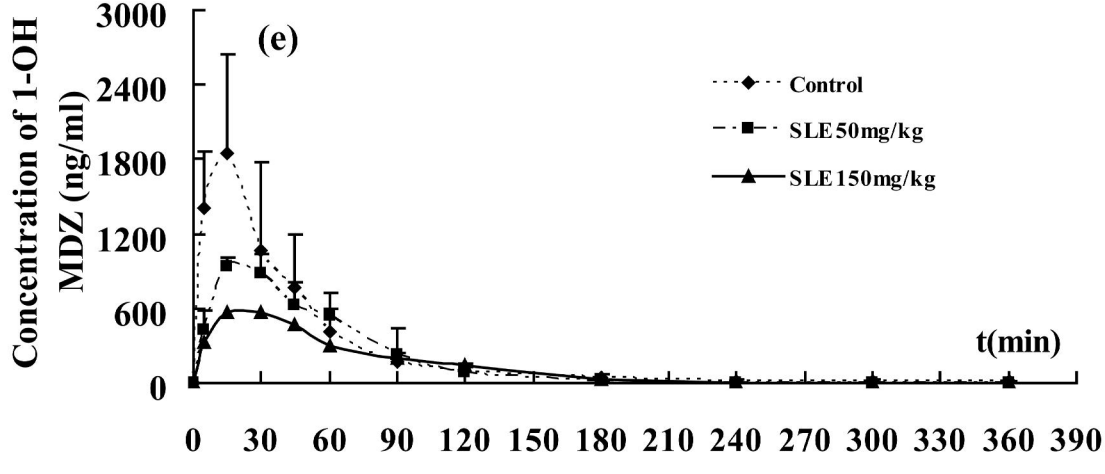

(C)
Concentration of MDZ (ng/ml)
7500
6000
4500
3000
1500
0
Control
SLE 50mg/kg
SLE 150mg/kg
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

Figure 1 (d-f)


(d)
Concentration of 4-OH MDZ (ng/ml)
3000
2400
1800
1200
600
0
Control
SLE 50mg/kg
SLE 150mg/kg
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390


(e)
Concentration of 1-OH MDZ (ng/ml)
3000
2400
1800
1200
600
0
Control
SLE 50mg/kg
SLE 150mg/kg
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

