

# Mechanism-Based Inhibitory and Peroxisome Proliferator-Activated Receptor $\alpha$ -Dependent Modulating Effects of Silybin on Principal Hepatic Drug-Metabolizing Enzymes<sup>S</sup>

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## ABSTRACT

Silybin, a major pharmacologically active compound in silymarin, has been widely used in combination with other prescriptions in the clinic to treat hepatitis and a host of other diseases. Previous studies suggested that silybin is a potential inhibitor of multiple drug-metabolizing enzymes (DMEs); however, the *in vitro* to *in vivo* translation and the mechanisms involved remain established. The aim of this study was to provide a mechanistic understanding of the regulatory effects of silybin on principal DMEs. Silybin (50 or 150 mg/kg/d) was administered to mice for a consecutive 14 days. The plasma and hepatic exposure of silybin were detected; the mRNA, protein levels, and enzyme activities of principal DMEs were determined. The results demonstrated that the enzyme activities of CYP1A2, CYP2C, CYP3A11, and UGT1A1 were significantly repressed, whereas little alteration of the mRNA and

protein levels was observed. Silybin inhibits these DMEs in a mechanism-based and/or substrate-competitive manner. More importantly, silybin was found to be a weak agonist of peroxisome proliferator-activated receptor (PPAR) $\alpha$ , as evidenced from the molecular docking, reporter gene assay, and the targeting gene expression analysis. However, silybin could significantly compromise the activation of PPAR $\alpha$  by fenofibrate, characterized with significantly repressed expression of PPAR $\alpha$  targeting genes, including L-FABP, ACOX1, and UGT1A6. This study suggests that silybin, despite its low bioavailability, may inhibit enzyme activities of multiple DMEs in a mechanism-based mode, and more importantly, may confer significant drug-drug interaction with PPAR $\alpha$  agonists via the repression of PPAR $\alpha$  activation in a competitive mode.

## Introduction

Silymarin, a flavonoid complex extracted from milk thistle plant *Silybum marianum*, has been widely used for centuries in the therapy of liver diseases and a host of other diseases (Pade and Stavchansky, 2008; Waldmann et al., 2012). Silybin is one of the major and pharmacologically active compounds in silymarin and has been developed and marketed as a drug in China for the therapy of liver diseases (Fong et al., 2013). The hepatoprotective effects of silybin and its purported utility in treating hepatitis, cholestasis, nonalcoholic fatty liver disease, and toxin-induced liver disease have been widely validated (Crocenzi et al., 2001; Ferenci et al., 2008; Wagoner et al., 2010; Loguercio et al., 2012; Marino et al., 2013). In addition to its well-known hepatoprotective effects, a panel of other pharmacological activities of silybin, such as anti-cancer, has been recently reported (Zhou et al., 2008; Rajamanickam et al., 2010; Ravichandran et al.,

2010; Ramasamy et al., 2011). Although silymarin and silybin are known to be safe and well-tolerated (Rutter et al., 2011; Marino et al., 2013; Zhu et al., 2013), hyperbilirubinemia caused by repressed UGT1A1 enzyme activity has been notified as a potential toxic effect (Flaig et al., 2007; Parveen et al., 2011; Rutter et al., 2011; Polyak et al., 2013; Sumida et al., 2013). Moreover, a critical concern about the use of silybin is the potential drug-drug interactions (DDI) because silybin is in most cases used in combination with a variety of other pharmaceuticals. From this consideration, it is definitely important to comprehensively verify the influence of silybin treatment in the regulation of drug-metabolizing enzymes (DMEs) to predict any potential DDI.

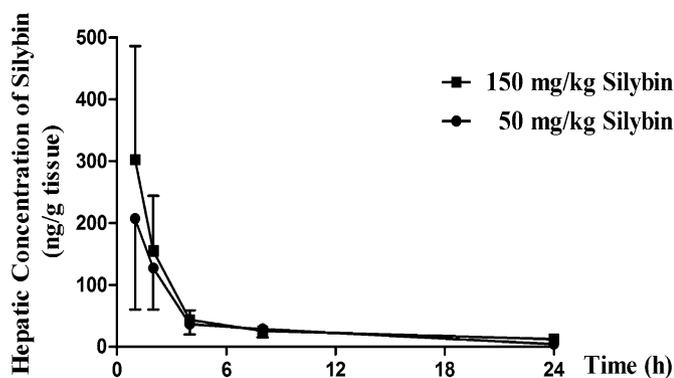
Although there are many previous reports contributing to the study of the regulatory effects of silybin in DMEs, the results and conclusions obtained are largely controversial. As concluded from most of the previous studies performed *in vitro*, silymarin and its major component silybin are likely to be potential inhibitors of a variety of P450 enzymes (Sridar et al., 2004), such as CYP3A4, CYP2C9, CYP2E1, and also a couple of UDP-glucuronosyltransferase (UGTs) (D'Andrea et al., 2005), such as UGT1A1, 1A6, 1A9, and 2B7. However, these *in vitro* observations failed to translate to the findings *in vivo*. A more recent study performed in volunteer human beings by consuming a standardized milk thistle supplement for a consecutive 14 days suggested no significant influence on the pharmacokinetics of probe drugs of CYP1A2, CYP2C9, CYP2D6, or CYP3A4/5. In contrast, an earlier

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**ABBREVIATIONS:** BCA, bicinechonic acid; DDI, drug-drug interaction; DME, drug-metabolizing enzyme; HLM, human liver microsome; HPLC, high performance liquid chromatography; i.g., intragastric; MLM, mouse liver microsome; 4-MU, 4-methylumbelliferone; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PXR, Pregnane X Receptor; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.



**Fig. 1.** Hepatic distribution of silybin in mice. Mice were orally administrated with silybin (50 mg/kg/d and 150 mg/kg/d, once daily) for a consecutive 14 days. The livers were collected at indicated time points (5 mice per time point) after the last administration. Exposure of silybin in livers was analyzed by a liquid chromatography tandem mass spectrometry system. The data are expressed as mean  $\pm$  S.D.

study performed in healthy volunteers indicated that a silymarin pretreatment significantly inhibited the metabolism of losartan, a CYP2C9 substrate. Such discrepancies from in vitro to in vivo and from various laboratories may be explained by complex constituents, low bioavailability, different experiment design, and the content variations of compounds contained in herbal extracts (Wen et al., 2008; Hao et al., 2011b; Calani et al., 2012; Zhu et al., 2013).

It might be proposed from previous studies that the consumption of rude extract of silymarin at typical dose is of low risk to metabolic herb-drug interactions because of the relatively low content and low systematic bioavailability of the compounds as potential DME inhibitors. However, because silybin has been already marketed as a therapeutic drug in China, it is important to perform a comprehensive study in verifying the regulatory effects of this drug in DMEs. Although it was previously shown that silybin is an inhibitor of multiple P450 and UGT isozymes in vitro, it remains unanswered about the in vivo effects and the underlying mechanisms. It is largely unclear, in addition to its direct enzyme activity-inhibitory effect, whether silybin could regulate the mRNA and protein expression of DMEs. In addition, DMEs are under the control of multiple nuclear receptors, such as Pregnane X Receptor (PXR) and peroxisome proliferator-activated receptor (PPAR $\alpha$ ) (Buckley and Klaassen, 2009; Runge-Morris and Kocarek, 2009; Zhou et al., 2014). More recently, silybin was identified as a potential antagonist of PXR that is a master regulator of CYP3A4 and other P450s (Mooiman et al., 2013). PPAR $\alpha$  is responsible for the induction of several UGTs and other genes involved in fatty acid

oxidation (Buckley and Klaassen, 2009; Runge-Morris and Kocarek, 2009; Azimzadeh et al., 2013). Because silybin, as a hepatoprotective agent, is highly possible used in combination with hypolipidemic drugs, such as PPAR $\alpha$  agonists, it is definitely important to evaluate the potential interactions via the regulation of PPAR $\alpha$ . On the basis of this consideration, we further determined the regulatory effects of silybin on PPAR $\alpha$  in both the basic and fibrate-activated conditions.

## Materials and Methods

**Chemicals and Reagents.** Silybin, quercetin, fenofibrate, glucose-6-phosphate (G-6-P), NADP<sup>+</sup>, glucose-6-phosphate dehydrogenase, 4'-hydroxydiclofenac, 4-hydroxymidazolam, 6-hydroxychlorzoxazone, UDP-glucuronic acid (UDPGA), D-saccharic acid 1, 4-lactone, alamethicin,  $\beta$ -estradiol,  $\beta$ -estradiol 3-O-glucuronide, 4-methylumbelliferone (4-MU), 4-MU O-glucuronide, and naloxone were all purchased from Sigma-Aldrich (St. Louis, MO). Midazolam, chlorzoxazone, diclofenac, phenacetin, and acetaminophen were obtained from National Institute for the Control of Pharmaceutical and Biologic Products (Beijing, China). Naloxone 3- $\beta$ -D-glucuronide was obtained from Toronto Research Chemicals (Ontario, Canada). Antibodies to CYP3A2, CYP2E1, and CYP1A2 were from Chemicon (Temecula, CA). Antibodies to CYP2C6, UGT1A1, UGT1A6, and UGT2B were from Santa Cruz (Santa Cruz, CA). Glyceraldehyde-3-phosphate dehydrogenase antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG, and goat anti-mouse IgG were purchased from Bioworld Technology (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit, SDS-PAGE, and sample loading buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Pooled human liver microsomes (HLMs), prepared from 10 Mongolian donors aged from 24 to 38, were purchased from Research Institute for Liver Disease (Shanghai, China). High performance liquid chromatography (HPLC)-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Billerica, MA).

