Cytochrome P450 dysregulations in thioacetamide induced liver cirrhosis in rats and
the counteracting effects of hepatoprotective agents

Yuan Xie, Guangji Wang, Hong Wang, Xilin Yao, Shan Jiang, An Kang, Fang Zhou,
Tong Xie, Haiping Hao

State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and
Pharmacokinetics, China Pharmaceutical University, Nanjing 210009, China
Running title: CYP450 dysregulations in TAA damaged liver

Corresponding author: Dr. Haiping Hao, State Key Laboratory of Natural Medicines, Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Tongjiaxiang 24, Nanjing 210009, China; Tel: +86-25-83271179; Fax: +86-25-83271060; E-mail: hhp_770505@yahoo.com.cn

Number of text pages: 27

Number of tables: 3

Number of figures: 6

Number of references: 21

Number of words in the abstract: 229

Number of words in the introduction: 494

Number of words in the discussion: 1121

**Abbreviations**

SLE, Schisandra Lignans Extract; DDB, dimethyl diphenyl bicarboxylate; TAA, thioacetamide; drug metabolizing enzymes (DMEs); CYPs, cytochrome P450s; UGTs, UDP-glucuronosyltransferases; NADP+, β-nicotinamide adenine dinucleotide phosphate; LC-MS, liquid chromatography mass spectrometry; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; Tbil, total bilirubin; TBST, Tris-buffered saline/Tween 20; PVDF, Polyvinylidene Fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RLM, rat liver microsome
Abstract:
Dysregulations of cytochrome P450s (CYPs) under liver injury have been extensively studied. However, little is known about the possible reversing effects of hepatoprotective agents, the understanding of which is of great importance in guiding clinical dosage adjustment for patients with liver injury. This study aims to investigate the dysregulation patterns of major CYPs in thioacetamide (TAA) induced liver cirrhosis rats, and the potential counteracting effects of hepatoprotective agents, schisandra lignan extracts (SLE) and dimethyl diphenyl bicarboxylate (DDB). TAA intoxications for 6 weeks induced apparent liver injury and dramatically reduced the hepatic protein expressions of CYP1A2, CYP2C6, CYP2E1, and CYP3A2 to 18%, 71%, 30%, and 21% of that in the normal control, respectively. Both SLE and DDB treatments could significantly reverse the TAA induced loss of CYPs’ protein levels, which may be ascribed to their hepatoprotective effects and direct CYPs inducing effects that have been confirmed in the healthy rats. However, the recovery of enzyme activities of most CYPs by SLE and DDB treatment was less evident than that for the protein expression levels. TAA exhibited a NADPH, time, and concentration dependent inactivating effects on all of the four major CYP isozymes; both DDB and GSH showed little effects on counteracting such an inactivation efficacy. These findings provided good explanation on the disproportional effects of hepatoprotective agents in recovering the protein levels and enzyme activities of TAA induced dysregulated CYPs.
Introduction

Thioacetamide (TAA), a potent selective hepatotoxin, is well known to induce both acute and chronic hepatic failure (Ishikawa et al., 2011; Steib et al., 2010; Zaldivar et al., 2010). Prolonged exposure to TAA always results in bile duct proliferation and liver cirrhosis histologically similar to that in human viral hepatitis infection (Hunter et al., 1977; Ledda-Columbano et al., 1991; Yeh et al., 2004). Because of this virtue, TAA has been widely applied to develop animal models of liver fibrosis and/or cirrhosis mimicking human nonbiliary liver diseases. From these animal models, many important pathological processes and involved mechanisms of liver fibrosis/cirrhosis had been disclosed, contributing to the development of novel diagnostic and therapeutic approaches and hepatoprotective drugs.

Human liver diseases caused by various factors usually lead to the dysregulations of most drug metabolizing enzymes (DMEs), especially cytochrome P450s (CYPs), which necessitates the dosage adjustment of therapeutic drugs in patients with severe liver diseases. Since TAA induced liver fibrosis/cirrhosis models have been widely applied to mimick human liver diseases, it is definitely important to understand TAA induced dysregulation patterns of drug metabolizing enzymes. Unfortunately, the present understanding of this critical issue is extremely limited. Increased expression of CYP2E1 and CYP2A5 was observed in TAA induced liver injury models (Camus-Randon et al., 1996; Avni et al., 2003); a previous report showed that nicotine metabolism was significantly decreased in the TAA induced fibrotic rat livers,
associated with the down-regulated protein levels of most CYPs (Nakajima et al., 1998). However, it remains unclear about the TAA induced potential regulation of enzyme activity of CYPs. TAA is a thiono-sulfur containing compound and readily bioactivated by CYP2E1 and FMO to produce acetamide and thioacetamide-S-dioxide. These reactive metabolites may covalently bind to various proteins, which is indeed the major mechanism of TAA on causing liver toxicity.

