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In vitro Assessment of the Effects of Silybin on CYP2B6-mediated Metabolism

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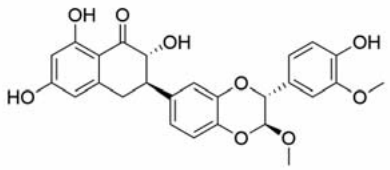
ABSTRACT

Silybin is a flavonol compound with a variety of physiological properties, such as hepatoprotective, anti-fibrogenic, and hypocholesterolemic effects. Although the *in vivo* and *in vitro* effects of silybin are frequently reported, studies on herb–drug interactions have yet to be performed. With the discovery of multiple important substrates of CYP2B6 recently, there is a growing body of evidence indicating that CYP2B6 plays a much larger role in human drug metabolism than previously thought.

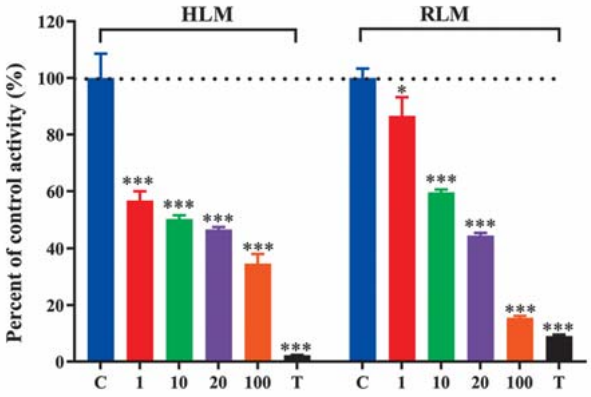
The purpose of this study is to determine how silybin affects the CYP2B6 enzyme's activity, as well as to clarify the molecular mechanisms for inhibition by silybin. The results showed that silybin inhibited CYP2B6 activity in liver microsomes in a non-competitive manner, with IC_{50} and K_i values of 13.9 µM and 38.4 µM, respectively. Further investigations revealed that silybin could down-regulate the expression of CYP2B6 protein in HepaRG cells. The hydrogen bond conformation of silybin in the active site of the CYP2B6 isoform was revealed by a molecular docking study. Collectively, our findings verify that silybin is an inhibitor of CYP2B6 and explain the molecular mechanism of inhibition. This can lead to a better understanding of the herb–drug interaction between silybin and the substrates of the CYP2B6 enzyme, as well as a more rational clinical use of silybin.

Introduction

Silybin is a primary component of the silymarin complex extracted from the milk thistle (*Silybum marianum* [L.] Gaertn. [Asteraceae]) [1], a commonly used herb to treat a range of liver diseases, including hepatitis and cirrhosis, and to protect the liver against poisoning from chemical and environmental toxins [2]. The structure is shown in ▶ **Fig. 1**. Silybin has recently received attention be-



▶ **Fig. 1** The chemical structure of silybin.



▶ **Fig. 2** The inhibitory effect of silybin on enzyme activity. Metabolites were generated by 60 min incubation of 100 μM bupropion with 0.25 mg/mL HLMs, 0.5 mg/mL RLMs. HLMs: human liver microsomes; RLMs: rat liver microsomes. T: ticlopidine. Error bars represent the mean ± SD, n = 3 (*: $p < 0.05$; ***: $p < 0.001$ compared with the control group)

cause of its other beneficial activities, such as anticancer, hypocholesterolemic, and neuroprotective properties [3,4]. Silybin has been widely used in clinical settings in combination with a variety of other drugs that are the substrates of human cytochrome P450 enzymes. It has been reported that silybin can inhibit the transcription factor NF-κB, which regulates and coordinates the expression of numerous genes involved in the drug metabolism enzymes and transporters. According to research, silybin had little effect on CYP3A4 *in vitro* and inhibited the drug transporters P-glycoprotein and breast cancer resistance protein [5–7]. CYP2B6, a human cytochrome P450 enzyme with broad substrate specificity, is primarily expressed in the liver and is involved in xenobiotic metabolism. According to current reports, CYP2B6 accounts for 2%~10% of total hepatic CYP content and is involved in the metabolism of a significant number of drugs, accounting for approximately 8% of all commercially available drugs [8]. The discovery of multiple important CYP2B6 substrates has increased interest in the xenobiotic factor that contributes to the expression and function of the enzyme. The substrates of CYP2B6 include several clinically utilized, such as central-nervous-system-acting drugs (bupropion, methadone, and mephenytoin), anticancer agents (cyclophosphamide and ifosfamide), and antiretroviral drugs (efavirenz) [9].