**Animal Experiments.** Male C57BL/6 mice, weighing 20–22 g, were obtained from Academy of Military Medical Sciences, China. They were kept in an air-conditioned animal quarter at a temperature of  $25 \pm 2^\circ\text{C}$  and a relative humidity of  $50 \pm 10\%$  with 12-hour light/dark cycles. Water and food were allowed ad libitum. Animals were acclimatized to the facilities for 1 week and then randomly divided into different groups for research. All animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

To assess the influence of silybin administration on DMEs, animals were randomly divided into three groups with different treatment of 2 weeks: control group (vehicle, intragastric i.g.), low-dose group (50 mg/kg/d silybin, i.g.), and high-dose group (150 mg/kg/d silybin, i.g.). All animals were sacrificed at indicated time points after the last administration. Livers were immediately harvested and stored at  $-80^\circ\text{C}$  until use.

In the study of regulatory effect of silybin on PPAR $\alpha$ , animals were randomly divided into six groups with different treatment of 2 weeks: control group (vehicle, i.g.), LS group (Low dose of Silybin, at 50 mg/kg/d, i.g.), HS group (High dose of Silybin, at 150 mg/kg/day, i.g.), fenofibrate group (100 mg/kg/d fenofibrate, i.g.), LSF group (Low dose of Silybin together with

TABLE 1

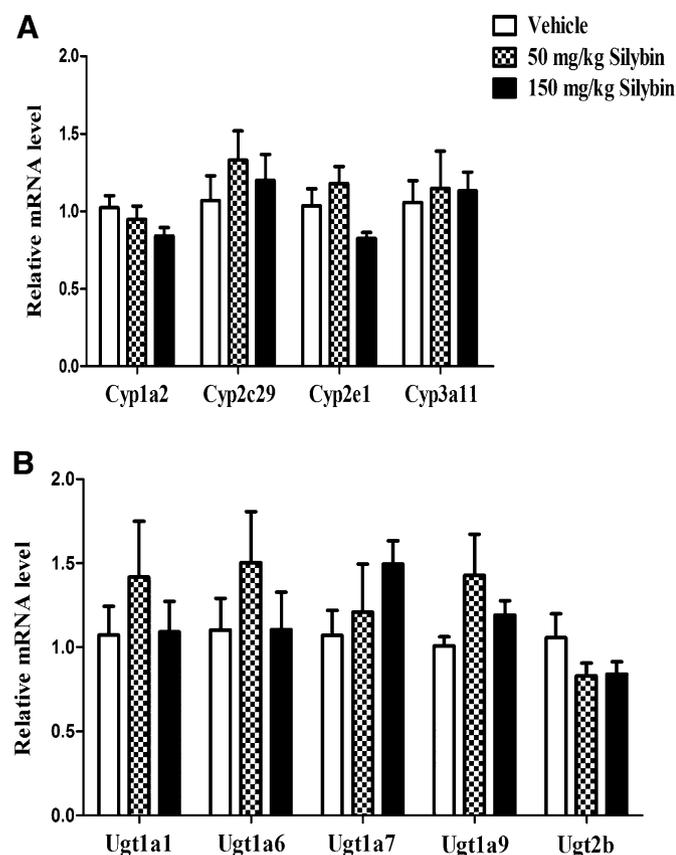
### Enzyme activities of main DMEs

Mice were treated with silybin (50 mg/kg or 150 mg/kg, once daily) for a consecutive 2 weeks. Twenty-four hours after the last administration, the mice were sacrificed and their livers were immediately removed to prepared microsomes. Enzyme activities of main P450s and UGTs were tested using the typical probe substrate approach. Data are shown as mean  $\pm$  S.D. ( $n = 3$ ).

Substrates	Activity of DMEs (pmol/min/mg microsome protein)		
	Vehicle	Silybin (50 mg/kg)	Silybin (150 mg/kg)
Phenacetin	516.23 $\pm$ 79.93	440.59 $\pm$ 33.02	319.10 $\pm$ 27.71 <sup>a</sup>
Diclofenac	159.06 $\pm$ 4.39	168.32 $\pm$ 8.92	126.97 $\pm$ 1.51 <sup>a</sup>
Chlorzoxazone	1446.31 $\pm$ 29.98	1553.31 $\pm$ 59.82	1332.36 $\pm$ 81.52
Midazolam	1291.11 $\pm$ 98.69	1305.59 $\pm$ 29.69	907.76 $\pm$ 60.01 <sup>b</sup>
$\beta$ -estradiol	718.71 $\pm$ 42.12	621.71 $\pm$ 16.36 <sup>a</sup>	419.42 $\pm$ 21.95 <sup>b</sup>
4-MU	75,459.46 $\pm$ 9478.70	109,474.65 $\pm$ 2324.02	83,348.31 $\pm$ 8070.37
Naloxone	286.74 $\pm$ 38.05	295.84 $\pm$ 31.74	289.04 $\pm$ 29.78

<sup>a</sup>P < 0.01 represents the comparison of activities of DMEs between Silybin treated groups and vehicle group.

<sup>b</sup>P < 0.001.



**Fig. 2.** The mRNA levels of principal P450s (A) and UGTs (B) in mice liver samples. Mice were intragastrically treated with 50 mg/kg or 150 mg/kg silybin for 2 weeks once per day. Twenty-four hours after the last administration, mice were sacrificed and the livers were immediately removed. The mRNA levels of Cyp1a2, Cyp2c29, Cyp2e1, Cyp3a11, Ugt1a1, Ugt1a6, Ugt1a7, Ugt1a9, and Ugt2b were determined via reverse-transcriptase PCR analysis; glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard to normalize all samples.

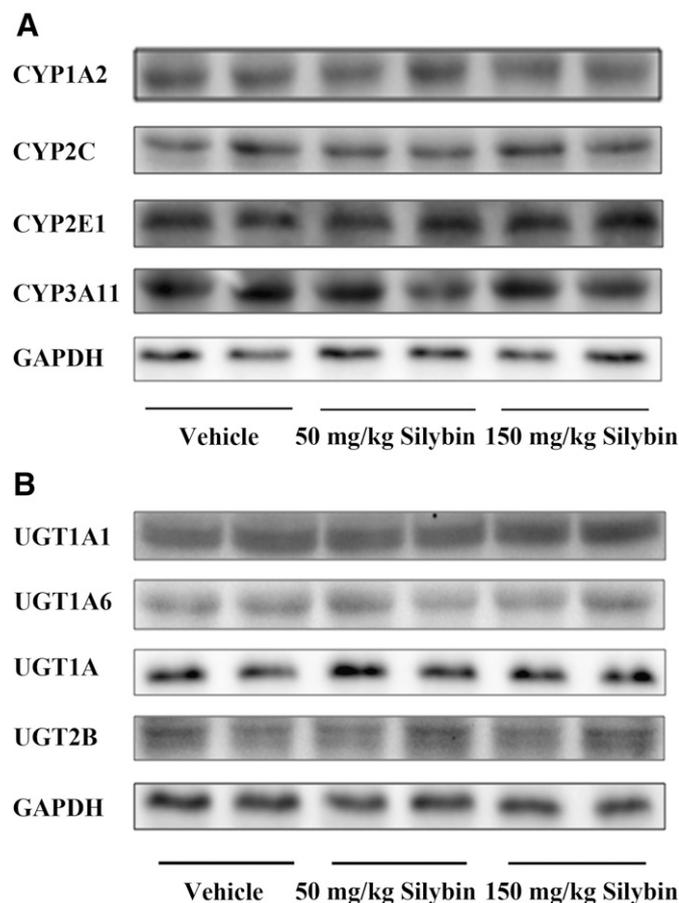
Fenofibrate, at 50 mg/kg/d and 100 mg/kg/d, respectively, i.g.), and HSF group (High dose of Silybin together with Fenofibrate, at 150 mg/kg/d and 100 mg/kg/d, respectively, i.g.). All of these mice were sacrificed 24 hours after the last administration, and livers were immediately harvested and stored.

**Analysis of Silybin.** Hepatic tissues collected in indicated time points were homogenized in 50% methanol. After the addition of ice-cold acetonitrile containing 1% formic acid and quercetin (internal standard) into the homogenation, the mixture was centrifuged at 18,000 rpm for 10 minutes at 4°C. The supernatants were transferred and then evaporated. The remaining residue was reconstituted in 100  $\mu$ l mobile phase, 5  $\mu$ l of which was analyzed by a liquid chromatography tandem mass spectrometry system consisting of a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) and an AB-Sciex 4500 QTRAP mass spectrometer with an electrospray source (AB Sciex, Foster City, CA). Separation was performed on a Phenomenex Luna 5u C18 column (100 A 250  $\times$  2 mm, 5  $\mu$ m; Torrance, CA). The HPLC conditions were the same as previously described (Zhu et al., 2013); the mass spectrometry (MS) parameters were as follows: declustering potential, -90 V; entrance potential, -13 V for silybin or -8 V for quercetin; collision energy, -30 eV; IonSpray voltage, -4500 V; collision cell exit potential, -10 V; curtain gas, 20 Arb; ion source gas 1, 50 Arb; ion source gas 2, 70 Arb; source temperature, 550°C. Multiple reaction monitoring was performed for silybin at m/z 481.1 to m/z 124.9 and quercetin (internal standard) at m/z 300.7 to m/z 150.1, respectively. The lower limit of quantification was 2 ng/ml, and the calibration curve was linear over the range of 2 to 500 ng/ml ( $r^2 > 0.99$ ).