Based on these characteristics, we hypothesized that TAA intoxication may lead to both the translational dysregulations and direct enzyme inactivations of CYPs. To test this hypothesis, both protein levels and enzyme activities of major CYPs were determined in the chronic TAA treated rat livers. Potential inactivating effects of TAA on CYPs were performed in the in vitro liver microsomal incubation systems.

In addition, it should be noted that the patients with liver diseases are usually inclined to consume hepatoprotective agents. Therefore, it is important to understand whether such hepatoprotective agents can influence the dysregulated CYPs in injured livers, which is of critical concern for adjusting the dosage regimen of co-administered drugs. Current understanding of such an important issue is extremely limited. We have recently found that the hepatoprotective agent, Schisandra lignan extrat (SLE), was effective on protecting the liver from CCl₄ induced damage and restoring the pharmacokinetic characteristics of its lignans components, indicating the recovery of some CYPs (Xie et al., 2010). Based on a TAA induced liver cirrhosis rat model that better mimics human liver cirrhosis, the present study was further designed to
determine whether SLE treatment would be effective on recovering TAA induced dysregulations of major CYPs.

Material and methods

Chemicals and regents

TAA was obtained from Jiahui medicine chemical limited-liability company (Anhui, China). Dimethyl diphenyl bicarboxylate (DDB) was purchased from Hangzhou Dengyun Pharm & Tech Co., Ltd pharmacy (Zhejiang, China). The ethanol extract of Schisandra chinensis Bail (SLE) was purchased from Nanjing Qingze Pharmaceuticals Company (Jiangsu, China). Midazolam, chlorzoxazone, diclofenac, phenacetin, and acetaminophen were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 4’-hydroxydiclofenac, 4-hydroxymidazolam, 6-hydroxychlorzoxazone, Glucose 6-phosphate, NADP+, and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile, methanol, and ethyl acetate were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA). Radio immunoprecipitation assay analysis buffer, phenylmethanesulfonyl fluoride, and SDS-PAGE sample loading buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Polyvinylidene difluoride membranes were obtained from Millipore (Shanghai, China). Rabbit anti-rat CYP3A2 polyclonal antibody, rabbit anti-rat CYP2E1 polyclonal antibody,
and mouse anti-rat CYP1A2 polyclonal antibody were from Chemicon Corporation (U.S.A). Mouse anti-rat CYP2C6 polyclonal antibody was from Santa Cruz (U.S.A). Mouse anti-rat β-actin polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Boster Biological Technology, Ltd (Wuhan, China). Enhanced chemiluminescence kit was purchased from Pierce Chemical Company (Rockford, USA).

Animals and Experimental design

Male Sprague-Dawley rats (180-220 g) were obtained from Academy of Military Medical Sciences, China. All rats were maintained in an air-conditioned animal quarter at a temperature of 25±2 °C and a relative humidity of 50±10%. Water and food were allowed ad libitum. The animals were acclimatized to the facilities for a week, and then fasted with free access to water for 12 h prior to each experiment. All animal experimental procedures were approved by the Animal Care and Use Committee of China Pharmaceutical University and have been carried out in accordance with the Declaration of Helsinki. Rats were randomly divided into five groups with six animals in each group. TAA was intraperitoneally administered (200 mg/kg, 3% in saline, twice a week for six weeks) to all groups of rats except the normal control group which received normal saline injectione. From the beginning of the fifth week, rats were intragastrically treated with CMC-Na suspension (group I and II), SLE (100 mg/kg/day, group III), SLE (400 mg/kg/day, group IV), or DDB (200 mg/kg/day, group V) for two weeks. To determine the effects of SLE and DDB
on regulating CYPs in healthy rats, three groups of healthy rats were received an intragastrical administration of SLE (100 mg/kg/day), SLE (400 mg/kg/day), or DDB (200 mg/kg/day) for two weeks, respectively. At the end of treatments, blood samples were collected; the rats were then euthanized and the liver samples were immediately removed and frozen in liquid nitrogen.