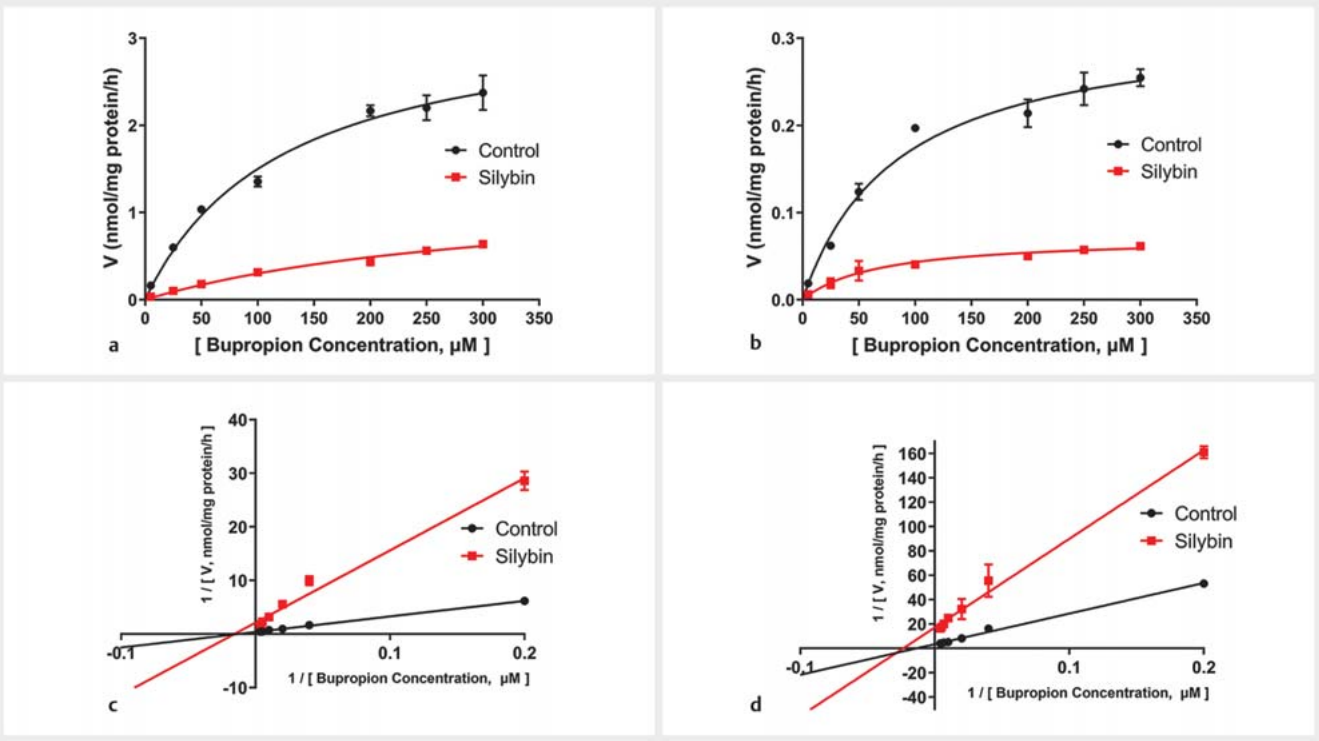
Silybin is used to treat chronic hepatitis as a component of traditional Chinese medicine and is often used in combination with other modern medicine in medical therapy [10]. Drug interactions can result from variations in CYP2B6 activity that impact the plasma level of its substrates. The inactivation of human CYPs may result in clinically significant drug–drug interactions. Currently, the inhibition potency of silybin on CYP2B6 and related molecular mechanisms has not been reported. Deciphering the inhibitory mechanism and testing the potential inhibition of silybin against CYP2B6 are therefore essential.

We chose bupropion, a CYP2B6-sensitive probe substrate [11], to examine the CYP2B6 activity *in vitro*. The objective of this study was to determine how much silybin inhibits CYP2B6 activity and protein expression in an *in vitro* study. For these purposes, a series of enzyme kinetic tests were carried out in human/rat liver microsomes to determine the inhibition assay of silybin against the CYP2B6 enzyme. A molecular docking study was carried out to investigate the binding site of silybin on the CYP2B6 isoform further. The effect of silybin on CYP2B6 mRNA and protein expression in HepaRG cells was investigated using RT-qPCR and Western blot analysis. We investigated the effect of silybin on CYP2B6 enzyme activity and expression, as well as the inhibitory type and potential mechanism of silybin on the CYP2B6 enzyme. All the findings presented in this paper are critical for understanding the interactions between silybin and CYP2B6 substrates, which will be useful in clinical applications of silybin.

Results and Discussion

The inhibitory effect of silybin on the activities of CYP2B6 in HLMs and CYP2B1 in RLMs was shown in ▶ **Fig. 2**. The inhibition degree of positive control, ticlopidine (10 μM), was 97.7% in HLMs and 90.9% in RLMs compared with the control group. In HLMs, the activity of the enzyme compared with the control was 36.6%, 46.6%, 50.4%, and 56.6% when the concentration of silybin was 100 μM, 20 μM, 10 μM, and 1 μM, respectively, and in RLMs, the activity was 15.5%, 44.6%, 59.5%, and 86.6%, respectively, at the same concentration of silybin compared with the control group.

The relationship between bupropion concentration and the rate (V) of hydroxy-bupropion (HBUP) formation incubated with HLMs and RLMs are shown in ▶ **Fig. 3a, b.** The same data are presented as the Lineweaver–Burk plot in ▶ **Fig. 3c, d**. The parameters of enzyme kinetics are summarized in ▶ **Table 1**. The V_{max} in HLMs was 3.339 nmol/h/mg protein (control group) and 1.293 nmol/h/mg protein (silybin group) with respective 95% confidence intervals of 3.061 to 3.688 nmol/h/mg protein and 1.062 to 1.7 nmol/h/mg protein. Compared with the control group, the value of K_m was significantly increased (122.7 μM vs. 329.9 μM) in HLMs ($p < 0.01$). Incubated with RLMs, the value of Vmax was significantly decreased (0.3217 nmol/h/mg protein vs. 0.072 nmol/h/mg protein) ($p < 0.01$). There was no significant difference in the value of K_m observed in RLMs. Lineweaver–Burk plots (▶ **Fig. 3c, d**) showed that the inhibition type of silybin on CYP2B6 was a noncompetitive inhibition model, and the type of CYP2B1 was mixed.



▶ **Fig. 3** The curve of Michaelis–Menten (**a, b**) and the Lineweaver–Burk plots (**c, d**) of the effect of silybin on CYP2B6 in HLMs (**a, c**) and CYP2B1 in RLMs (**b, d**). Data are obtained from 60 min incubation with bupropion (5–300 μM) in the absence or presence of silybin (20 μM). Error bars represent the mean ± SD, n = 3.

