SILYBIN INACTIVATES CYTOCHROMES P450 3A4 AND 2C9 AND INHIBITS MAJOR HEPATIC GLUCURONOSYLTRANSFERASES

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ABSTRACT:

Silybin, a major constituent of the milk thistle, is used to treat several liver disorders. Silybin inactivated purified, recombinant cytochromes P450 (P450) 3A4 and 2C9 in a mechanism-based manner. The inactivations were time-, concentration-, and NADPH-dependent. The inactivation of the 7-benzyloxy-4-(trifluoromethyl-)coumarin O-debenzylation activity (P450 3A4) was characterized by a $K_i$ of 32 $\mu$M, a $k_{inact}$ of 0.06 min$^{-1}$, and a $t_{1/2}$ of 14 min. Testosterone metabolism to 6-$\beta$-hydroxytestosterone (P450 3A4) was also inactivated with a $K_i$ of 166 $\mu$M, a $k_{inact}$ of 0.08 min$^{-1}$, and a $t_{1/2}$ of 9 min. The 7-ethoxy-4-(trifluoromethyl)coumarin O-deethylation activity of purified human P450 2C9 was inactivated with a $K_i$ of 5 $\mu$M, a $k_{inact}$ of 0.14 min$^{-1}$, and a $t_{1/2}$ of 7 min. Parallel loss of heme was observed with both P450s. Activity of both P450 enzymes was not recovered after removal of silybin either by dialysis or by spin gel filtration. In addition, silybin inhibited the glucuronidation of 7-hydroxy-4-trifluoromethylcoumarin catalyzed by recombinant hepatic UDP-glucuronosyltransferases (UGTs) 1A1, 1A6, 1A9, 2B7, and 2B15, with $IC_{50}$ values of 1.4 $\mu$M, 28 $\mu$M, 20 $\mu$M, 92 $\mu$M, and 75 $\mu$M, respectively. Silybin was a potent inhibitor of UGT1A1 and was 14- and 20-fold more selective for UGT1A1 than for UGT1A9 and UGT1A6, respectively. Thus, careful administration of silybin with drugs primarily cleared by P450s 3A4 or 2C9 is advised, since drug-drug interactions cannot be excluded. The clinical significance of in vitro UGT1A1 inhibition is unknown.

Silybum marianum, a biennial herb commonly known as milk thistle, has been used for decades as a herbal remedy and also in extracted formulation as a prescription drug (Sweetman, 2002) for the treatment of a variety of liver disorders (Flora et al., 1998). The principal active components of milk thistle extract are the flavonolignans, collectively termed silymarin, present as three isomers, namely silybin (INN silibinin), silychristin, and silydianin. Silybin (Fig. 1) is present as two diastereoisomers and represents about 50 to 70% of the silymarin extract (USP, 2002).

Evaluation of the clinical efficacy of silymarin is complicated by the scale of clinical trials, heterogeneity of diagnoses, lack of standardized preparations, and inconsistent dosing and outcome parameters, and should be done with care. Nevertheless, silymarin (Legalon) is administered for the treatment of alcoholic liver disease (Salmi and Sarna, 1982) and chronic hepatitis or cirrhosis (Ferenci et al., 1989). Silymarin has further been evaluated most extensively for the management of acute Amanita phalloides mushroom poisoning, with reported success in animals (Vogel et al., 1984) and humans (Hruby et al., 1983). Both silymarin and silybin have been reported to exert hepatoprotective effects in animal models against various chemicals such as ethanol (Valenzuela et al., 1989), CCl$_4$ (Mourelle et al., 1989), and acetaminophen (Muriel et al., 1992). The proposed mechanisms of action relate to its functions as an antioxidant (Valenzuela et al., 1986), and as an inhibitor of lipid peroxidation and free radical scavenger (Carini et al., 1992). Silymarin also increases hepatocyte protein synthesis by stimulating RNA polymerase (Sonnenbichler et al., 1986), inhibits nitric oxide production (Dehmlow et al., 1996), and exhibits cancer-chemopreventative properties (Singh and Agarwal, 2002).

Although the administration of silymarin is considered safe with minimal adverse events, the competitive interaction of silybin with cytochromes P450 (P450s$^1$) and the potential for drug interactions were recently reported (Beckmann-Knopp et al., 2000). The importance of phase I biotransformation, mainly facilitated by cytochromes P450, in the oxidative metabolism and eventual detoxification of drugs and xenobiotics is well recognized (Guengerich, 1991). Importantly, glucuronidation facilitated by UDP-glucuronosyltransferases (UGTs) is increasingly recognized as a major phase II detoxification pathway in humans (Fisher et al., 2001), and silybin may exert effects on both phase I and phase II metabolic reactions. For instance, silybin has been shown to noncompetitively inhibit denitronifedipine oxidation, mediated by P450 3A4 ($K_i = 11 \mu$M), in human liver microsomes (Beckmann-Knopp et al., 2000). Competitive inhibition was reported for S(–)-warfarin 7-hydroxylation (P450 2C9; $K_i = 19 \mu$M).