**Blood biochemical analysis and histopathological study**

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and total bilirubin (Tbil) were determined by an automatic blood biochemical analyzer (Beckman Counter LX20, USA). Slices of the same part of the livers were cut off and fixed in phosphate buffered 10% formaldehyde solution and then embedded in paraffin wax. Sections of liver tissue (6mm²*5μm) were cut and stained with haematoxylin–eosin and examined for histopathological changes under the microscope (Olympus BH2, Japan). The images were taken using Nicon coolpix990 camera at original magnification of 100× and 200×.

**CYPs enzyme assays**

Rat liver microsomes were prepared as described previously (Kamataki and Kitagawa, 1974; Kaul and Novak, 1987) and stored at -80°C until use. Protein concentrations were measured by the method of Lowry *et al* (Lowry et al., 1951). Enzymatic activities of CYPs were characterized based on the following reactions: phenacetin...
O-deethylation for CYP1A2, diclofenac 4-hydroxylation for CYP2C6, chlorzoxazone 6-hydroxylation for CYP2E1, and midazolam 4-hydroxylation for CYP3A2, as described previously with minor modifications (Qiu et al., 2008). A 200 μl incubation mixture for a regular assay procedure were prepared with 0.2 mg/ml liver microsomes, 10 mM glucose 6-phosphate, 0.5 mM NADP, 10 mM MgCl₂, 1 unit of glucose 6-phosphate dehydrogenase, 100 mM phosphate buffer (pH 7.4), and the specific probe substrate (phenacetin, 5-400 μM; diclofenac, 1-400 μM; chlorzoxazone, 1-200 μM; or midazolam, 1-200 μM) of each CYP isoenzyme. The incubation was conducted at 37 °C for 20 min for CYP1A2, 10 min for CYP2C6, 10min for CYP2E1, and 5 min for CYP3A2, respectively. The incubation time and the microsomal protein concentrations were optimized to ensure the linear formation of metabolites. The reaction was terminated by cold acetonitrile. All incubations were performed in duplicates. The Cl int values were determined by nonlinear regression analysis of the enzyme activity-substrate concentration data using the Michaelis-Menten model.

Mechanism based inactivations of TAA on microsomal CYPs were assessed using a two-step procedure. Primary incubations were performed in a media containing 0.5 mM NADPH, 5 mg/ml RLM, and various concentrations of TAA in 100 mM phosphate buffer (pH 7.4) in a final volume of 200 μl. At various time points (0, 5, 10, 15, and 30min), 20 μl of the primary reaction media was removed and added into a secondary reaction media containing specific CYPs substrate, NADPH regenerating system, and buffer as usual. All the experimental procedures of the secondary incubations were the same to that described above for the enzyme activity assay of
CYPs. All incubations were performed in triplicates.

Western blot analyses

Protein levels of the major rat CYP isozymes were determined by a western blot analysis. Liver protein samples (10 μg of protein per lane) were separated by SDS polyacrylamide gel electrophoresis with an 8% polyacrylamide gel and transferred to a PVDF membrane by electroblotting. The PVDF sheets were blocked in 5% nonfat dry milk in TBST (0.05% Tween 20 in TBS) at 37 °C for one hour. The blots were incubated with primary antibodies diluted in 5% nonfat dry milk in TBST overnight at 4 °C followed by incubation with goat anti-rabbit (CYP2E1 and CYP3A2) or goat anti-mouse (CYP1A2 and CYP2C6) secondary antibodies for one hour. The primary antibodies against CYP1A2, CYP3A2, CYP2C6, and CYP2E1 were diluted 1:1500, 1:1500, 1:1000, and 1:1000, respectively. For reference, β-actin was detected using a polyclonal antibody (1:200) for 1 hour and then incubated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1500) for 1 h at 37°C. The immunoblots were developed with the enhanced chemiluminescence detection method with reagents from Bioworld Technology inc. USA. Proteins were detected by enhanced chemiluminescence on Kodak film (Sigma-Aldrich, St. Louis, MO, U.S.A.). Quantification of relative CYP levels was done by laser densitometry of the X-ray films. The density of the immunoreactive bands was analyzed using Image J software (NIH).
Statistical analysis

The values of the experimental data were expressed as mean ± standard deviation (S.D.). Statistical significances were evaluated using one-way ANOVA, followed by the post-hoc LSD method for multiple comparisons. The difference was considered significantly significant when the probability value was less than 0.05 ($P<0.05$).