▶ **Table 1** The enzyme kinetics parameters.

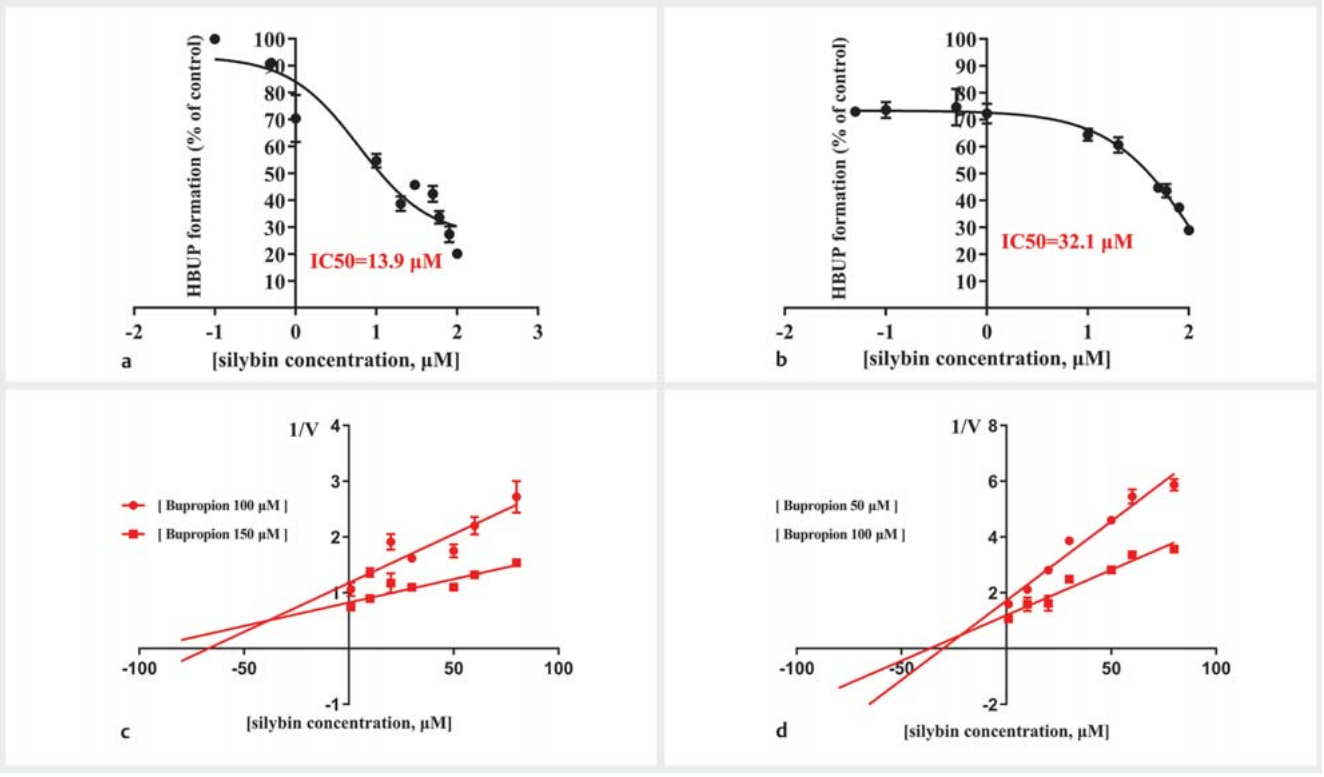
	Control		Silybin	
Parameters	**V_{max} (nmol/h/mg protein)**	**K_m (μM)**	**V_{max} (nmol/h/mg protein)**	**K_m (μM)**
HLM	3.339 (3.061–3.688)[a]	122.7 (98.15–155.3)	1.293** (1.062–1.7)	329.9** (232.7–505.4)
RLM	0.3217 (0.2988–0.3494)	84.42 (67.43–106.8)	0.072** (0.0645–0.0818)	67.49 (46.91–98.32)

[a] 95 % confidence interval; ** $P < 0.01$ compared with the control group

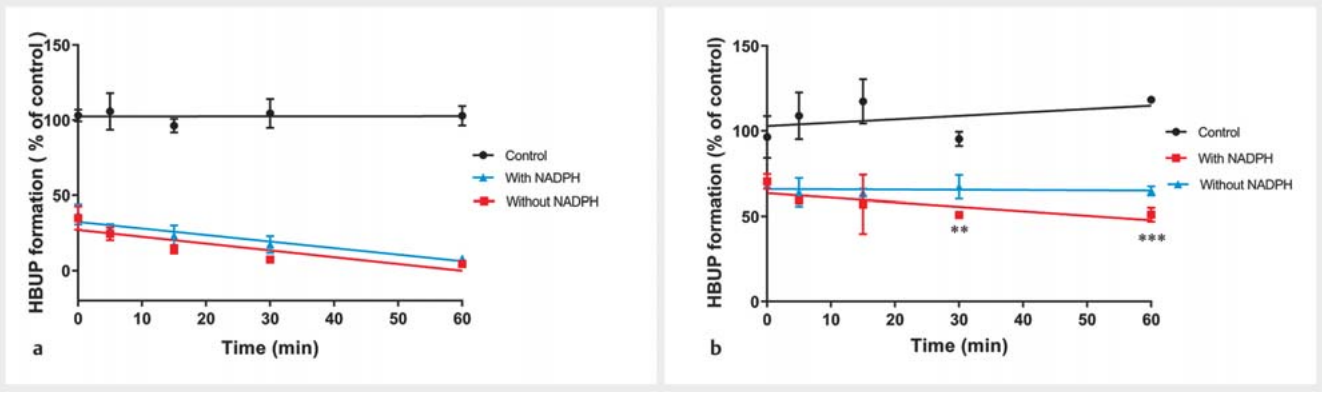
The inhibitory effects of silybin against CYP2B6 in HLMs and CYP2B1 in RLMs were investigated at different concentrations of silybin to explore the value of IC_{50}, as depicted in ▶ **Fig. 4**. Silybin concentration-dependently inhibited the catalytic activities of CYP2B6 and CYP2B1 with IC_{50} values of 13.9 μM and 32.1 μM, respectively. The K_i values for CYP2B6 and CYP2B1 were determined to be 38.4 μM and 58.4 μM, respectively.