**P450 Enzyme Assays.** Mice were sacrificed 24 hours after the last administration, and the livers were immediately harvested to prepare microsomes using differential centrifugation, as previously described (Leblond et al., 2001). Protein concentrations were measured using BCA protein assay kit, according to the manufacturer's instructions. The probe substrates for CYP1A2, CYP2C,

CYP2E1, and CYP3A11 are phenacetin, diclofenac, chlorzoxazone, and midazolam, respectively (Lofgren et al., 2004). For the test of activities of CYPs, a typical incubation mixture in a total volume of 200  $\mu$ l contained 0.5 mM NADPH-generating system (10 mM G-6-P, 0.5 mM NADP<sup>+</sup>, 10 mM MgCl<sub>2</sub>, 1 U glucose 6-phosphate dehydrogenase), 100 mM phosphate buffer (pH 7.4), different amount of liver microsomes, and the specific probe substrate (100  $\mu$ M phenacetin, 100  $\mu$ M diclofenac, 400  $\mu$ M chlorzoxazone, or 10  $\mu$ M midazolam). The incubation was conducted at 37°C for 30 minutes for CYP1A2, 30 minutes for CYP2C, 10 minutes for CYP2E1, and 5 minutes for CYP3A11, respectively. The incubation time and the microsomal protein concentrations were optimized to ensure the linear formation of metabolites. The reaction was terminated by addition of cold acetonitrile. After centrifugation, the supernatant was injected into a HPLC or liquid chromatography mass spectrometry system (Shimadzu) for analysis, as described previously (Lofgren et al., 2004; Qiu et al., 2008). All incubations were performed in triplicates.

**Mechanism-Based Inhibition of P450s.** Mechanism-based inhibition of silybin on microsomal CYPs was conducted in both mouse liver microsomes (MLMs) and HLMs using a method as previously described (Xie et al., 2012). In NADPH-dependent inhibition assays, primary incubations were performed in a media containing 3 mg/ml MLMs or 2 mg/ml HLMs and 100  $\mu$ M silybin in 100 mM phosphate buffer (pH 7.4) in a final volume of 200  $\mu$ l in the presence or absence of the NADPH-generating system. In preincubation time- and concentration-dependence test, primary incubations were performed in a media containing 0.5 mM NADPH-generating system, microsomes, and different concentrations of silybin (0, 10, 50, and 100  $\mu$ M) in 100 mM phosphate buffer (pH 7.4) in a final volume of 200  $\mu$ l. At various time points (0, 2, 4, and 8 minutes), 20  $\mu$ l primary reaction media was moved to a secondary reaction



**Fig. 3.** Protein levels of principal P450s (A) and UGTs (B) in mice liver samples. Protein levels of CYP1A2, CYP2C, CYP2E1, CYP3A11, UGT1A1, UGT1A6, UGT1A, and UGT2B were determined via Western blot analysis; glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard to normalize all samples.

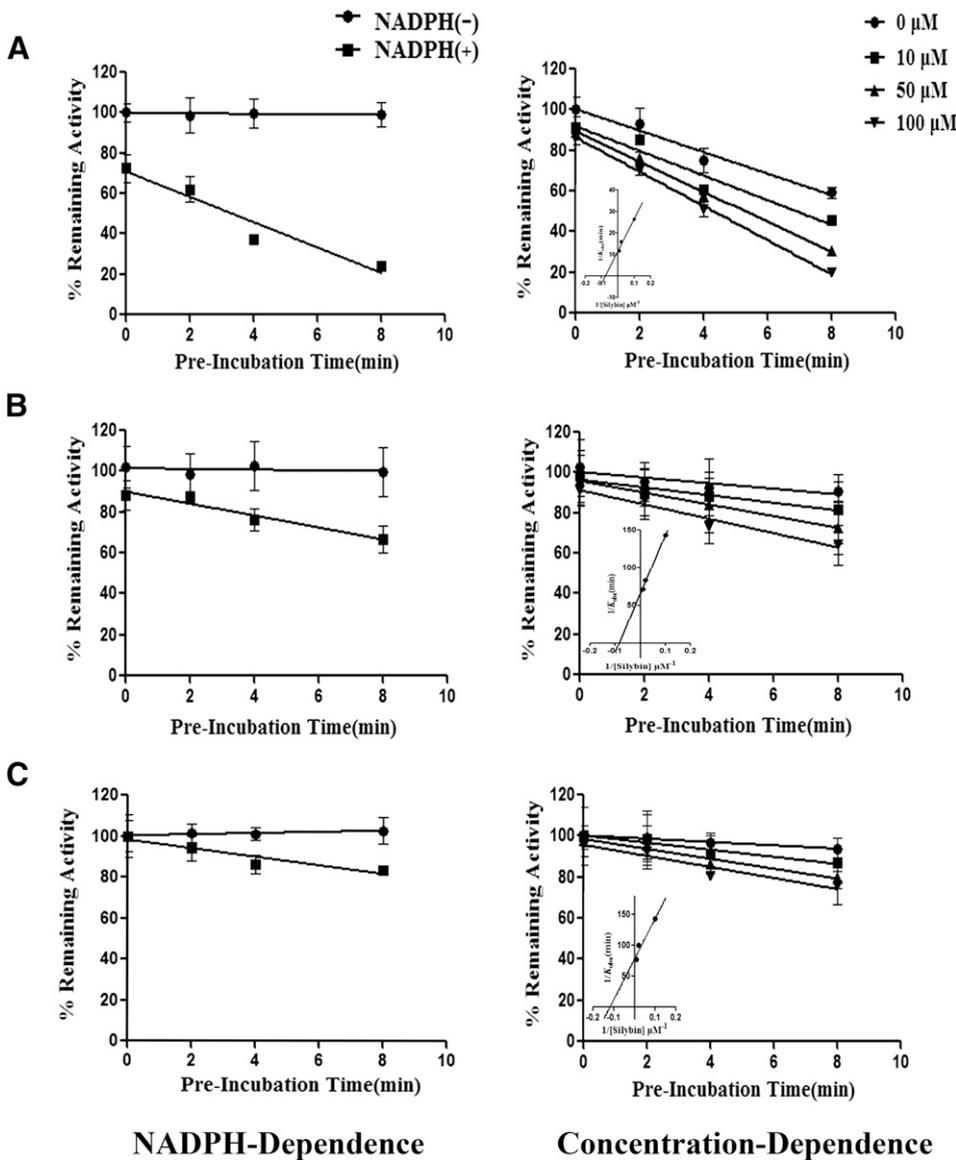
media containing specific CYP probe substrate, NADPH-generating system, and PBS, as usual. All of the experimental procedures of the secondary incubations were the same as that described above for the enzyme activity assay of CYPs. For NADPH-dependent inhibition assays, activities were normalized to the activity at 0 minutes without NADPH generating system, and, for preincubation time-dependent and concentration-dependent inhibition assays, activities were normalized to the activity at 0 minutes without silybin so that the percent decrease in CYP activities reflected activity loss due to only inactivation. All incubations were performed in triplicates.

**UGT Enzyme Assays Ex Vivo.**  $\beta$ -estradiol, 4-MU, and naloxone were used as substrates to test the enzyme activities of UGT isoforms in mouse livers. A typical incubation mixture contained 50 mM Tris-HCl buffer (pH 7.4), 10 mM  $MgCl_2$ , 25  $\mu g/ml$  alamethicin, 20 mM UDPGA, microsomes, and a selective substrate (100  $\mu M$   $\beta$ -estradiol, 1 mM 4-MU, and 125  $\mu M$  naloxone). Microsomes were preincubated with alamethicin for 30 minutes at 4°C. The incubation was conducted at 37°C for 30 minutes for  $\beta$ -estradiol, 10 minutes for 4-MU, and 60 minutes for naloxone, respectively. Reactions were terminated by the addition of ice-cold acetonitrile or perchloric acid. All incubations were performed in duplicates. The substrates and their glucuronides were detected by HPLC or liquid chromatography mass spectrometry (Shimadzu), as described previously (Shiratani et al., 2008; Hao et al., 2011a; Jiang et al., 2011).

**UGT Inhibitory Effects of Silybin In Vitro.** To determine the competitive inhibitory effects of silybin in UGTs, different concentrations of silybin (0, 100, 200, and 500  $\mu M$ ) and  $\beta$ -estradiol (40, 100, 200, and 400  $\mu M$ ) are contained in the incubation system. MLMs or HLMs were preincubated with alamethicin for 30 minutes at 4°C. Then silybin,  $\beta$ -estradiol, and UDPGA were added to the system, followed by incubation in 37°C for 30 minutes or 10 minutes. Other procedures were the same as described in UGT enzyme activity assay.

Mechanism-based inhibition of silybin on microsomal UGTs was conducted as previously reported. In brief, different concentration of silybin (0, 10, 50, and 100  $\mu M$ ) was preincubated with MLMs or HLMs for 0.5 hour with or without the addition of NADPH before the addition of UDPGA and probe substrates to initiate the UGT-catalyzed reactions.