Results

TAA induced liver cirrhosis and hepatoprotective effects of SLE and DDB

We have recently reported that TAA intoxication for 8 weeks caused severe liver damage in rats (Hao et al., 2011). The present study showed that TAA intoxication for 6 weeks could also induce apparent hepatic injury as evidenced by the significantly enhanced levels of serum ALT, AST, ALP, and Tbil ($p\leq 0.01$). SLE and DDB treatment for 2 weeks could partially reverse TAA induced alternations of serum ALT, AST, and Tbil levels, supporting the hepatoprotective effects of both SLE and DDB (Supplemental Fig.1). TAA induced extensive changes in liver morphology including steatosis, inflammation, fibrosis, centrilobular necrosis, and scattered lymphomononuclear infiltration in hepatic parenchyma. Treatment with SLE exhibited dose-dependent reversal effects on TAA induced liver fibrosis, characterized with decreased necrotic zones and hepatocellular degeneration (Fig.1).

Expression of hepatic CYPs in TAA induced cirrhotic livers

Hepatic CYPs protein levels were determined by western blot analyses. TAA
Intoxication significantly decreased CYP1A2, CYP2C6, CYP2E1, and CYP3A2 protein levels to 18%, 71%, 30%, and 21% of that of the normal control, respectively. SLE and DDB treatment markedly attenuated TAA intoxication caused downregulation of all these CYPs' protein levels, especially for CYP3A2 and CYP2E1 (Fig. 2). It was noted that the protein levels of some CYPs in SLE and DDB treated rats were even higher than those in the normal control group; CYP3A2 protein levels in SLE (100mg/kg and 400mg/kg) and DDB (200mg/kg) treated rats were 1.09-fold, 1.37-fold, and 1.56-fold of that in the normal control; CYP2E1 protein level in DDB treated rats was 2.26-fold of that in the normal control (Fig. 2).

Activity of hepatic CYPs in TAA induced cirrhotic livers
Liver microsomes were prepared from each group of rats to test the enzyme activities of CYPs using the typical probe substrate approach. Results are shown in table 1. As evidenced from intrinsic clearance (Cl_int) values, TAA intoxication markedly decreased the activity of CYP1A2, CYP2C6, CYP3A2, and CYP2E1 to 49%, 33%, 69%, and 43% of that in the normal control, respectively. SLE and DDB treatment partially reversed TAA induced loss of CYP1A2, CYP2E1, and CYP3A4 activity. In contrast, CYP2C6 activity was less affected by SLE and DDB treatment. Correlation analysis showed that the activity and protein expression were consistent for CYP1A2 and CYP3A2, but not for CYP2E1 and CYP2C6 (Fig. 3).

Activity and expression of hepatic CYPs in healthy rats treated with SLE/DDB
To clarify the effects of SLE and DDB on regulating normal rat liver CYPs, rats were treated with SLE and DDB for 2 weeks and the activity and expression of CYP1A2, CYP2C6, CYP3A2, and CYP2E1 were determined. As shown in Table 2, SLE and DDB showed inhibitory effects on CYP1A2; to the contrary, both SLE and DDB largely enhanced the activity of CYP2C6, CYP3A2, and CYP2E1. Consistently, the results of western blot analysis showed the decrease of CYP1A2 but the increase of CYP2C6, CYP3A2, and CYP2E1 protein levels in the SLE and DDB treated healthy rats’ livers (Figure 4).

Mechanism based inhibitory effects of TAA on CYPs

In view of the bioactivation characteristics of TAA, we further investigated the potential mechanism based inhibitory effect of TAA on CYPs. TAA (0, 5, 50, 200, and 400 μM) was incubated with normal rat liver microsomes by adding the NADPH regenerating system; the remaining enzyme activity at indicated time points was determined by a typical dilution approach (Brady et al., 1987; Li et al., 2009).