To ascertain if silybin's inhibition of CYP2B6/CYP2B1 was time dependent and that its action was NADPH dependent, an NADPH-dependent inhibition experiment was carried out. The time-dependent inhibition (TDI) refers to a change in CYP inhibitor potency that occurs during an *in vitro* incubation. Experimentally, the inhibitory effects of various pathways could be compared between those obtained in the presence and absence of NADPH during a preincubation period [12]. As shown in ▶ **Fig. 5**, compared with the control group, the inhibitory effect of silybin on CYP2B6 was gradually enhanced with the prolongation of co-incubation time (▶ **Fig. 5a**), and there was no difference between with the NADPH group and without the NADPH group ($p > 0.05$). This finding demonstrated that TDI is mostly treated by its spontaneous oxidative products and does not require NADPH. The inhibitory effect of silybin on CYP2B1 was gradually increased with increasing co-incubation time in the NADPH group (▶ **Fig. 5b**), and there were significant differences between with the NADPH group and without the NADHP group ($p < 0.05$).

Molecular docking analysis of the interaction between silybin and CYP2B6 was performed to confirm the inhibitory mechanism at the molecular level. As shown in ▶ **Fig. 6**, silybin could be tightly docked into the catalytic cavity of the CYP2B6 protein. For identifying and quantifying activity pockets in the CYP2B6 protein



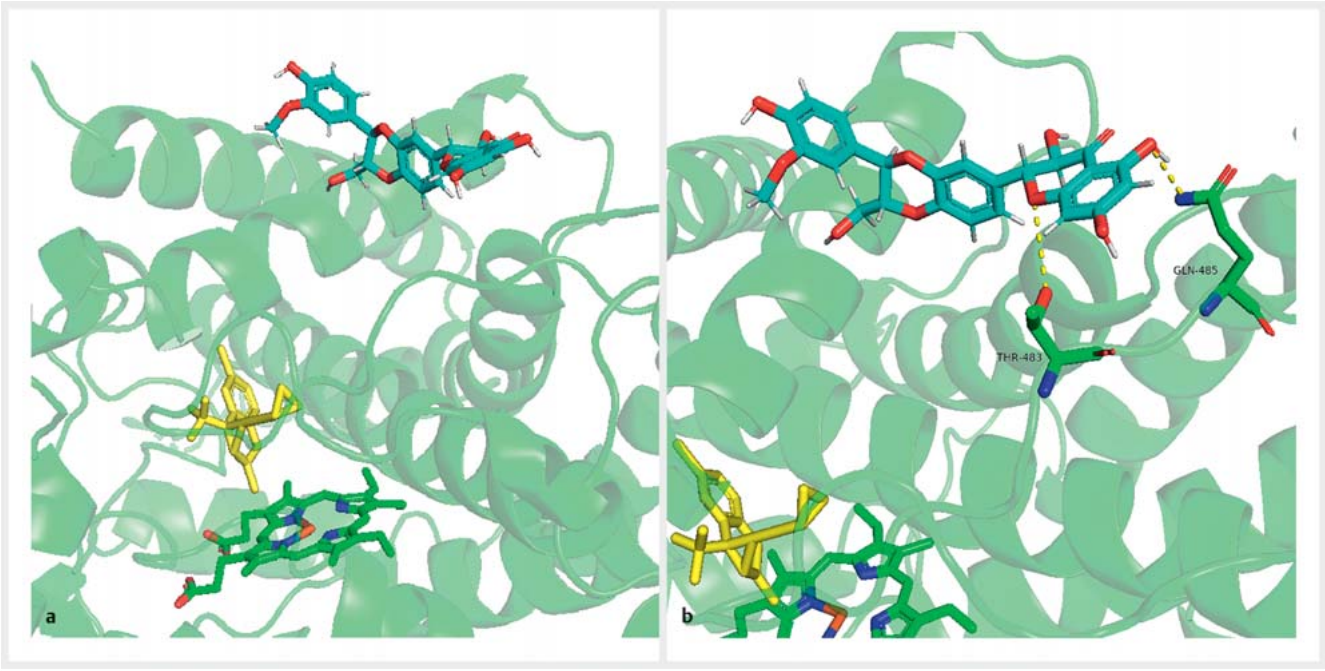
▶ **Fig. 4** The inhibition curve of silybin on CYP2B6 (HLMs, **a**) and CYP2B1 (RLMs, **b**) and the Dixon plots for CYP2B6 (HLMs, **c**), CYP2B1 (RLMs, **d**). The activity of CYP2B6 and CYP2B1 were tested using probe substrate in HLMs and RLMs. Error bars represent the mean ± SD, n = 3.