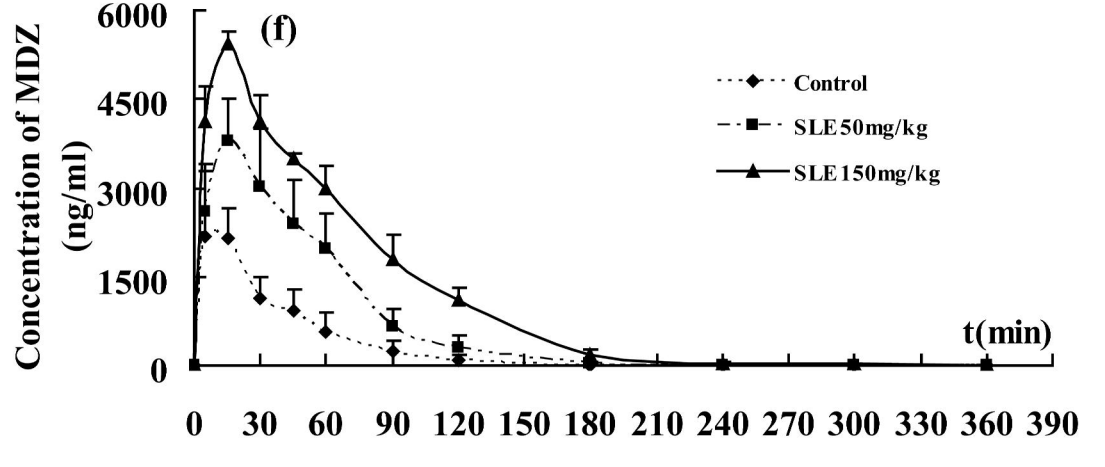
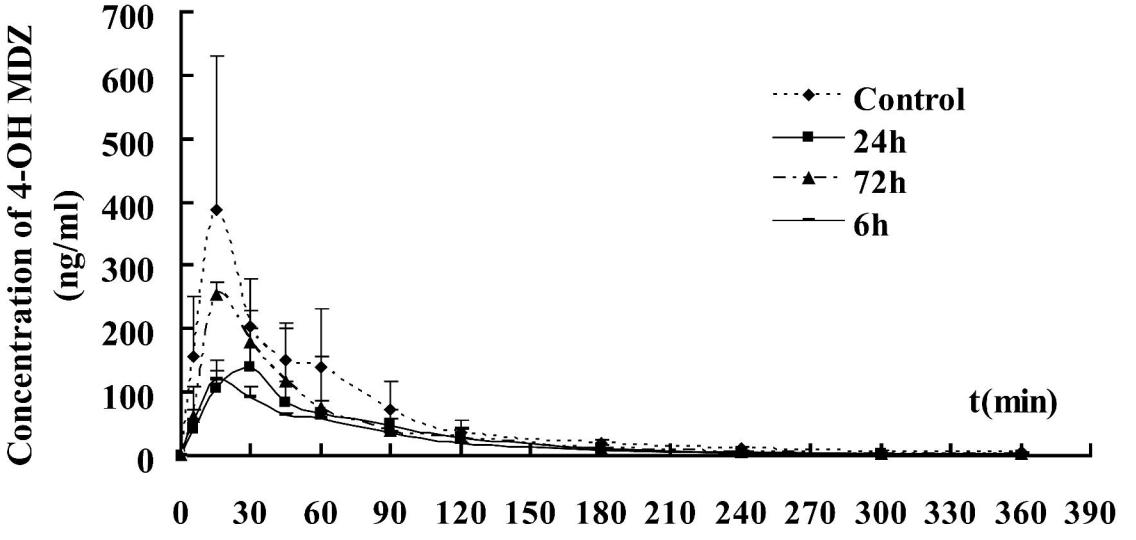
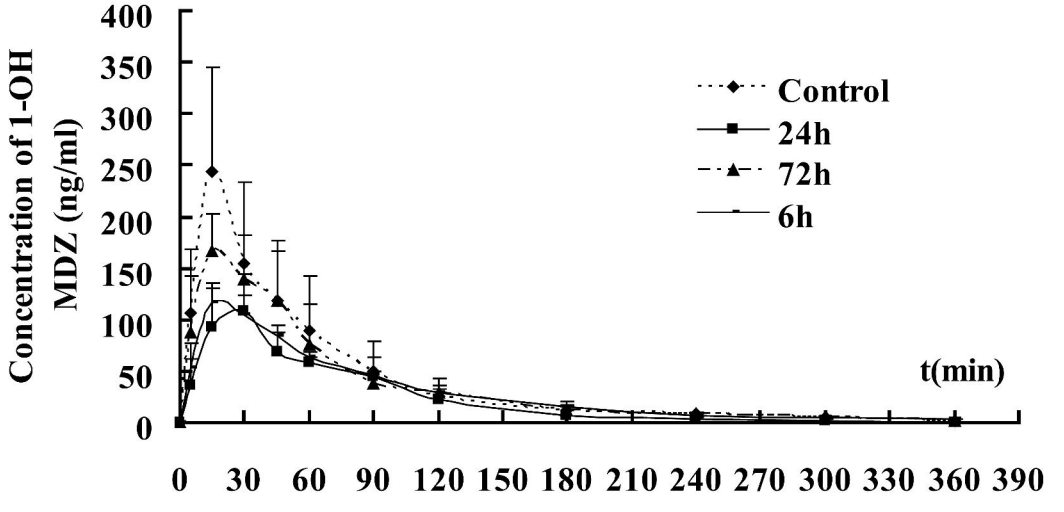

(f)
Concentration of MDZ (ng/ml)
6000
4500
3000
1500
0
Control
SLE 50mg/kg
SLE 150mg/kg
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