Appropriate controls, lacking TAA, were included to assure that the loss of activity was not due to TAA irrelevant inactivation. Without the addition of NADPH, TAA has little effect on inactivating CYPs (data not shown). TAA inhibited CYP1A2, CYP2C6, CYP3A2, and CYP2E1 activity in a concentration and time dependent manner when NADPH was included in the incubations (Fig. 5). The observed first-order rate constants ($k_{obs}$) for the inactivation of CYPs by individual
concentrations of TAA were obtained from the slope of individual lines. These slopes were fit to a Kitz-Wilson plot, which is shown in the inset of Fig. 5. The calculated $K_I$ and $k_{\text{inact}}$ values are shown in Table 3. As evidenced from the $k_{\text{inact}}/K_I$ values, it seems that CYP3A2 and CYP2C6 were more susceptible than CYP1A2 and CYP2E1 to the TAA induced mechanism-based enzyme inactivations. The addition of DDB and reduced glutathione (GSH) to the primary incubation systems had little effect on protecting against TAA induced enzyme inactivations of CYPs (Fig. 6).

**Discussion**

Intensive understanding of CYPs dysregulation patterns in liver injury is of critical concern because of its significance in guiding clinical dosage regimen adjustment in patients with liver diseases. Considering that patients with liver diseases are inclined to consume hepatoprotective agents, it is important to understand the mixed effects of pathological factors and hepatoprotective agents on regulating CYPs. The present study contributes to confirm that the protein levels and enzyme activities of CYPs were decreased in TAA induced rat cirrhotic livers. Upon NADPH dependent bioactivation, TAA were able to inactivate enzyme activities of CYPs in a time and concentration dependent manner. Hepatoprotective agents SLE and DDB treatment could partially restore TAA induced protein loss of CYPs but had little direct effect in antagonizing enzyme inactivating effects of TAA.

Dosage regimen adjustment for many therapeutic drugs is necessary for the patients
with severe liver diseases, because of the dysregulations of various DMEs in damaged livers. However, it remains still a difficult issue for designing an ideal dosage adjustment regimen because various DMEs are not altered in a uniform extent in the damaged livers. The case may be further complicated by the concomitant ingestion of hepatoprotective agents which may directly or indirectly regulate the protein expression and/or enzyme activities of DMEs. Taking SLE and DDB as examples, the present study highlighted the importance of studying the regulations of drug metabolizing enzymes by hepatoprotective agents in damaged livers. In accordance with a previous report (Nakajima et al., 1998), we confirmed that the protein levels of all the four major CYP isozymes were significantly reduced by chronic TAA intoxication, albeit to a varying extent for different isozymes. Results revealed that the hepatoprotective agents SLE and DDB largely attenuated TAA induced protein loss of most CYP isozymes. The protein levels of CYP2E1 and CYP3A2 in TAA intoxicated rats with SLE and DDB treatment were even higher than that in the normal control of rats. These results suggest that SLE and DDB may have direct inducing effects on CYPs. For this consideration, both protein levels and enzyme activities of major CYPs were determined in the healthy rats treated with SLE and DDB for 2 weeks. Results showed that SLE and DDB treatment could significantly enhance the protein expressions and enzyme activities of most CYP isozymes except CYP1A2, for which an inhibitory effect was observed instead. Previously, we had also confirmed that SLE after long term consumption could induce the protein expression of CYP3A2 in both the rat intestine and liver (Lai et al., 2009). Schisandra
had been proved to be a strong inducer of CYP3A4 and CYP2C9 through PXR in normal hepatocyte cultures (Mu et al., 2006). Together, these results indicate that SLE and DDB have direct inducing effects on CYPs, which provides good explanation on why the protein expression levels of CYP3A2 and CYP2E1 in TAA intoxicated and SLE/DDB treated rats are upregulated to a level higher than that in the normal control. Although SLE and DDB shows inhibitory effects in CYP1A2, the loss of CYP1A2 in TAA damaged livers could be largely restored by hepatoprotective agents’ treatment, suggesting a differential regulating effect in between healthy and damaged livers by hepatoprotective agents. These results in combination suggest that the restoration of CYPs in damaged livers by hepatoprotective agents may be ascribed to both the direct enzyme inducing effects and the hepatoprotective-driving consequent effects.