▶ **Fig. 5** NADPH-dependent inhibition of CYP2B6 by silybin (HLMs, **a**) and CYP2B1 (RLMs, **b**). Error bars represent the mean ± SD, n = 3.

structure, the DoGSiteScore server was used. The DoGSiteScore server is a grid-based function prediction method to identify potential pockets on the protein surface. The binding energy of the lowest energy conformation was predicted as − 8.0 kcal/mol. In addition, the critical interaction between silybin and CYP2B6 was also investigated. As depicted in ▶ **Fig. 6**, GLN-458 and THR-483 were involved in forming hydrogen bonds (the yellow lines on the right of ▶ **Fig. 6**) with the CYP2B6 protein structure, which may include the primary inhibitory mechanism of silybin.

The result of the MTT viability assay in the HepaRG cell is shown in ▶ **Fig. 7**, and the IC_{50} value of silybin against HepaRG cells is 142.2 μM (**Fig. S2**, Supporting Information). The gene expression of CYP2B6 and the reference gene, GAPDH, were determined by RT-qPCR; the results are shown in ▶ **Fig. 8 a, c**. Compared with the control group, the expression of CYP2B6 mRNA was of no significant difference in the low-dose group ($p > 0.05$). In contrast, CYP2B6 mRNA expression in the medium (50 μM) and high (100 μM) dose groups showed an apparent downward trend, with



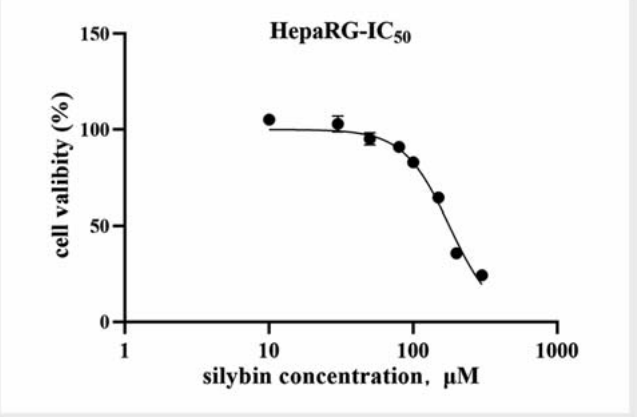
▶ **Fig. 6** The binding pose of silybin in the active pocket of CYP2B6. Silybin is shown as blue sticks, the heme of CYP2B6 show as green sticks, the substrate of CYP2B6 show as yellow sticks in left (**a**). The detailed part of the interaction is shown as the right side (**b**).

reductions of 47.6% and 56.5%, respectively ($p < 0.05$). HepaRG cells were treated with 100 µM silybin at different times, and there was a significant decrease after being treated 24 h ($p < 0.05$).

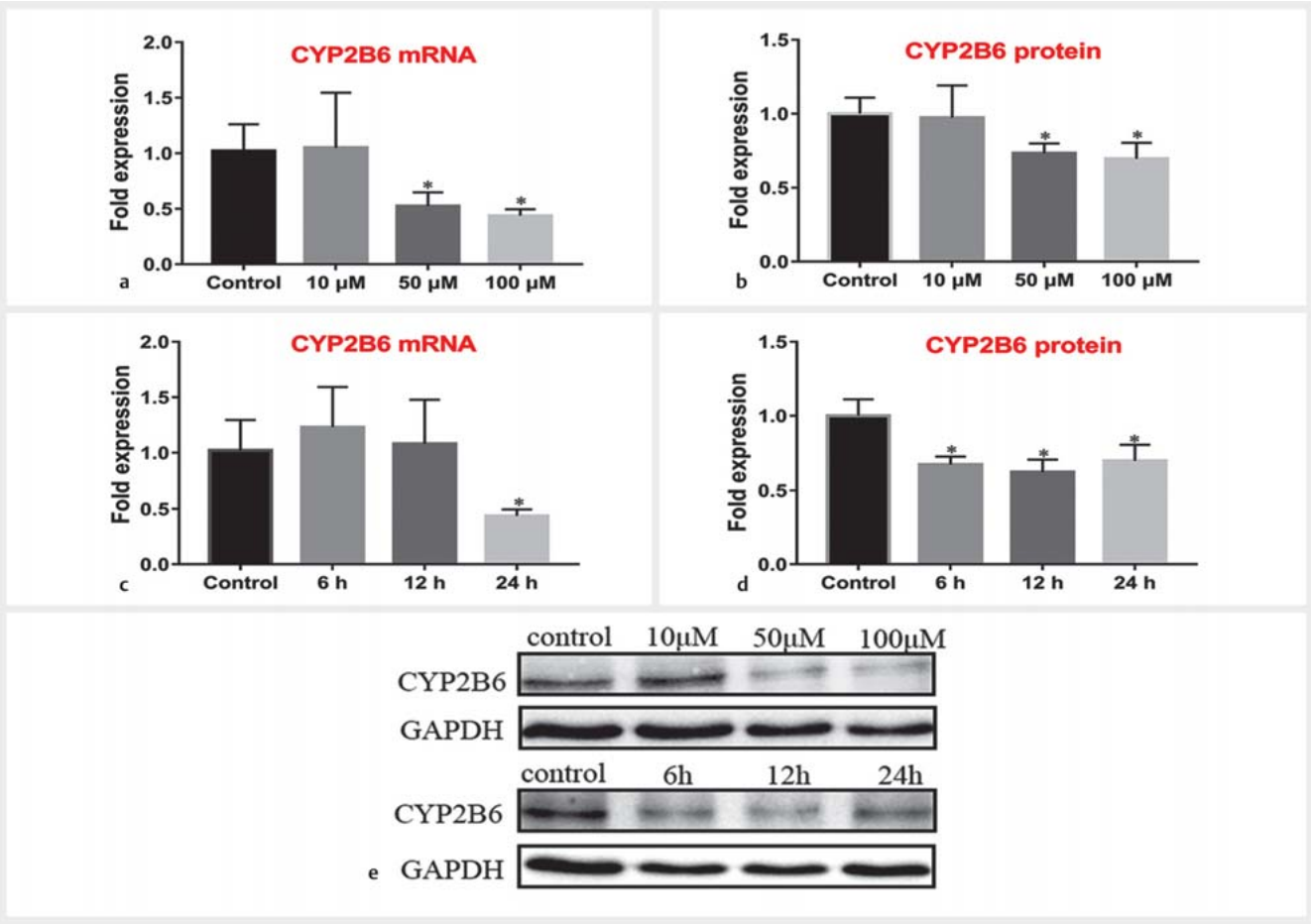
Western blotting was performed to quantify CYP2B6 protein expression in the HepaRG cells treated with silybin. CYP2B6 and the GAPDH protein were detected as a band of about 60 kDa and 35 kDa among the proteins extracted from the HepaRG cells. Each protein band was normalized to the band of reference protein, GAPDH. The results are shown in ▶ **Fig. 8 b, d, e**. The data showed that there was no significant difference in CYP2B6 protein expression in HepaRG cells when treated with 10 µM silybin for 24 h; CYP2B6 protein expression was significantly decreased while the concentration of silybin was 50 µM and 100 µM ($p < 0.05$). In HepaRG cells treated with 100 µM silybin after 6, 12, and 24 h, CYP2B6 protein expression all decreased significantly ($p < 0.05$).