Figure 2

(a)


Concentration of 4-OH MDZ (ng/ml)
700
600
500
400
300
200
100
0
Control
24h
72h
6h
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

(b)


Concentration of 1-OH MDZ (ng/ml)
400
350
300
250
200
150
100
50
0
Control
24h
72h
6h
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

(c)

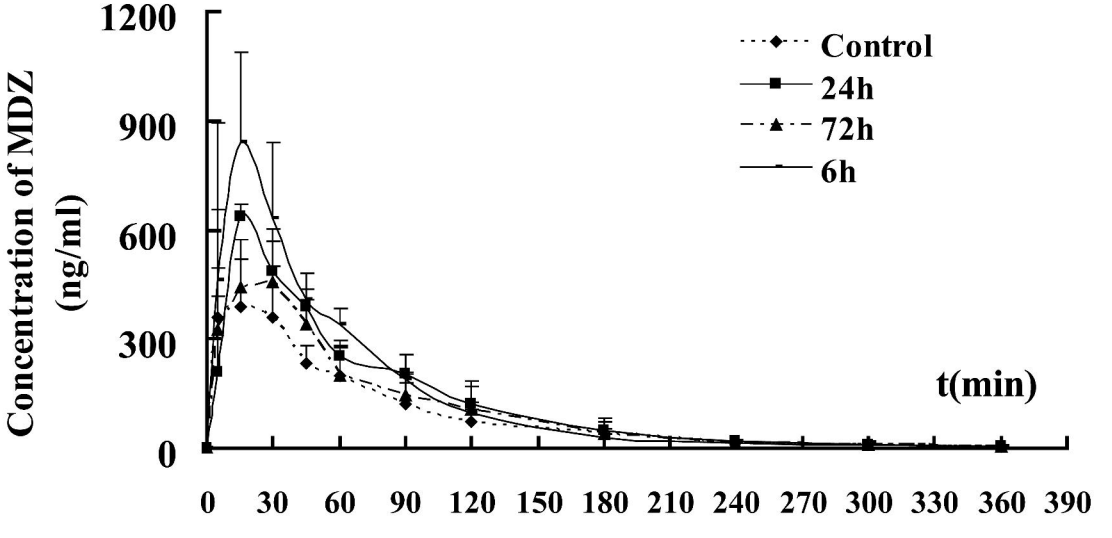

Concentration of MDZ (ng/ml)
1200
900
600
300
0
Control
24h
72h
6h
t(min)
0 30 60 90 120 150 180 210 240 270 300 330 360 390