Using the typical probe substrate approach, we found that the enzyme activities of all the major CYPs were significantly decreased in TAA induced cirrhotic livers. To our surprise, the enzyme activities of most CYPs were not restored in proportion to the protein levels by hepatoprotective agents’ treatment. In view that TAA can be bioactivated by CYP2E1 to produce highly reactive metabolite di-S-oxide (Kang et al., 2008), we hypothesized that TAA upon bioactivation may inactivate CYPs. Our results showed that TAA exhibited both time and concentration dependent inhibitory effect on all the four CYP isozymes when NADPH was added to initiate bioactivations. In contrast, TAA had little effect on inhibiting CYPs when NADPH was omitted from the incubation systems. In accordance, we have recently found that
TAA could also inactivate UDP-glucuronosyltransferases (Hao et al., 2011). In addition, DDB and GSH could not counteract such an inactivation; this result may suggest that the oxidative stress produced from the procedure of TAA bioactivation has little effect on inactivating CYPs. It is thus understandable why the recovery effects of hepatoprotective agents in the enzyme activities are less evident than that in the protein levels of CYPs in TAA intoxicated rats. We have recently showed that both SLE and DDB had little effect on inhibiting TAA metabolism. Furthermore, the results from the present study suggest that SLE and DDB cannot inhibit CYP2E1, which was identified as the major enzyme responsible for TAA bioactivation and hepatotoxicity. Together, our results suggest the hepatoprotective effects of SLE and DDB are not likely resourced from the direct influence on the TAA’s metabolic bioactivation. In combination with our previous report (Hao et al., 2011), our results provide novel evidence on supporting the previous assumption of that TAA hepatotoxicity is mainly caused by TAA's reactive metabolites on covalently modifying macromolecules (Chieli and Malvaldi, 1984). However, it is important to note that the TAA chronic intoxication leads to differential regulating mode in between CYPs and UGTs, although both can be inactivated by TAA bioactivation. In addition, the effects of hepatoprotective agents on regulating CYPs and UGTs are also different. It may deserve further research to delineate the direct effects and underlying mechanisms of TAA's reactive metabolites on inactivating various drug metabolizing enzymes; thus providing strong scientific evidence on explaining the diverse regulating patterns of drug metabolizing enzymes in TAA induced cirrhotic livers.
Based on the TAA induced liver fibrosis/cirrhosis model and the study of SLE and DDB, the present study highlights the importance of investigating the mixed effects of hepatic pathological factors and hepatoprotective agents treatment on regulating CYPs. Our results show that TAA intoxication causes a tremendous loss of CYPs while the hepatoprotective agents SLE and DDB treatments attenuate the protein loss of CYPs. SLE and DDB have a direct inducing effect on most of the CYP isozymes except CYP1A2 in healthy rats. TAA exhibits a NADPH, time, and concentration dependent inactivating effect on all of the four CYP isozymes and both DDB and GSH have little effect on counteracting such an inactivation effect, providing good explanation on the disproportional recovery of protein levels and enzyme activities of CYPs by hepatoprotective agents’ treatment. Hopefully, our results will stimulate wide interest in investigating the effects of various hepatoprotective agents on regulating drug metabolizing enzymes in injured livers; the understanding of which is pivotal in guiding the design of dosage adjustment regimen for patients with severe liver diseases.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Hao, G. Wang, Y. Xie

Conducted experiments: Y. Xie, Hao, H. Wang, Jiang, Yao, Kang, Zhou, T. Xie

Performed data analysis: Y. Xie, Hao

Wrote or contributed to the writing of the manuscript: Hao, Y. Xie
References


R and Xie W (2006) Traditional Chinese medicines Wu Wei Zi (Schisandra chinensis Baill) and Gan Cao (Glycyrrhiza uralensis Fisch) activate pregnane X receptor and increase warfarin clearance in rats. *J Pharmacol Exp Ther* **316**:1369-1377.


Footnotes

This work was supported by the National Natural Science Foundation of China [Grants 91029746, 30801422]; the Program for New Century Excellent Talents in University of China [Grant NCET-09-0770]; and a Foundation for the Author of National Excellent Doctoral Dissertation of China [Grant 200979].
Legends for figures

Fig. 1. Representative photomicrographs of histopathological studies of livers stained with haematoxylin and eosin. Rats were treated with TAA (200 mg/kg) intraperitoneally twice a week for 6 weeks; SLE and DDB was administered to rats intragastrically for 2 weeks once a day at the beginning of fifth week. Left panel A-E:100x; right panel a-e: 200x; (A, a) Normal, (B, b) TAA treated rats, (C, c) rats treated with SLE 100 mg/kg + TAA, (D, d) rats treated with SLE 400 mg/kg + TAA, (E, e) rats treated with DDB 200 mg/kg + TAA.