There is growing evidence that CYP2B6 is more important to human drug metabolism. Since silybin is frequently used to treat hepatic disease, it is important to be aware of the possible risks of drug–drug interactions. Inhibition of cytochrome P450 enzymes is a central mechanism, resulting in clinically significant drug–drug interactions [13]. Silybin could affect the activity or expression of drug-metabolizing enzymes that are responsible for the elimination of co-administrated therapeutic agents. Unfortunately, little research has been done so far on how silybin and the CYP2B6 enzyme interact. Thus, in this study, we used a combination of *in vitro* and molecular docking studies to examine the effect of silybin on the CYP2B6 enzyme and the molecular mechanism.

In this study, we first demonstrated that silybin is an inhibitor of the CYP2B6 enzyme. Rat CYP2B1 and human CYP2B6 share approximately 80% nucleotide sequence identity [14], and rats are often used as rodent species in an investigation of the metabolism rates and routes. Silybin is the primary active constituent of silymarin, and it has been used as a therapeutic agent in many liver diseases in clinical practice [15]. As silybin possesses numerous pharmacological activities, it is essential to determine the effects of silybin on the CYP2B6 enzyme. In this study, silybin was revealed to be a noncompetitive inhibitor of CYP2B6 with an IC_{50} value of 13.9 µM and a K_i value of 38.4 µM. In addition, silybin was a mixed inhibitor of CYP2B1 in RLM with an IC_{50} value of 32.1 µM and a K_i value of 58.4 µM. Based on these results, silybin showed a more substantial inhibitory effect on CYP2B6 in HLM



▶ **Fig. 7** The MTT curve of HepaRG cells.



▶ **Fig. 8** The effect of silybin on CYP2B6 mRNA and protein expression. The expression of CYP2B6 mRNA in HepaRG cells (**a, c**); the expression of CYP2B6 protein expression in HepaRG cells (**b, d**); the protein blots of HepaRG cells treated with silybin (**e**). The data are presented as a fold change in the silybin-treated group relative to the control group. Error bars represent the mean ± SD, n = 3.

than CYP2B1 in RLM. The reason for the difference between the two types of inhibition is most likely due to species differences; this hypothesis needs to be explored after perfecting the crystal structure of the CYP2B1 protein in the future. To further understand the interaction of silybin and CYP2B6, molecular docking analysis was performed to confirm the drug binding conformation. According to our molecular docking study, silybin, when it is co-administrated with other substrates of CYP2B6 enzyme, may decrease the activity of CYP2B6 enzyme by forming hydrogen bonds with GLN-458 and THR-483, which could cause suppressing or reducing hepatic clearance of substrates.

Our results of this study also indicate that silybin can down-regulate the expression of the CYP2B6 enzyme in HepaRG cells. This down-regulation effect is reflected in the mRNA and protein levels of CYP2B6. HepaRG cells, a human-origin cell line, have been proven to be a classical model to investigate the expression level of mRNA and protein of CYP450 enzyme in the human liver [16]. Studies have shown that the pregnant X receptor (PXR) is one of the major transcriptional regulators of CYP2B6 [8], and silybin is a stronger inhibitor of PXR [17]. Thus, silybin may down-regulate CYP2B6 protein expression by inhibiting the PXR.

Regarding the substrates of CYP2B6, it should be noted that inhibition of the CYP2B6 enzyme is a double-edged sword, which may bring beneficial effects or side effects on human health. Efavirenz and cyclophosphamide are two of the better-studied drugs concerning CYP2B6 metabolism. These widely used drugs have very narrow therapeutic indices, and variations in CYP2B6 expression and activity lead to significantly altered drug plasm concentrations [18]. Cyclophosphamide is a prodrug. Increased expression and activity of CYP2B6 may result in an increase in the circulating concentration of active moiety, which is a beneficial effect [19]. On the contrary, in the case of efavirenz, increased CYP2B6 enzyme metabolism may lead to an insufficient plasma concentration and to not achieving anti-viral therapy [20]. According to our results, silybin can inhibit the activity and down-regulate protein expression of the CYP2B6 enzyme, and it is easily conceivable that silybin may be beneficial for decreasing the toxicity of cyclophosphamide when co-administered. Unfortunately, inhibiting activity and expression can also significantly increase the drug plasm concentration of efavirenz, which could increase the neurotoxicity of efavirenz.