**Reverse-Transcriptase Polymerase Chain Reaction.** Total RNA was isolated from mouse livers or HepG2 cells with the RNAiso Plus reagent (TakaRa Biotechnology, Dalian, China), according to the manufacturer's protocol. Purified total RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (TakaRa Biotechnology). Real-time polymerase chain reaction (PCR) was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA) and SYBR Green Reagent Kit (TakaRa Biotechnology) to determine the mRNA expressions. Primer sequences are list in Supplemental Table 1, and each primer's specificity was monitored using product-melting curves in each reaction well.



**Fig. 4.** Mechanism-based inhibition of P450s by silybin in MLM. In NADPH-dependent inhibitory assays, 100  $\mu M$  silybin was incubated with normal mouse liver microsomes in the presence or absence of NADPH-generating system. In time- and concentration-dependent inhibitory assays, silybin (0, 10, 50, and 100  $\mu M$ ) was preincubated for different periods (0, 2, 4, and 8 minutes) in the presence of NADPH, followed by incubation with each probe substrate. The remaining enzyme activities were then determined, as described in *Materials and Methods*. The  $k_{obs}$  was obtained from the slope of the individual lines, and these slopes were fit to a Kitz-Wilson plot (inset). (A) CYP3A11, (B) CYP2C, (C) CYP1A2.

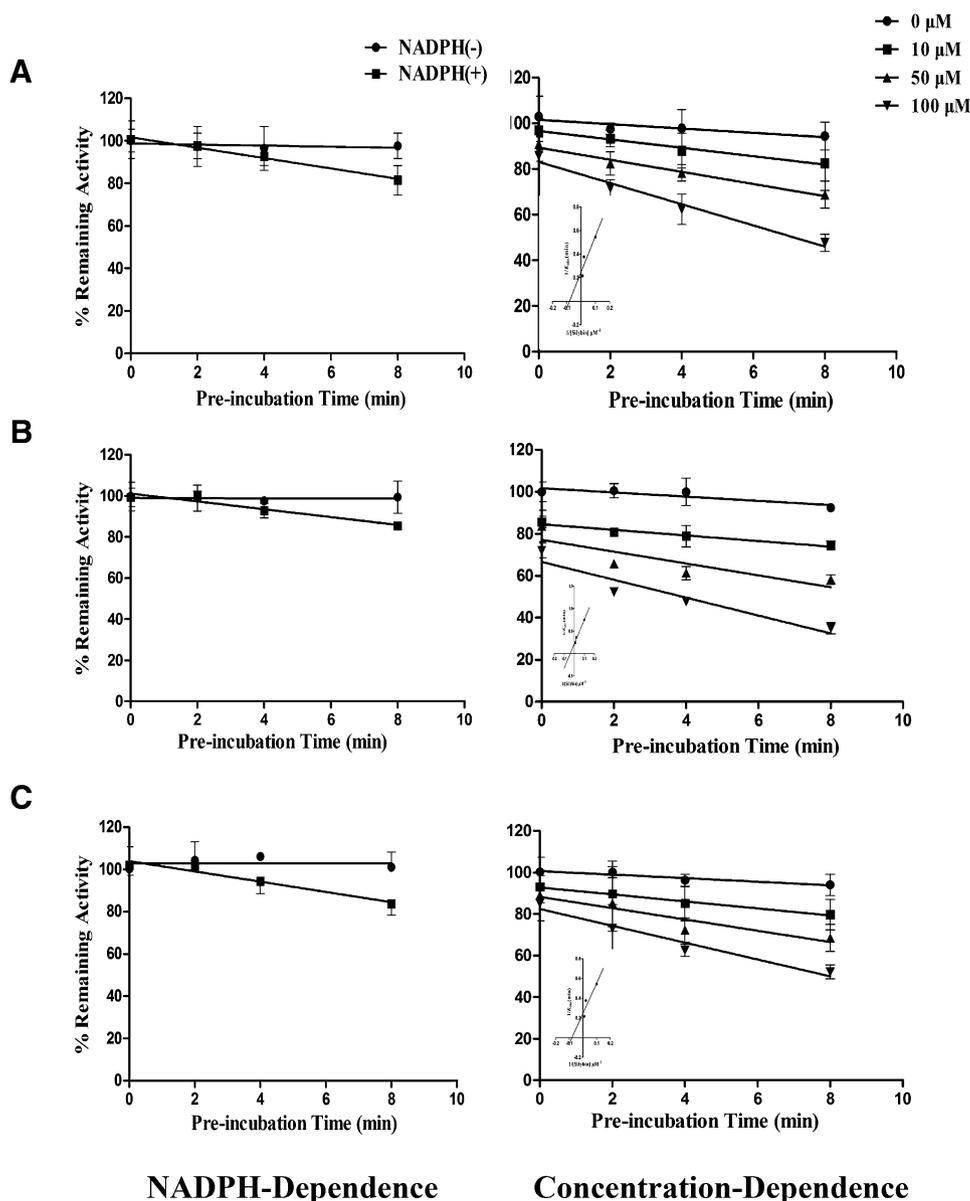
**Western Blot Analysis.** Total proteins of mouse livers were extracted with ice-cold radioimmunoprecipitation assay lysis buffer containing 1% protease inhibitor cocktail (Beyotime Institute of Biotechnology, Haimen, China). Protein concentration was measured by BCA assay. Protein lysates (40  $\mu\text{g}$  per lane) were separated by SDS-PAGE with an 8% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane by electroblotting. The polyvinylidene fluoride member was blocked in 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 at 37°C for 1 hour. The blots were incubated with primary antibodies diluted in 5% nonfat milk in Tris-buffered saline/0.1% Tween 20 overnight at 4°C, followed by incubation with appropriate second antibodies at 37°C for 1 hour. The dilution of all the antibodies was optimized to ensure linearity. Proteins were detected by enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA). The signal was captured using a ChemiDoc XRS<sup>+</sup> System (Bio-Rad Laboratories, Hercules, CA). The protein levels of DMEs were normalized to that of the reference band glyceraldehyde-3-phosphate dehydrogenase.

**Cell Culture and Treatment.** HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone, Logan, Utah) and antibiotics (100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin)

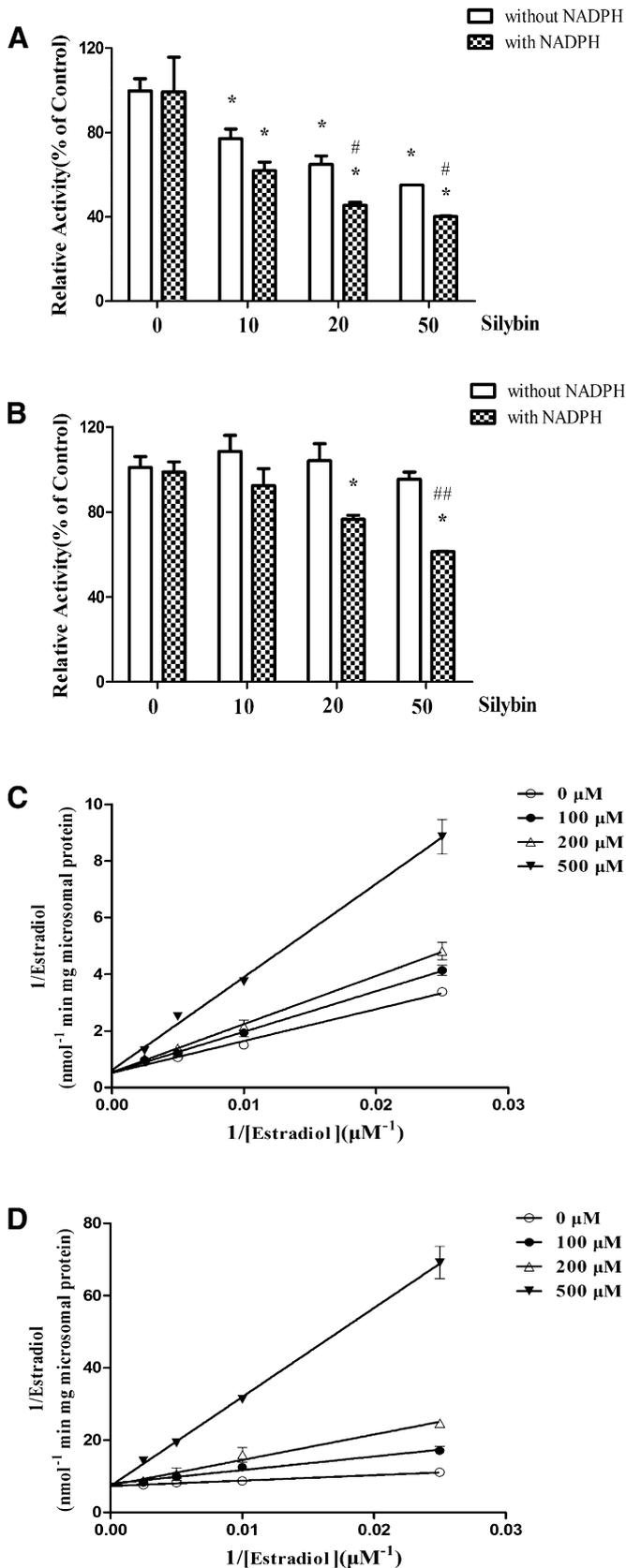
at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For reverse-transcriptase PCR studies, cells were seeded in 6-well plates in complete Dulbecco's modified Eagle's medium, and, 24 hours after incubation, the cells were treated with silybin (25  $\mu\text{M}$  or 50  $\mu\text{M}$ ) with or without addition of known PPAR $\alpha$  agonist (fenofibrate, 50  $\mu\text{M}$ ) for 24 hours.