Figure 3

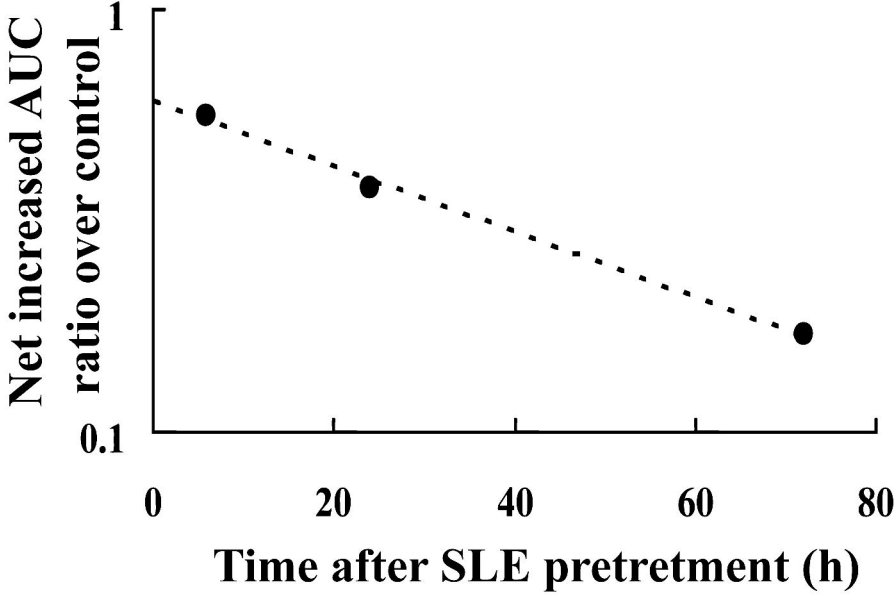
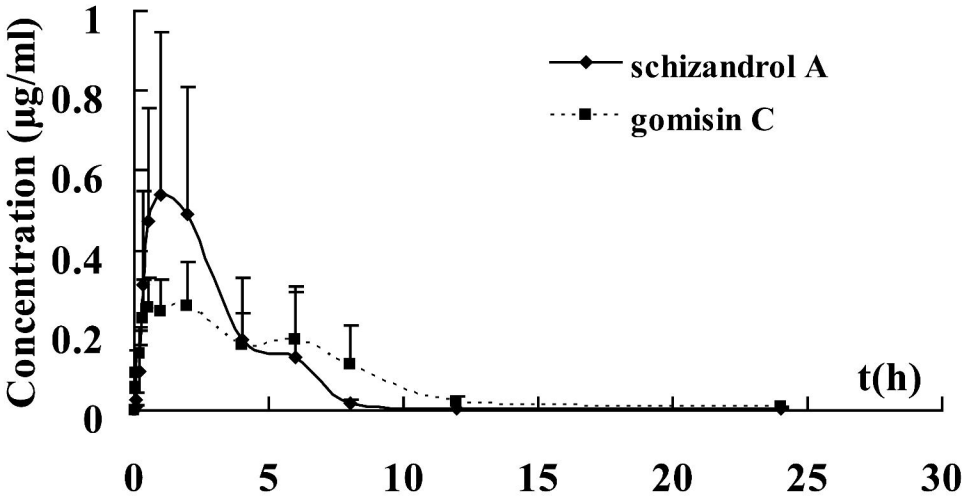

Net increased AUC ratio over control
1
0.1
0
20
40
60
80
Time after SLE pretretment (h)

Figure 4

(a)


Concentration (μg/ml)
1
0.8
0.6
0.4
0.2
0
schizandrol A
gomisin C
t(h)
0
5
10
15
20
25
30

(b)