It is well-known that *in vitro* data is essential for understanding potential enzyme inhibition and drug–drug interaction *in vivo*. However, many factors might influence drug interactions mediated by CYP450 inhibition, including the extent of drug absorption, the bioavailability, and the total clearance of the affected drug. Therefore, further *in vivo* studies are needed to identify the interactions of silybin and the substrates of the CYP2B6 enzyme, which can provide more guidance for the clinical use of silybin.

In conclusion, in this study, we systematically examined how silybin inhibits the the activity and expression of the CYP2B6 enzyme. The results showed that silybin could inhibit the activity of the CYP2B6 enzyme in a noncompetitive manner in an HLMs assay, as well as down-regulate the expression of the CYP2B6 protein in HepaRG cells. These findings suggested a possible drug–drug interaction between silybin and drugs metabolized by the CYP2B6 enzyme, which has to be confirmed by further research.

Material and Methods

Materials

Bupropion (≥ 99 % purity), carbamazepine (≥ 99 % purity), silybin (≥ 99 % purity), and ticlopidine (≥ 96 % purity) were obtained from Meilun Biotech Corporation. TRIzol was obtained from Tiangen Biotech Corporation. Hydroxybupropion (≥ 99.9 % purity) was purchased from Cerilliant Corporation. Antibodies against CYP2B6 were purchased from Abcam Corporation, and an antibody against GAPDH was purchased from Proteintech Corporation. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Meilun Biotech Corporation. Human liver microsomes (HLMs, 50-donor pooled) and rat liver microsomes (RLMs, 10 mg) were purchased from Bioreclamation-IVT Corporation. Methanol, acetonitrile (liquid chromatography-mass spectrometry grade), and formic acid (≥ 96 % purity) were purchased from Meilun Biotech Corporation.

Methods

Determination of CYP450 enzymatic activity

Bupropion (100 µM) was used as selective probe substrates for CYP2B6 in human liver microsomes (HLMs) and CYP2B1 in rat liver microsomes (RLMs). The reaction mixture consisted of 0.25 mg/mL HLMs protein or 0.5 mg/mL RLMs protein, bupropion, and an NADPH regenerating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 1 U/mL glucose-6-phosphate dehydrogenase) in 100 µL 50 mM KPI buffer solution (9.74 g $K_2HPO_4{\cdot}3\text{-}H_2O$, 0.398 g Na_2HPO_4, and 0.280 g ethylenediaminetetraacetic acid was dissolved in 500 mL ultrapure water), performed in 96-well plates. The silybin was dissolved in dimethylsulfoxide, and the solvent volume did not exceed 1 % (vol/vol) compared to the total incubation system volume. The ticlopidine (10 µM) was selected as the control of the CYP2B6 inhibitor [21]. The concentration of silybin was 1, 10, 20, and 100 µM in the incubation system. The reaction was initiated by adding NADPH and then incubated at 37 °C for 60 min. The reaction was terminated with an equal volume of ice-cold acetonitrile. After the end of the reaction, 100 µL of the reaction solution was taken out and added to 20 µL internal standard solution (carbamazepine, 612.4 ng/mL), vortexed, and centrifuged at 15 294×g for 5 min, and the supernatant was injected for HPLC/MS-MS analysis of hydroxybupropion. All incubation samples were conducted in triplicate.

Time-dependent inhibition tests

Time-dependent inhibition (TDI) tests were conducted according to the previous reported [22]. The mixture containing KPI buffer, NADPH regenerating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 1 U/mL glucose-6-phosphate dehydrogenase), HLMs or RLMs, and silybin preincubation at 37 °C for 0, 5, 15, 30, and 60 min, and then, the reaction was initiated by adding bupropion. The reaction was performed at 37 °C for an additional 60 min. The sample preparation was performed according to the section above.

Kinetic analyses for inhibition

To determine the types of inhibition and the value of kinetic parameters, the final substrate concentrations were from 5 µM to 300 µM (5 µM, 25 µM, 50 µM, 100 µM, 200 µM, 250 µM, 300 µM). The incubation time was 60 min with HLMs (0.25 mg/mL) and RLMs (1 mg/mL) with or without silybin (20 µM) preincubation. The sample preparation was performed according to the section above. The maximum reaction velocity (V_{max}) and the Michaelis constant of CYP450 substrate (K_m) values were calculated from nonlinear regression analysis of experimental data according to the Michaelis–Menten equation. The Dixon and Lineweaver–Burk plots were adapted to determine the inhibition type, and the second plot of slopes from Lineweaver–Burk plot versus the compounds' concentrations was utilized to calculate the K_i value. All kinetic data were fitted by the following kinetic equations [23]:

- Competitive: $V= (V_{max}S)/[K_m (1 + I/K_i) +S]$ (1)
- Noncompetitive: $V= (V_{max}S)/[(K_m + S)\times(1 + I/K_i)]$ (2)
- Mixed-inhibition: $V= (V_{max}S)/[(K_m + S)\times(1 + I/\alpha K_i)]$ (3)

V is the velocity of HBUP formation and Vmax is the maximum velocity. S and I are substrate (bupropion) and inhibitor (silybin) concentrations. K_i is the inhibition constant of the silybin against the target CYP2B6; Km is the Michaelis constant for HBUP formation in HLM.

Next the silybin was incubated with HLMs (0.25 mg/mL) in different concentrations (1, 10, 20, 30, 50, 60, 80 µM) and incubated with RLMs (0.5 mg/mL) in different concentrations (0.05, 0.1, 0.5, 1, 10, 20, 30, 50, 60, 80, 100 µM) to explore the values of half inhibition concentration (IC_{50}).