**Molecular Docking.** A computer-based modeling program (DISCOVERY STUDIO; Accelrys, San Diego, CA) was employed to generate docking models to assess whether silybin could bind with PPAR $\alpha$  to transactivate it. The chemical structure of silybin was modified to follow the stereochemistry, as shown previously (Jancova et al., 2007). In addition, the structure of fenofibrate was also from previous publication (Gregus et al., 1998). The conformer with least energy for silybin or fenofibrate was chosen for subsequent docking. Crystal structure of human PPAR $\alpha$  ligand binding domain with BMS-631707 (Protein Data Bank ID: 2REW) disclosed in 2007 was used. Repeated dockings were carried out until no further refinement in clustering or binding energy of conformer was achieved. Based on population size and binding energy, the best conformation was chosen for further analysis of protein-ligand interaction.

**Reporter Gene Assay.** For reporter gene assay, HepG2 cells were seeded in 24-well plates in complete Dulbecco's modified Eagle's medium. After 24 hours of incubation, the cells were transfected with PPAR luciferase reporter



**Fig. 5.** Mechanism-based inhibition of P450s by silybin in HLM. In NADPH-dependent inhibitory assays, 100  $\mu\text{M}$  silybin was incubated with normal mouse liver microsomes in the presence or absence of NADPH-generating system. In time- and concentration-dependent inhibitory assays, silybin (0, 10, 50, and 100  $\mu\text{M}$ ) was preincubated for different periods (0, 2, 4, and 8 minutes) in the presence of NADPH, followed by incubation with each probe substrate. The remaining enzyme activities were then determined, as described in *Materials and Methods*. The  $k_{\text{obs}}$  was obtained from the slope of the individual lines, and these slopes were fit to a Kitz-Wilson plot (inset). (A) CYP3A4/5, (B) CYP2C9, (C) CYP1A2.



**Fig. 6.** Mechanism-based inactivation (A, B) and competitive inhibition (C, D) of silybin on UGT1A1. Silybin was preincubated in normal mouse (A) and human (B) liver microsomes for 0.5 hour before the enzyme activity assay of UGT1A1 in the presence or absence of NADPH-generating system. Data are expressed as percentage of control, and bars represent mean  $\pm$  S.D. ( $n = 3$ ). \* $P < 0.05$ , compared

construct using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manual instruction. After 24 hours of exposure, the medium was removed and the cells were treated with silybin (0, 25, or 50  $\mu\text{M}$ ) in the presence or absence of fenofibrate (50  $\mu\text{M}$ ) for 24 hours. Cells were then washed with PBS and lysed with Cell Lysis Buffer (Genomeditech, Shanghai, China) for 15 minutes. The cell lysates were transferred to a 96-well plate for reporter activities of firefly luciferase activity assay, according to the manufacturer's instructions.

**Data and Statistical Analysis.** The values of the experimental data were expressed as mean  $\pm$  S.D. Statistical significances were evaluated using one-way analysis of variance, followed by the Newman-Keuls method for multiple comparisons. The difference was considered significant when the probability value was less than 0.05 ( $P < 0.05$ ).

## Results

**Hepatic Distribution of Silybin in Mice.** After intragastric administration of silybin (50 mg/kg/d and 150 mg/kg/d) for a consecutive 14 days, mice were then sacrificed at indicated time points (5 mice per time point), and plasma and liver samples were collected to analyze the concentration. Silybin was undetectable in the plasma, except for the first time point (data not shown). However, the hepatic exposure levels of silybin were found much higher than that in the plasma (Fig. 1), albeit still at relatively low levels.

**Silybin Inhibits Activities of CYP1A2, CYP2C, CYP3A11, and UGT1A1.** Liver microsomes from mice treated with different doses of silybin for 2 weeks were prepared to test the enzyme activities of P450s and UGTs using the typical probe substrate approach *ex vivo* (Table 1). With silybin treatment at high dose (150 mg/kg), the enzyme activity of CYP1A2 (deethylation of phenacetin), CYP2C (diclofenac hydroxylation), and CYP3A11 (midazolam hydroxylation) was significantly repressed, characterized with an enzyme activity of 60%, 80%, and 70% of that in the control group, respectively. In contrast, no significant enzyme inhibition was found in the low-dose-treated group. These results suggest that a relatively high dose of silybin is necessary to exert efficient enzyme-inhibitory effect on the activities of P450s. However, it was of interest to note that silybin at a relatively low dose (50 mg/kg) could significantly inhibit the enzyme activity of UGT1A1 (glucuronidation of  $\beta$ -estradiol) and showed a dose-dependent effect. No significant influence was found for other UGT isozymes even at high-dose treatment by silybin.

**Silybin Has No Influence on the Expression of DMEs.** To elucidate the possible mechanism of enzyme-inhibitory effects of silybin in DMEs, we first asked whether silybin could regulate the enzyme expression of DMEs. For this purpose, the mRNA levels and protein levels of the dominant P450s and UGTs were determined by reverse-transcriptase PCR and Western blot analyses, respectively. The results showed that silybin treatment of a consecutive 14 days had little influence on both the mRNA levels (Fig. 2) and protein levels (Fig. 3) of DMEs, suggesting that the enzyme-inhibitory effects of silybin are unlikely caused by the decreased enzyme expression.

**Mechanism-Based Inhibitory Effects of Silybin on CYP1A2, CYP2C, and CYP3A11.** Because silybin has no direct influence on the enzyme expression and its hepatic exposure level is relatively low, we hypothesized that the loss of enzyme activities might be caused by direct inactivation of P450s. We thus performed mechanism-based

with control group (with or without NADPH, respectively). # $P < 0.05$ , ## $P < 0.01$ , compared with relative group treated with the same dose of silybin without NADPH. Silybin was coincubated with  $\beta$ -estradiol in normal mouse liver microsomes (C) and human liver microsomes (D). Double reciprocal plots for the kinetics of inhibition of  $\beta$ -estradiol glucuronidation by silybin in normal mouse liver microsomes.

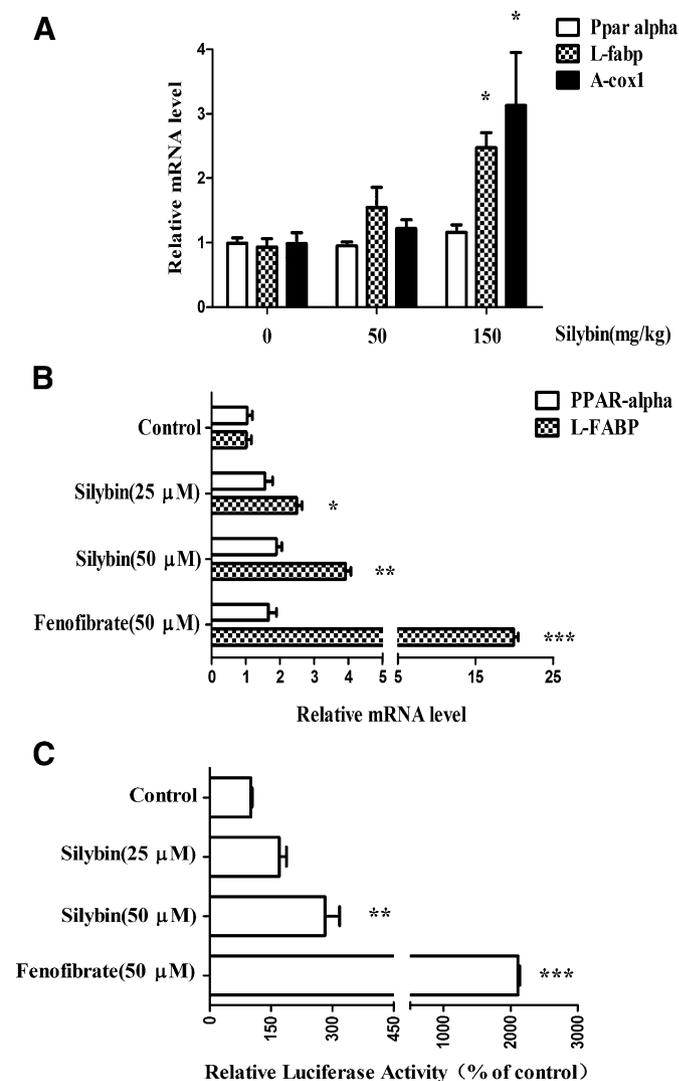
inhibition assays to test whether the enzyme-inhibitory effects of silybin were NADPH-, time-, and concentration-dependent in the *in vitro* microsomes incubating system. In NADPH-dependent inhibitory test, 100  $\mu\text{M}$  silybin was incubated with MLMs in the presence or absence of NADPH-generating system. In time- and concentration-dependent inhibitory test, silybin (0, 10, 50, and 100  $\mu\text{M}$ ) was preincubated for different times (0, 2, 4, and 8 minutes) in the presence of NADPH, followed by incubation with each probe substrate. The results showed that silybin inhibited the activities of CYP1A2, CYP2C, and CYP3A11 in a NADPH-dependent manner (Fig. 4). Moreover, the enzyme-inhibitory effect of silybin was found also concentration- and preincubation time-dependent (Fig. 4). The observed first-order rate constants ( $k_{obs}$ ) for the inactivation of P450s by individual concentrations of silybin were obtained from the slope of individual lines. As shown in the inset of Fig. 4, the slopes were fit to a Kitz-Wilson plot, with the  $K_{inact}$  values at 0.01, 0.02, and 8.40  $\text{minutes}^{-1}$ , and  $K_i$  at 8.4, 11.8, and 3.9  $\mu\text{M}$  for CYP1A2, CYP2C, and CYP3A11, respectively, in MLM. The mechanism-based inactivation of silybin on these P450 enzymes was also validated in HLMs (Fig. 5). The estimated  $K_{inact}$  values of silybin are 3.95, 4.64, and 4.06  $\text{minutes}^{-1}$ , and  $K_i$  values are 13.6, 25.0, and 12.4  $\mu\text{M}$  for CYP1A2, CYP2C9, and CYP3A4/5, respectively, in HLM. All of these results support that silybin exerts a mechanism-based inhibitory effect on CYP1A2, CYP2C, and CYP3A11.