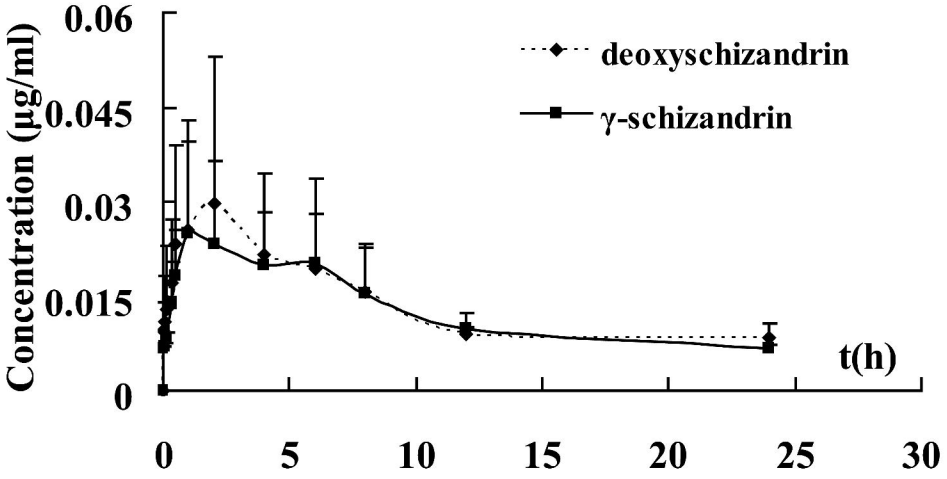
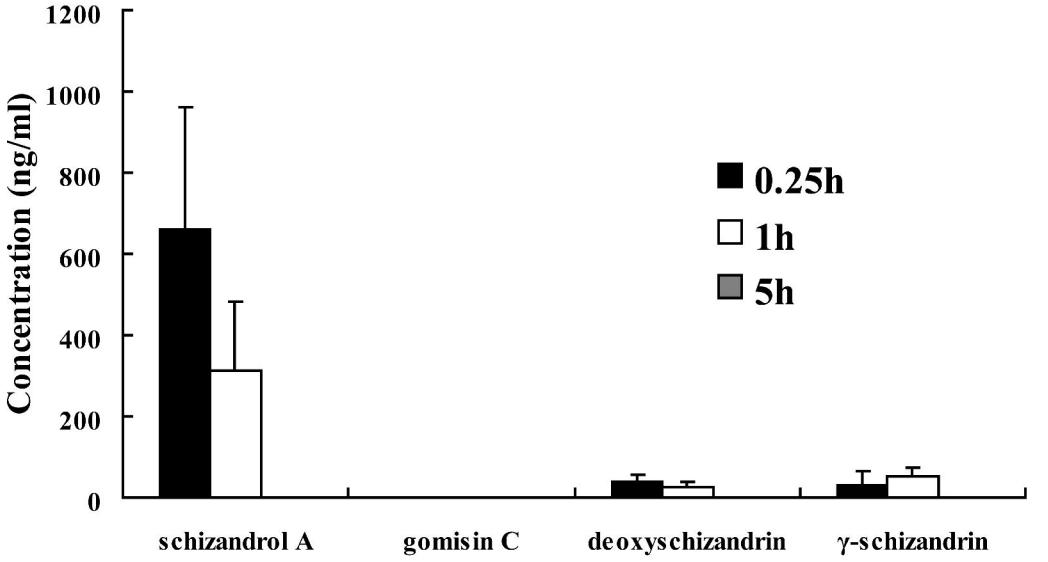

Concentration (μg/ml)
0.06
0.045
0.03
0.015
0
deoxyschizandrin
γ-schizandrin
t(h)
0
5
10
15
20
25
30

Figure 5

(a)


1200
1000
800
600
400
200
0
Concentration (ng/ml)
0.25h
1h
5h
schizandrol A
gomisin C
deoxyschizandrin
γ-schizandrin

(b)

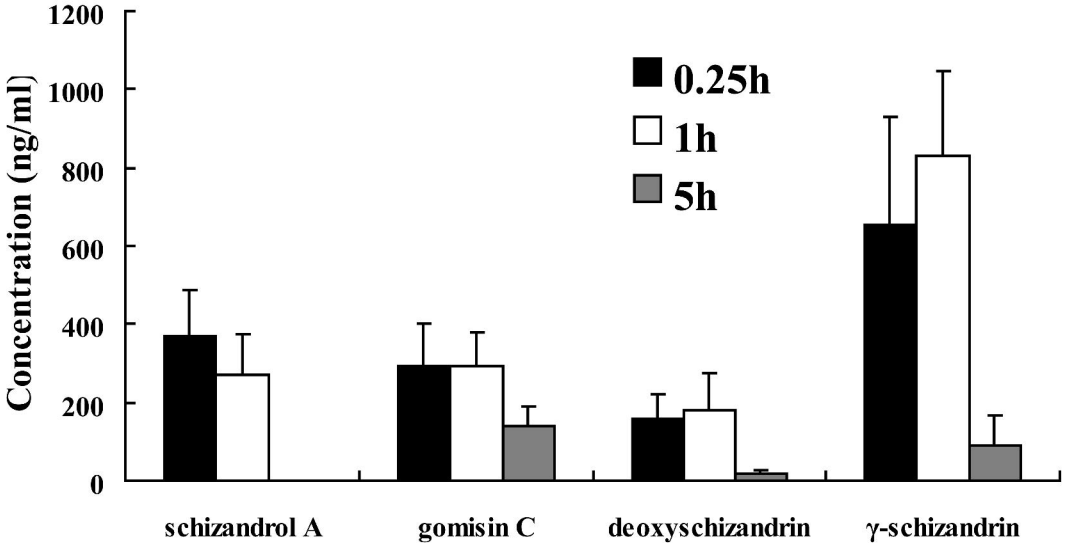

1200
1000
800
600
400
200
0
Concentration (ng/ml)
0.25h
1h
5h
schizandrol A
gomisin C
deoxyschizandrin
γ-schizandrin