Fig. 2. Western blot analyses of major CYPs in rat liver samples. Rats were treated with TAA for 4 weeks and then co-treated with or without SLE or DDB for another 2 weeks. TAA was intraperitoneally administered (200 mg/kg, twice a week for six weeks) to all groups of rats except the normal control group which received normal saline injection. SLE (100 and 400 mg/kg) and DDB (200 mg/kg) were administered intragastrically once a day for 2 weeks. Protein expression levels of CYP1A2, CYP2C6, CYP3A2, and CYP2E1 were determined via western blot analysis; β-actin was used as an internal standard to normalize all samples. * p≤0.05, ** p≤0.01, vs. normal group; † p≤0.05, †† p≤0.01, vs. TAA group.
Fig. 3. Correlation analysis of protein levels and enzyme activities of CYPs.

Fig. 4. Western blot analyses of CYPs in the liver samples of healthy rats treated with SLE and DDB. Rats were intragastrically treated with SLE and DDB once a day for 2 weeks. Protein expression levels of CYP1A2, CYP2C6, CYP3A2, and CYP2E1 were determined via western blot analysis; β-actin was used as an internal standard to normalize all samples.

Fig. 5. Time- and concentration-dependent inactivation of CYPs by TAA. TAA (0, 5, 50, 200, and 400 μM) was incubated with normal rat liver microsomes by adding the NADPH regenerating system; the remaining enzyme activity was then determined by a typical dilution method. The $k_{obs}$ was obtained from the slope of the individual lines and these slopes were fit to a Kitz-Wilson plot (inset). (A) CYP2C6, (B) CYP1A2, (C) CYP3A2, and (D) CYP2E1.

Fig. 6. The effects of DDB and GSH on TAA induced inactivation of CYPs. TAA (50 μM) was incubated with normal rat liver microsomes containing a NADPH regenerating system with or without the addition of DDB (2, 10, and 50 μM) or GSH (0.2, 1, 5 mM); the remaining enzyme activity of CYP2C6, CYP1A2, CYP3A2 and CYP2E1 was then determined by a typical dilution method. * $p \leq 0.05$ vs. control.
Table 1. Intrinsic clearance ($Cl_{int}$) of liver micosomal CYPs in normal, TAA induced hepatic injury rats treated with or without SLE and DDB.

<table>
<thead>
<tr>
<th></th>
<th>CYP1A2</th>
<th></th>
<th></th>
<th>CYP2C6</th>
<th></th>
<th></th>
<th>CYP3A2</th>
<th></th>
<th></th>
<th>CYP2E1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clint</td>
<td>times</td>
<td></td>
<td>Clint</td>
<td>times</td>
<td></td>
<td>Clint</td>
<td>times</td>
<td></td>
<td>Clint</td>
<td>times</td>
</tr>
<tr>
<td></td>
<td>$\mu$L/min/mg</td>
<td>of</td>
<td></td>
<td>$\mu$L/min/mg</td>
<td>of</td>
<td></td>
<td>$\mu$L/min/mg</td>
<td>of</td>
<td></td>
<td>$\mu$L/min/mg</td>
<td>of</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>0.3741</td>
<td>1.00</td>
<td></td>
<td>0.0079</td>
<td>1.00</td>
<td></td>
<td>0.0412</td>
<td>1.00</td>
<td></td>
<td>0.0095</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>TAA</strong></td>
<td>0.1847</td>
<td>0.49</td>
<td></td>
<td>0.0026</td>
<td>0.33</td>
<td></td>
<td>0.0283</td>
<td>0.69</td>
<td></td>
<td>0.0041</td>
<td>0.43</td>
</tr>
<tr>
<td>SLE(100mg/kg)+TAA</td>
<td>0.2351</td>
<td>0.63</td>
<td></td>
<td>0.0029</td>
<td>0.36</td>
<td></td>
<td>0.0388</td>
<td>0.94</td>
<td></td>
<td>0.0080</td>
<td>0.84</td>
</tr>
<tr>
<td>SLE(400mg/kg)+TAA</td>
<td>0.3185</td>
<td>0.85</td>
<td></td>
<td>0.0029</td>
<td>0.36</td>
<td></td>
<td>0.0397</td>
<td>0.96</td>
<td></td>
<td>0.0133</td>
<td>1.40</td>
</tr>
<tr>
<td>DDB(200mg/kg)+TAA</td>
<td>0.2930</td>
<td>0.78</td>
<td></td>
<td>0.0028</td>
<td>0.35</td>
<td></td>
<td>0.0411</td>
<td>1.00</td>
<td></td>
<td>0.0080</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Rats were treated with TAA for 4 weeks and then co-treated with or without SLE/DDB for another 2 weeks. Liver microsomes were prepared and the enzyme kinetics of CYPs was determined using the specific substrate for each of the isozyme. Intrinsic clearance ($Cl_{int}$) was calculated by $V_{max}/K_{m}$. 