**Mechanism-Based and Substrate-Competitive Inhibitory Effects of Silybin on UGT1A1.** The results above indicated that silybin significantly inhibits the enzyme activities of UGT1A1, but had little influence on the mRNA levels and protein levels. Moreover, it was previously reported that silybin could be metabolized by various P450s and UTGs. Thus, we extended to determine the possible mechanism-based and substrate-competitive effects of silybin on inhibiting the enzyme activity of UGT1A1. Preincubation of different concentrations of silybin with MLMs dramatically inactivated the activity of UGT1A1 in the presence of NADPH-generating system (Fig. 6A). In the absence of NADPH, silybin preincubation could also inhibit the activity of UGT1A1 concentration-dependently in MLMs, albeit to a lesser extent than that in the presence of NADPH. Besides, coincubation of different concentration of silybin with  $\beta$ -estradiol in MLMs in the absence of NADPH produced double reciprocal plots that intercepted in the  $1/v$  axis, suggesting a competitive inhibitory effect of silybin on UGT1A1 (Fig. 6C). To provide a translational link to human beings, HLMs were also used to determine the enzyme-inhibitory effects of silybin in UGT1A1. The results collected from HLM study also suggested a mechanism-based inactivation (Fig. 6B) and a substrate-competitive inhibition (Fig. 6D) of silybin on human UGT1A1. These results suggest that, in addition to a mechanism-based inhibitory effect, silybin may exert a substrate-competitive inhibitory effect on UGT1A1.

**Silybin Is a Moderate PPAR $\alpha$  Agonist.** More recently, silybin was found to be a potential PXR antagonist; however, little is known about its regulatory effects on other key nuclear receptors. PPAR $\alpha$  is another important nuclear receptor in controlling the expression of versatile enzymes including UGTs that is an important class of enzymes of drug metabolism. Although silybin treatment has little effect on the expression of UGTs, the typical PPAR $\alpha$  targeting genes L-Fabp and Acox1 were significantly upregulated with silybin treatment at a high dose in mice (Fig. 7A). In HepG2 cells, silybin treatment also induced a concentration-dependent activation of PPAR $\alpha$ , characterized with significant upregulation of L-FABP, but little alteration of the mRNA level of PPAR $\alpha$  itself (Fig. 7B). To further validate whether silybin is a PPAR $\alpha$  agonist, we performed a reporter gene assay and a molecular docking study. The results from the reporter gene assay clearly indicated that silybin could significantly increase the PPAR $\alpha$ -regulated luciferase activity. However, the activation of

PPAR $\alpha$  by silybin was found much lower than that by fenofibrate, a typical PPAR $\alpha$  agonist (Fig. 7C). Computational molecular docking was enrolled to predict the interaction between silybin, as well as fenofibrate, with human PPAR $\alpha$ . As compared with fenofibrate, many less poses were docked for silybin, which might be explained by the much bigger molecular size of silybin than fenofibrate. Optimal conformers were selected for further analysis. It was found that silybin could bind to PPAR $\alpha$  with a mode similar to fenofibrate. As shown in Fig. 8, silybin binds to the cavity of PPAR $\alpha$  via the hydrogen bonds to TYR314 and TYR464, similar to that observed in the complex with fenofibrate. Together, these results support that silybin is a weak agonist of PPAR $\alpha$ .

**Silybin Attenuates the PPAR $\alpha$  Activation by Fenofibrate.** Because the molecular docking analysis suggests silybin binds to PPAR $\alpha$  on the similar binding sites with fenofibrate, we hypothesized



**Fig. 7.** Effect of silybin on PPAR $\alpha$  signal. (A) mRNA levels of Ppar $\alpha$  and its target genes (L-Fabp and Acox1) in the liver of mice treated with silybin. Mice were treated with 50 mg/kg or 150 mg/kg silybin for 2 weeks. The mRNA levels of Ppar $\alpha$ , L-Fabp, and Acox1 were determined via reverse-transcriptase PCR analysis. (B) The mRNA levels of PPAR $\alpha$  and L-FABP and (C) luciferase activities of PPAR $\alpha$  in HepG2 cells treated with silybin or fenofibrate. HepG2 cells were treated with dimethylsulfoxide (0.1%), silybin (25 and 50  $\mu\text{M}$ ), or fenofibrate (50  $\mu\text{M}$ ) for 24 hours. Cells were then collected for reverse-transcriptase PCR assay and reporter gene assays. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with respective control.

that silybin may interact with fenofibrate in binding and the subsequent activation of PPAR $\alpha$ . Both silybin and fenofibrate had no significant influence in the mRNA level of PPAR $\alpha$  (Fig. 9A). The upregulation of PPAR $\alpha$  targeting genes by fenofibrate was significantly attenuated by the cotreatment with silybin (Fig. 9B). Reporter gene assay also supported that silybin could significantly attenuate the activation of PPAR $\alpha$  by fenofibrate (Fig. 9C). To further validate this result, an *in vivo* study performed in mice was conducted. As shown in Fig. 10, fenofibrate treatment resulted in an obvious upregulation of L-fabp and Acox1, although these effects were significantly attenuated by silybin cotreatment. All of these data collected from cultured HepG2 cells and the *in vivo* study in mice demonstrated that silybin could significantly repress the activation of PPAR $\alpha$  by fenofibrate.

Because a panel of UGT isozymes is targeting genes of PPAR $\alpha$  (Runge-Morris and Kocarek, 2009), we extended this research to investigate whether fenofibrate-mediated regulation of the expressions and activities of UGTs was also influenced by silybin. Fenofibrate treatment alone resulted in an obvious upregulation of the mRNA levels of several UGT isozymes in HepG2 cells. The cotreatment with silybin significantly repressed the induction of these UGTs by fenofibrate (Supplemental Figs. 1–3). However, fenofibrate treatment in the mice led to a significant inducing effect only on UGT1A6, as evidenced from both the mRNA and enzyme activity test. Silybin cotreatment significantly repressed the upregulation of UGT1A6 induced by fenofibrate (Fig. 11). Likewise, the upregulation of UGT1A6 induced by WY14643, another classic agonist of PPAR $\alpha$ , was also significantly repressed by silybin cotreatment (data not shown). Together, these results suggest that silybin, a weak PPAR $\alpha$  agonist itself, may repress the activation of PPAR $\alpha$  induced by strong agonists such as fenofibrate and WY14643 and thereby may confer potential DDI with PPAR $\alpha$  agonists.

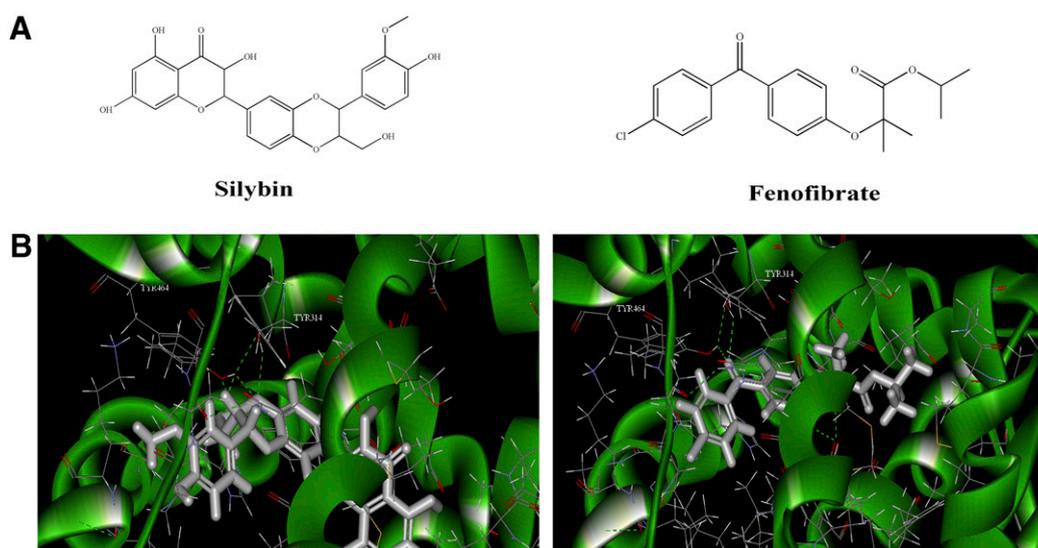
## Discussion

A panel of previous studies performed *in vitro* demonstrated that silybin could directly inhibit the enzyme activities of some P450s and UGTs (Venkataraman et al., 2000; Sridar et al., 2004; Brantley