Figure 6

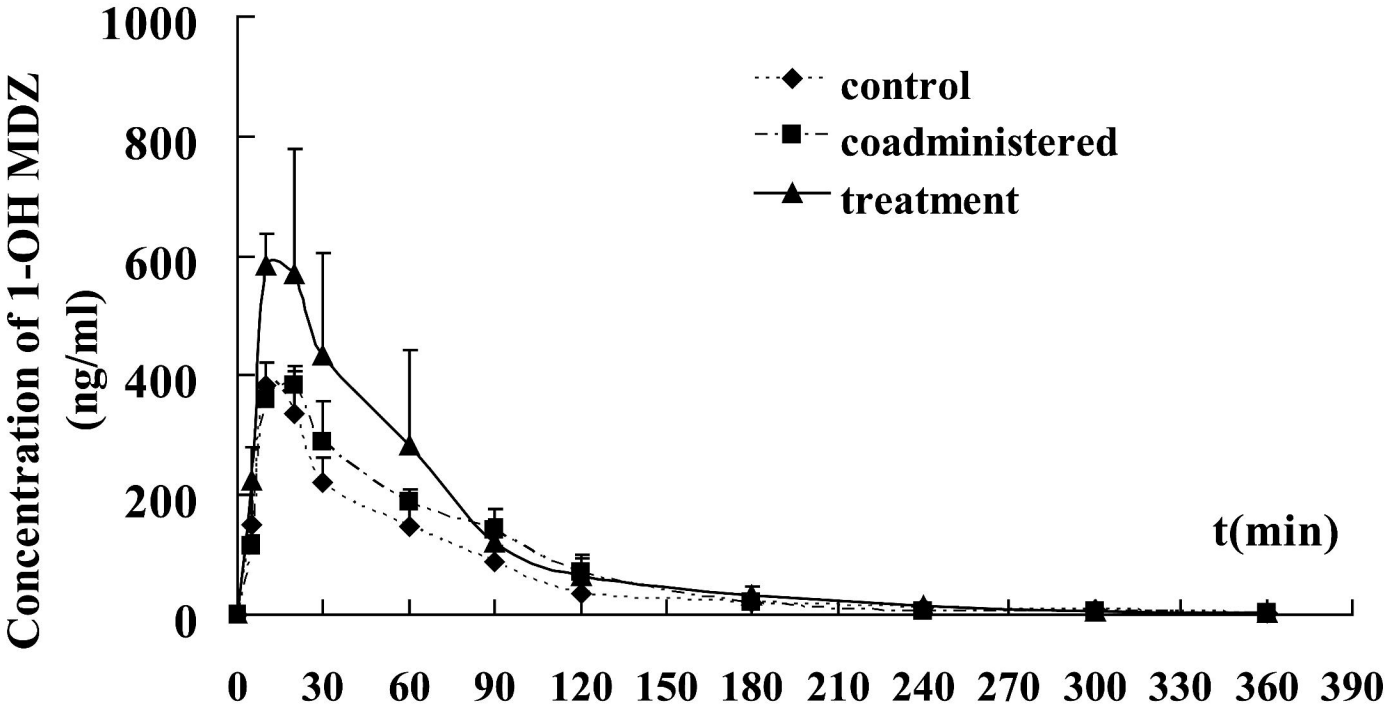
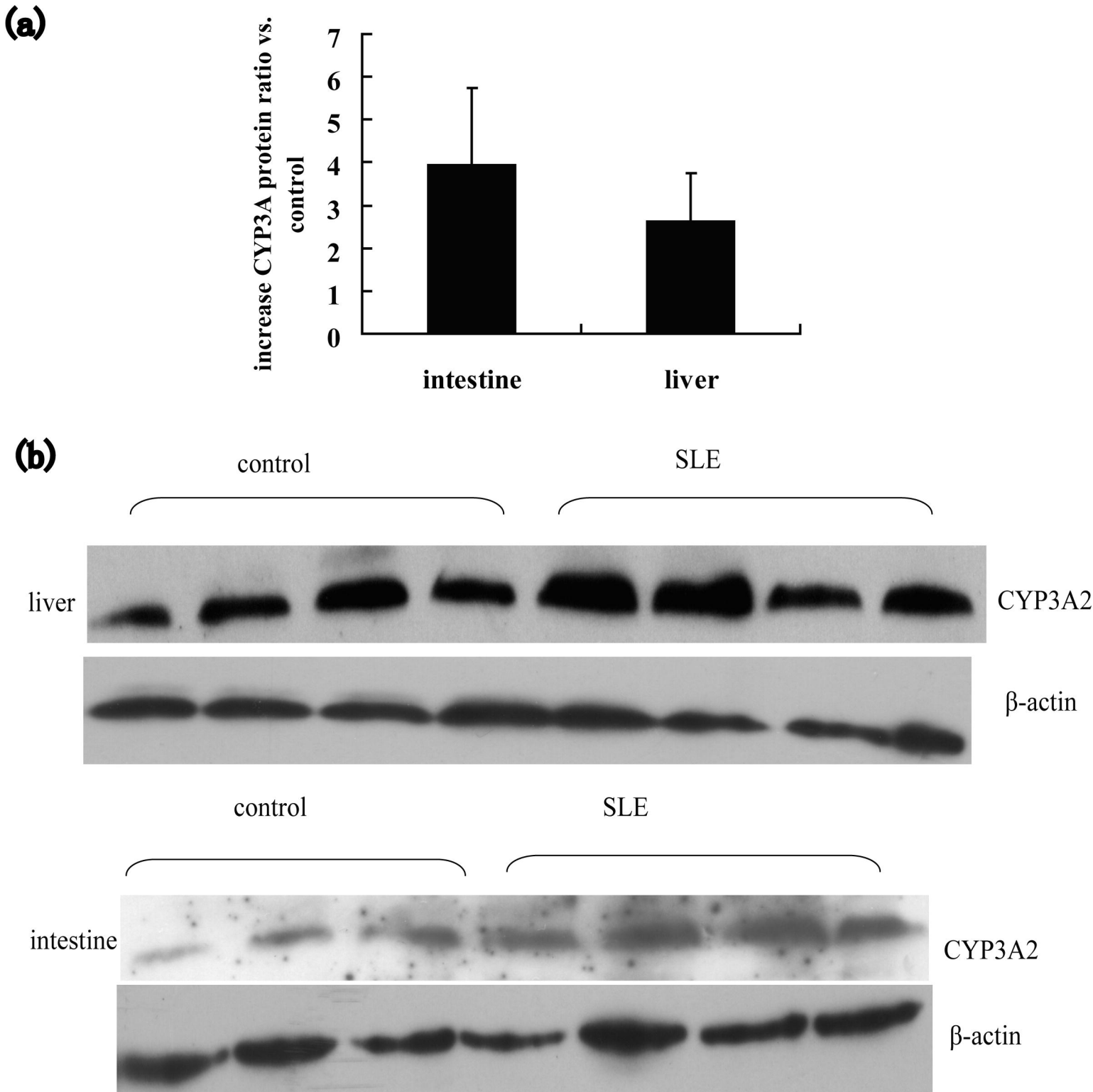

1000
800
600
400
200
0
Concentration of 1-OH MDZ (ng/ml)
control
coadministered
treatment
t(min)
0
30
60
90
120
150
180
210
240
270
300
330
360
390

Figure 7


(a)
increase CYP3A protein ratio vs. control
7
6
5
4
3
2
1
0
intestine
liver
(b)
control
SLE
liver
CYP3A2
β-actin
control
SLE
intestine
CYP3A2
β-actin