Table 2. Intrinsic clearance (Cl\textsubscript{int}) of liver microsomal CYPs in healthy rats treated with SLE and DDB.

<table>
<thead>
<tr>
<th></th>
<th>CYP1A2</th>
<th>CYP2C6</th>
<th>CYP3A2</th>
<th>CYP2E1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clint</td>
<td>Clint</td>
<td>Clint</td>
<td>Clint</td>
</tr>
<tr>
<td></td>
<td>(\mu\text{L/min/mg}) times of</td>
<td>(\mu\text{L/min/mg}) times of</td>
<td>(\mu\text{L/min/mg}) times of</td>
<td>(\mu\text{L/min/mg}) times of</td>
</tr>
<tr>
<td></td>
<td>pro)</td>
<td>control</td>
<td>pro)</td>
<td>control</td>
</tr>
<tr>
<td>Control</td>
<td>0.3741</td>
<td>1.00</td>
<td>0.0079</td>
<td>1.00</td>
</tr>
<tr>
<td>SLE(100mg/kg)</td>
<td>0.2728</td>
<td>0.73</td>
<td>0.0142</td>
<td>1.79</td>
</tr>
<tr>
<td>SLE(400mg/kg)</td>
<td>0.2027</td>
<td>0.54</td>
<td>0.0252</td>
<td>3.18</td>
</tr>
<tr>
<td>DDB(200mg/kg)</td>
<td>0.3502</td>
<td>0.94</td>
<td>0.0216</td>
<td>2.72</td>
</tr>
</tbody>
</table>

Rats were treated with SLE (100 and 400 mg/kg) or DDB (200 mg/kg) intragastrically once a day for 2 weeks. Liver microsomes were prepared and the enzyme kinetics of CYPs was determined using the specific substrate for each of the isozyme. Intrinsic clearance (Cl\textsubscript{int}) was calculated by V\textsubscript{max}/K\textsubscript{m}. 
Table 3. Mechanism based inactivation effects of TAA in CYPs in vitro.

<table>
<thead>
<tr>
<th></th>
<th>$K_I$ (μM)</th>
<th>$k_{\text{inact}}$ (min$^{-1}$)</th>
<th>$k_{\text{inact}}/K_I$ (min$^{-1}$·μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>10.558</td>
<td>0.018</td>
<td>0.0017</td>
</tr>
<tr>
<td>CYP2C6</td>
<td>22.812</td>
<td>0.074</td>
<td>0.0033</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>58.914</td>
<td>0.056</td>
<td>0.0010</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>6.700</td>
<td>0.026</td>
<td>0.0039</td>
</tr>
</tbody>
</table>

TAA (0, 5, 50, 200, and 400 μM) was incubated with normal rat liver microsomes containing a NADPH regenerating system; the remaining enzyme activity was then determined by a typical dilution method. $K_I$ and $k_{\text{inact}}$ values were calculated from the Kitz-Wilson plot; $k_{\text{inact}}/K_I$ values were calculated to indicate the inactivation rate.
### Figure 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>TAA</th>
<th>TAA SLE(400mg/kg)</th>
<th>TAA SLE(100mg/kg)</th>
<th>TAA DDB(200mg/kg)</th>
</tr>
</thead>
</table>

![](image)

- CYP1A2
- CYP2C6
- CYP3A2
- CYP2E1
- β-Actin

*Note: The figure shows the expression levels of CYP1A2, CYP2C6, CYP3A2, and CYP2E1 under different conditions.*
Figure 3
Figure 4
Figure 5
Figure 6