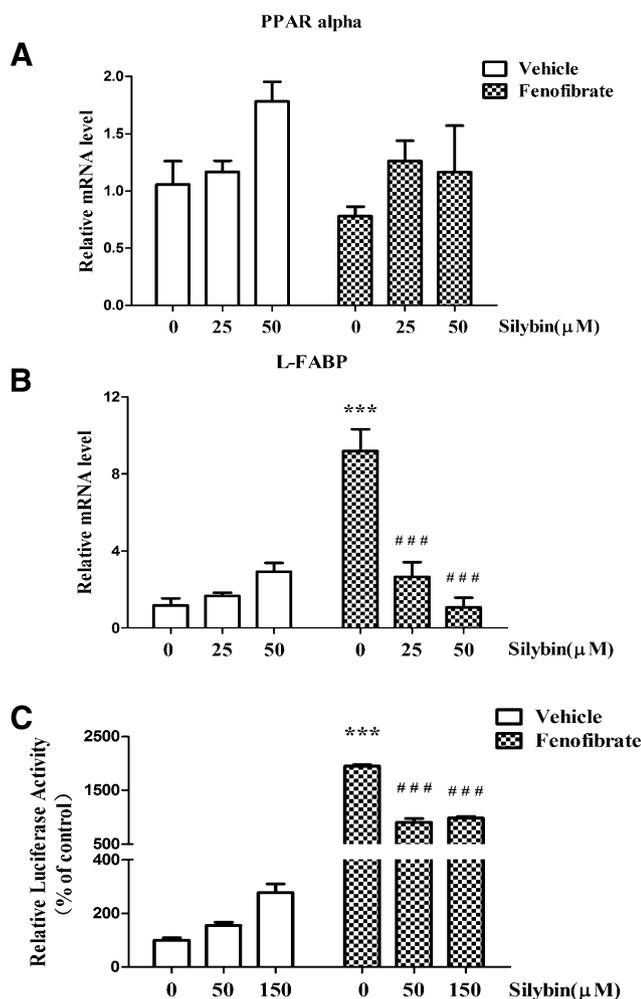
et al., 2013). However, most of the *in vivo* studies indicated that silybin has negligible effects on DMEs. Notably, silybin has been recently identified as a novel PXR antagonist that can prevent PXR-mediated induction of CYP3A4 (Mooiman et al., 2013). We found in this study that silybin, despite very low exposure levels in the plasma and liver, exerts moderate inhibitory effects on CYP1A2, CYP2C, CYP3A11, and UGT1A1 largely in a mechanism-based mode. More importantly, silybin has been identified as a weak PPAR $\alpha$  agonist, but can strongly repress the activation of PPAR $\alpha$  induced by typical agonists like fenofibrate.

After a consecutive treatment of 14 days, the plasma level of silybin is almost undetectable (<5 ng/ml) even at a relatively high dose (150 mg/kg). However, it is important to note that the hepatic exposure level of silybin is much higher than that in the plasma, albeit still in a relatively low level. This result suggests that, although the oral bioavailability of silybin is extremely poor, the possibility of hepatic enzyme regulatory effects *in vivo* cannot be excluded because of its specific extraction and accumulation in the liver. To develop a direct *in vitro* to *in vivo* link, we performed an *ex vivo* study by harvesting the livers of mice after a 14-day treatment with silybin to determine the enzyme activities of principal DMEs. With low-dose treatment (50 mg/kg), silybin has little effect on the enzyme activities of all the P450s, but has a significantly inhibitory effect on UGT1A1. When the dose increased to 150 mg/kg, significantly inhibitory effects were observed for CYP1A2, CYP2C, CYP3A11, and UGT1A1. These results indicated that, because of the extremely low bioavailability of silybin, a relatively high dose is necessary to exert significant activity-inhibitory effects on DMEs.

Because the maximum hepatic exposure level of silybin is only about 300 ng/g, we argued that such a low level of silybin is unlikely to exert its enzyme-inhibitory effects in a substrate-competitive mode on the basis of previous reports about its relatively high  $K_i/IC_{50}$  values *in vitro* (Sridar et al., 2004; D'Andrea et al., 2005). To provide a mechanistic understanding, we checked the mRNA and protein levels of dominant DMEs in mice. Surprisingly, silybin treatments have little effect in the expression of DMEs, suggesting that the inhibitory effects observed *ex vivo* are unlikely originated from either the transcriptional or translational level. Besides, the activities of all



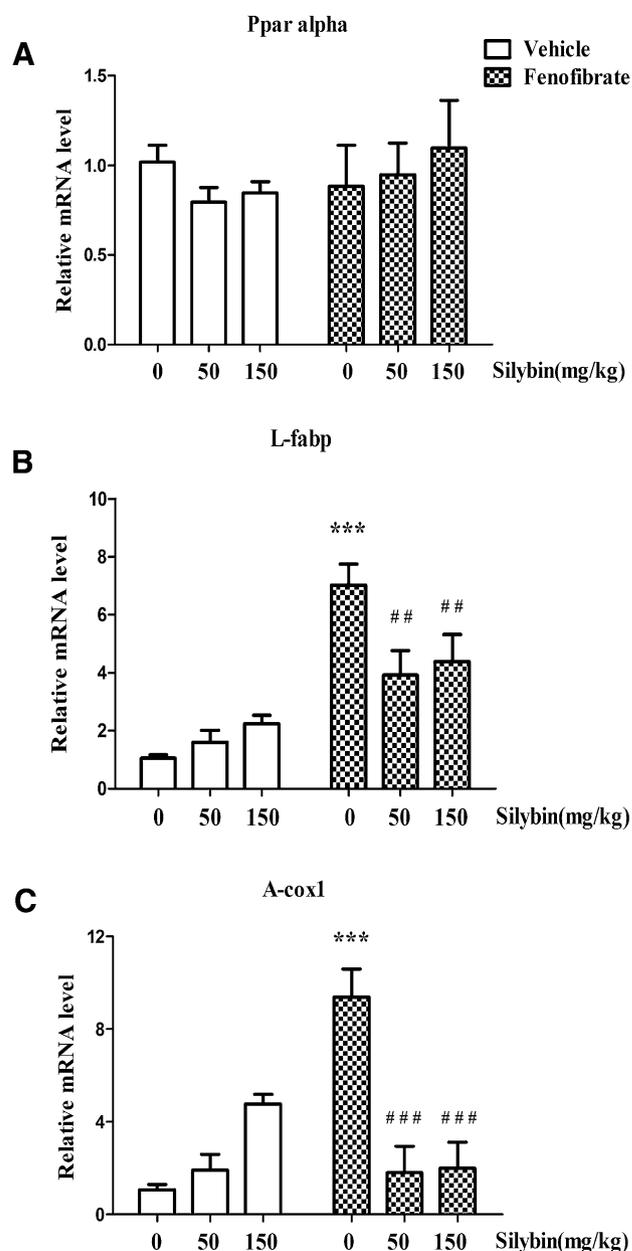
**Fig. 8.** Computational molecular docking of silybin and fenofibrate to the ligand binding domain of PPAR $\alpha$ . (A) Chemical structure of silybin and fenofibrate. The conformer with least energy for silybin or fenofibrate was chosen for subsequent docking. Crystal structure of human PPAR $\alpha$  was obtained from Protein Data Bank, and the docking analysis was conducted by DISCOVERY STUDIO. Repeated dockings were carried out until no further refinement in clustering or binding energy of conformer was achieved. Based on population size and binding energy, the best conformation was chosen for further analysis. Amino acid residues predicted to interact with the ligands are shown (B).



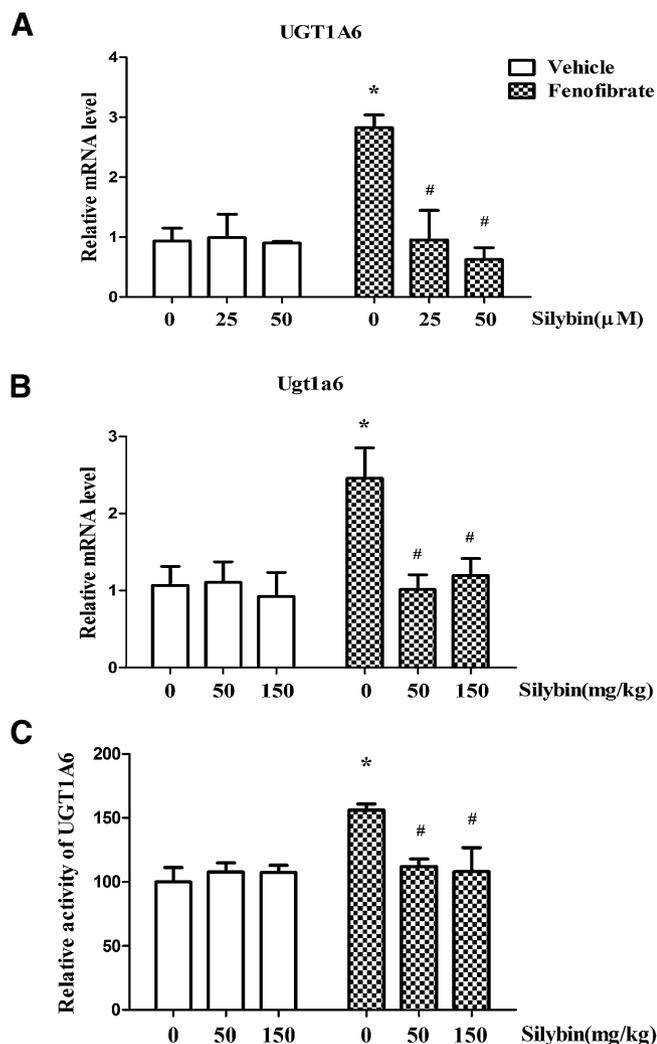
**Fig. 9.** Effect of silybin on PPAR $\alpha$  activation in the presence or absence of fenofibrate in HepG2 cells. HepG2 cells were treated with dimethylsulfoxide (0.1%) or silybin (25 and 50  $\mu$ M) in the presence or absence of fenofibrate (50  $\mu$ M) for 24 hours. Cells were then collected to detect the mRNA level of PPAR $\alpha$  (A) and L-FABP (B), as well as the luciferase activities of PPAR $\alpha$  (C). \*\*\* $P$  < 0.001 compared with dimethylsulfoxide (vehicle) group; ### $P$  < 0.001 compared with fenofibrate group.