Cell line and culture conditions

HepaRG cells were obtained from the National Collection Authenticated Cell Cultures, Chinese Academy of Sciences. The procedures for induction and maintaining HepaRG cells were carried out as reported previously [24]. The HepaRG cells were maintained at 37 °C in humidified air incubator with 5 % CO_2. HepaRG cells were detached by gentle trypsinization containing ethylene diamine tetra-acetic acid (EDTA) and seeded at a density of 2.6×10^4 cells in each well. They were first incubated in the DMEM (Dulbecco's modified Eagle medium) supplemented with 10 % fetal bovine serum (FBS). For differential HepaRG cells, they were first seeded in the growth medium composed of DMEM with 10 %

FBS. After 2 weeks, they were shifted to the same culture medium supplemented with 2% dimethyl sulfoxide (differentiation medium) for 2 weeks. The medium was renewed every 1 to 2 days.

Dose-Response Curve based on MTT

HepaRG cells were seeded in a 96-well plate at a density of 2.6×10^4 cells in each well, incubated in DMEM medium at 37 °C for 24 h. Silybin (dissolved in 0.2% DMSO) was formulated in DMEM with 1% FBS in concentrations of 10, 30, 50, 80, 100, 150, 200, and 300 µM, and six replicates were used for each concentration, with a final volume of 100 µL. After 24 h, the medium was removed, and the cells were PBS washed. 0.5 mg/mL MTT (dissolved in PBS) was added and incubated for 4 h at 37 °C. Following 4 h of treatment incubation, the solution was removed and 200 µL DMSO was added to each well, and then the absorbance was measured at 490 nm.

Total RNA extraction, reverse transcription, and RT-qPCR analysis

Differentiated HepaRG cells were seeded in six-well plates and treated with different concentrations of silybin (dissolved in 0.2% DMSO); 10, 50, and 100 µM were chosen, as were different incubation times. Using TRNsol (total RNA solution), total RNA was isolated from HepaRG cells. The concentration of total RNA was quantified by NanoDrop 2002, and the total RNA (500 ng/µL – 600 ng/µL) was reverse transcribed using Strand cDNA Synthesis SuperMix for qPCR kit. cDNA was then diluted five times with RNase-free distilled water, and RT-qPCR was performed using a qPCR SYBR mix kit. Primer sequences of related genes are shown in Table S1. RT-qPCR conditions are as follows: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. All data were normalized to the average of the housekeeping genes GAPDH. Changes in gene expression between control and silybin-treated liver tissue or HepaRG cells were calculated using the following formulation: ΔCt=Ct gene – Ct reference, and the fold change in gene expression was calculated with the comparative Ct ($2^{-\Delta\Delta Ct}$) method [25].

Western blot analysis

The effect of silybin on the protein expression of CYP2B6 in HepaRG cells was determined by Western blot analysis. Total protein was extracted from HepaRG cells using RIPA cell-lysis buffer containing protease inhibitor PMSF. After protein concentration had been measured using the BCA assay, protein extracts were added to 5× loading buffer and denatured by heating at 100 °C for 10 min. The same amount of proteins was loaded on and separated by 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with 5% skimmed milk for 2 h at 37 °C. The primary antibodies GAPDH (diluted in TBST 1 : 2000) and antibodies CYP2B6 (diluted in TBST 1 : 2000) were incubated at 4 °C overnight. The horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit in TBST diluted 1 : 10 000) was incubated at 37 °C for 1 h and enhanced chemiluminescence (ECL) substrate was used for detection. Image J analysis software was used for quantitative analysis of the blots.

Molecular Docking

The Protein Data Bank (PDB) database was used to retrieve the 3D structure of CYP2B6 with PDB ID:5WBG. The two-dimensional structure of the silybin was downloaded from PubChem in SDF format, Chem 3D was used to transform the dimension of silybin from 2D to 3D, and conversion of file format to. pdb. Water molecules in crystal structures were eliminated by PyMOL software, and hydrogen atoms were added to the protein. A docking study was performed using AutoDock Vina. AutoDock Vina takes the. pdbqt file format for receptor and ligand as an input. The size of the grid box in AutoDock Vina was kept as 23.25, 15.75, and 15 for x, y, and z, respectively, and its grid centered at dimensions (x, y, and z): 55.099, -1.739, and 25.688, respectively. The binding affinity of a ligand is a negative score with Kcal/mol as a unit. AutoDock Vina script can generate nine poses of a ligand with different binding energy for each ligand input. In all of these poses, the preferred conformations were the ones that have the lowest binding energy with the active site.

HPLC-MS/MS analysis

HPLC separation was performed on a C18 column (CAPCELL PAK C18, 50 × 2.00 mm, 5 µM) coupled with a Security Guard cartridge (C18, 4 × 3.00 mm i. d., Phenomenex). For *in vitro* metabolism analysis, the mobile phase consists of methanol (solvent A) and water (containing 5 mM ammonium formate, solvent B). Mass data were acquired in positive ion mode, and for the optimized parameters and conditions see the support information.

Statistical analysis

All statistical analyses were performed using SPSS 25.0 software (IBM, USA). The main pharmacokinetic parameters were calculated using Phoenix WinNonlin 7.0 software (Certara, USA) with a noncompartmental model. AutoDock Vina 1.2.2 (Molecular Graphics Lab, USA) were used for the docking study. The data are expressed as the means ± standard error of the mean. Significant differences between the control and experimental groups were determined by Student's *t*-test using SPSS. A two-tailed P value < 0.05 was considered statistically significant.

Contributors' Statement

Ling Wang and Xuehua Jiang conceived the study; Wenwen Zhang and Yice Zhang performed literature search; Wenwen Zhang wrote the initial draft; Wenwen Zhang, Chengmin Wen and Ling Wang revised the draft and prepared the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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