these DMEs in the mice treated with a single dose of silybin (50 mg/kg and 150 mg/kg)-treated mice were slightly altered (Supplemental Fig. 4). Thus, we asked whether the mechanism-based inhibitory effect is involved. The NADPH, time-, and concentration-dependent inhibitory effects of silybin in CYP1A2, CYP2C, and CYP3A11 strongly indicate that silybin may act as a mechanism-based inhibitor of these P450 isozymes. Analysis of structure-function relationships for the inhibition of human P450s by flavonoid derivatives indicated that the number and position of hydroxyl and/or methoxy groups highly influence the inhibitory action of flavonoids toward P450s (Shimada et al., 2010; Kotewong et al., 2014). Silybin contains five hydroxyl groups, and the metabolites formed by P450s were found to be O-demethylated and mono- or dihydroxy products (Jancova et al., 2007), and this may be related to its ability to cause inhibition of some P450s and UGTs. Notably, our results showed that addition of NADPH in the incubation system further aggravated the enzyme-inhibitory effect of silybin in UGT1A1. Together, we proposed that silybin itself and the metabolites produced from P450 metabolism may confer an ability to inactivate the activity of UGT1A1, thus explaining a mechanism-based inhibitory effect observed from both MLM and HLM incubations. Besides, UGT1A1 is a dominant enzyme responsible for the glucuronidation

of silybin. Because of the high affinity of silybin to UGT1A1, it may be proposed that the substrate-competitive inhibition, in addition to the mechanism-based mode, explains the in vivo inhibitory effect on UGT1A1. Silybin has negligible effects on UGT2B subfamily, which is in line with the fact that silybin has a poor affinity with UGT2B enzymes. Results collected from the present study suggest that the long treatment with silybin at high dose may exert strong enzyme-inhibitory effect on UGT1A1 in both the mechanism-based and substrate-competitive modes and thus may impair the metabolism of its endogenous and xenobiotic substrates. It is well known that UGT1A1



**Fig. 10.** Effect of silybin on PPAR $\alpha$  activation in the presence or absence of fenofibrate in mice. C57BL/6 mice were treated with vehicle or silybin (50 and 150 mg/kg) together with or without fenofibrate (100 mg/kg) for a consecutive 14 days. Twenty-four hours after the last administration, the mice were sacrificed and their livers were immediately removed. mRNA levels of Ppar $\alpha$  (A), L-Fabp (B), and Acox1 (C) were determined via reverse-transcriptase PCR analysis; glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard to normalize all samples. \*\*\* $P$  < 0.001 compared with dimethylsulfoxide (vehicle) group; ## $P$  < 0.01, ### $P$  < 0.001 compared with fenofibrate group.



**Fig. 11.** Effect of coadministration of silybin and fenofibrate on the expression and activity UGT1A6. (A) mRNA levels of UGT1A6 in HepG2 cells. After being treated with dimethylsulfoxide (0.1%) or silybin (25 and 50  $\mu$ M) in the presence or absence of fenofibrate (50  $\mu$ M) for 24 hours, HepG2 cells were collected for reverse-transcriptase PCR assay. (B) mRNA levels and (C) activities of Ugt1a6 in the livers of mice. C57BL/6 mice were treated with vehicle or silybin (50 and 150 mg/kg) together with or without fenofibrate (100 mg/kg) for a consecutive 14 days. Twenty-four hours after the last administration, the mice were sacrificed and their livers were immediately removed to detect the mRNA levels and activities of UGT1A6. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard to normalize all samples in reverse-transcriptase PCR analysis. \* $P < 0.05$  compared with dimethylsulfoxide (vehicle) group; # $P < 0.05$  compared with fenofibrate group.

is an important enzyme in the metabolism of bilirubin, and that the inherited mutation of UGT1A1 resulted in complete or partial enzyme inactivation, which is the principal cause of newborn Crigler-Najjar syndrome and Gilbert syndrome, characterized with severe and mild hyperbilirubinemia, respectively (Flaig et al., 2007; Parveen et al., 2011; Rutter et al., 2011; Polyak et al., 2013). Indeed, it has been previously reported that patients receiving silybin treatment at high dose were characterized with increased plasma levels of bilirubin. Moreover, it is important to note that UGT1A1 is also an important enzyme in the metabolism of a panel of drugs such as irinotecan, a topoisomerase I inhibitor that is widely used in the treatment of metastatic colorectal cancer. Because the compromised glucuronidation is a principal cause of underlying irinotecan toxicity, it is better to avoid the coadministration with silybin and the crude extracts containing this compound.

The present study indicates that silybin has little effect on the basal expression of DMEs. However, it is important to note that DMEs are inducible via the activation of certain nuclear receptors. Silybin and isosilybin were recently identified as novel PXR antagonists, which could inhibit PXR agonist-induced CYP3A4 expression (Mooiman et al., 2013). Considering that PPAR $\alpha$  is a master regulator of UGTs (Zhou et al., 2014) and that silybin has a high affinity with UGT isozymes, we asked whether silybin could regulate the function of PPAR $\alpha$ . All of the evidence collected from the targeting gene analysis, reporter gene assay, and molecular docking assay support that silybin per se is a weak PPAR $\alpha$  agonist. However, silybin could significantly antagonize the activation of PPAR $\alpha$  by typical agonists, including fenofibrate and WY14643. Molecular docking analysis suggests that silybin shares the same binding sites of PPAR $\alpha$  with fenofibrate. Thus, silybin may competitively prevent the binding of typical agonists to the cavity of PPAR $\alpha$  due to its much bigger size, thereby resulting in a compromised activation. In this study, treatment of fenofibrate in HepG2 cells significantly upregulated the expression of a panel of UGT isozymes, whereas it only upregulated Ugt1a6 in mice. The cotreatment of silybin could significantly attenuate the upregulation of UGTs, especially UGT1A6, induced by fenofibrate. This result is in line with previous findings that UGT1A6 is the most sensitive, targeting UGT isozymes of PPAR $\alpha$ . Because PPAR $\alpha$  agonists such as fenofibrate are widely used in the therapy of nonalcoholic fatty liver disease, it is highly possible that they may be coadministered with silybin because of its well-known hepatoprotective effects. Therefore, the present finding about the potential interaction of silybin with typical PPAR $\alpha$  agonists is of clinical significance.

An important concern is whether silybin treatment is at risk to significant drug-drug interactions in human beings. Because the oral bioavailability of silybin is very poor, it is unlikely to reach the level that can induce significant competitive inhibitory effect in the liver when silybin is administered at physiologically relevant dose. However, using an ex vivo test, we have shown that the hepatic enzyme activities of CYP1A2, CYP2C, CYP3A11, and UGT1A1 are significantly reduced upon high dose and long-time administration of silybin, although the hepatic exposure level is still much lower than the effective concentration tested in vitro. Because mechanism-based enzyme inhibition is typically characterized with time dependency, the data obtained from the present study suggest that the long-time consumption of silybin is at risk to induce drug-drug interactions via mechanism-based inactivation of multiple enzymes. Moreover, in view that a relatively low dose (50 mg/kg) of silybin treatment significantly repressed PPAR $\alpha$  activation induced by exogenous agonists in mice, the potential interaction between silybin and PPAR $\alpha$  agonists should be closely monitored in the clinic.

In summary, we conclude that silybin, despite its poor systematic exposure, upon long-time consumption may exert inhibitory effects on a panel of principal DMEs in a mechanism-based mode. More importantly, silybin is per se a weak PPAR $\alpha$  agonist but can competitively attenuate the activation of PPAR $\alpha$  induced by its typical agonists such as fenofibrate. Our study suggests that potential interactions induced by silybin, and in particular with PPAR $\alpha$  agonists, should be closely monitored when it is administered at high dosage and for a long time.

#### Authorship Contributions

*Participated in research design:* Hao, G. Wang, H. Wang.  
*Conducted experiments:* H. Wang, Yan, Zhao, Che, Zhang, Cheng, Ya. Xie.  
*Contributed new reagents or analytic tools:* Cao, Liu, Li, Qi.  
*Performed data analysis:* H. Wang, Hao, Yu. Xie.  
*Wrote or contributed to the writing of the manuscript:* H. Wang, Hao, G. Wang.

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