$^1$ Abbreviations used are: P450, cytochrome P450; GSH, glutathione; BSA, bovine serum albumin; BFC, 7-benzoyloxy-4-(trifluoromethyl)coumarin; 7EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; reductase, NADPH-cytochrome P450 reductase; UDPGA, uridine diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; SN-38, 7-ethyl-10-hydroxycamptothecin.
Dextromethorphan (P450 2D6) was characterized as a competitive inhibitor, with high inhibition potency observed for a low binding site (K_i) and vice versa (Beckmann-Knopp et al., 2000). Negligible effects on the metabolism of erythromycin (P450 3A4), chloroxazone (P450 2E1), S(-)-mephenytoin (P450 2C19), caffeine (P450 1A2), and coumarin (P450 2A6) were observed. A previous report indicated no inhibition of P450 2E1 (Miguez et al., 1994). A recent report has, however, indicated that silybin is a potent inhibitor of β-naphthoflavone (P450 1A; IC_50 = 0.9 μM) metabolism in cultured hepatoma cells (Gerhauser et al., 2003). Treatment of human hepatocytes with 0.25 mM silymarin inhibited the P450 3A4 activity as measured by the formation of testosterone 6β-hydroxylation (Venkataramanan et al., 2000). In addition, acute and chronic treatment of hepatocytes with silymarin significantly inhibited glucuronidation of 4-methylumbelliferyl (UGT1A6/9) (Venkataramanan et al., 2000). One study in humans demonstrates that silymarin administration decreased the excretion of bilirubin glucuronide in patients with liver cirrhosis due to alcohol abuse (Salmi and Sarna, 1982), although clinical significance was not clearly indicated and other reports on clinical hyperbilirubinemia or interactions between silybin and drugs are lacking.

It is also of interest to determine whether the inhibition of the major hepatic P450s also involves irreversible enzyme inactivation and whether there is evidence for the in vitro inhibition of major hepatic UGTs. The aim of this report was to further investigate the effects of silybin on key human phase I and phase II metabolic enzymes. Silybin was evaluated for its ability to inactivate human P450s 3A4 and 2C9 using purified recombinant enzymes in a reconstituted system. The inhibition of relevant human hepatic UGTs was also investigated in recombinant cDNA-expressed enzyme preparations.

Materials and Methods

Chemicals and Reagents. Silybin (CAS 22888-70-6), α,α-dilaurylphosphatidylcholine, α,α-dioleoyl-sn-glycero-3-phosphocholine, phosphatidylserine, testosterone, 7-hydroxy-4-(trifluoromethyl)coumarin glucuronide, bovine serum albumin (BSA), glutathione (GSH), NADPH, Triton, catalase, sodium dithionite, Hepes, uridine diphosphoglucuronic acid (UDPGA), and alamethicin were purchased from Sigma-Aldrich (St Louis, MO). 7-Benzylxoy-4-(trifluoromethyl)coumarin (BFC) was obtained from BD Gentest (Woburn, MA). 7-Ethoxy-4-(trifluoromethyl)coumarin (7EFC) was obtained from Molecular Probes (Eugene, OR). 7-Hydroxy-4-(trifluoromethyl)coumarin (HFC) was purchased from FluorChem USA (San Leandro, CA). Recombinant UGTs 1A1, 1A6, 1A9, 2B7, and 2B15 were purchased from BD Gentest. All recombinant UGTs were expressed using baculovirus-infected Sf-9 insect cells. HPLC-grade methanol and acetonitrile were purchased from Mallinckrodt (St. Louis, MO). Slide-A-Lyzer cassettes were obtained from Pierce Chemical (Rockford, IL).

Purification of Enzymes. P450 3A4 was expressed in Escherichia coli MV1304 cells and purified to homogeneity by the method of Gillam et al. (1993). P450 2C9 was expressed in E. coli DH5α cells and purified as previously described (Sandhu et al., 1993). P450 NADPH-reductase was expressed in E. coli TopP3 cells and purified according to a previously published procedure (Hanna et al., 1998). P450s 2B6, 2D6, and 2E1 were expressed and purified according to previously published protocols (Hanna et al., 2000).

P450 3A4/2C9 Inactivation Assays. Purified P450s 3A4/2C9 were preincubated with or without silybin in a reconstituted system in the presence of NADPH to determine the BFC O-debenzylation/7EFC O-deethylation activities, respectively. In the case of P450 3A4, 0.5 nmol of the purified enzyme was reconstituted with 1.5 nmol of reductase and lipid mixture (α,α-dilaurylphosphatidylcholine, α,α-dioleoyl-sn-glycero-3-phosphocholine, and phosphatidylserine in a ratio of 1:1:1). The reconstitution mixture was allowed to sit at room temperature for 15 min and brought up to a volume of 0.8 ml by adding 50 mM Hepes buffer (pH 7.5), catalase (500 U), GSH (2 mM), and MgCl₂ (30 mM). With P450 2C9, 0.2 nmol of purified enzyme was reconstituted with 0.4 nmol of reductase and 20 μg of α,α-dilaurylphosphatidylcholine at 4°C for 45 min. The reaction mixture was brought up to a volume of 400 μl with potassium phosphate buffer (pH 7.4) containing catalase (100 U/ml). The samples were equilibrated at 37°C for 15 min and the reactions were initiated by adding NADPH to a final concentration of 1.2 mM. Aliquots (9 pmol of each enzyme) were transferred at the indicated times to a secondary reaction mixture (P450 3A4 secondary buffer contained 1 mM NADPH, 50 μM BFC, 3.3 mM MgCl₂, 40 μg/ml BSA in 200 mM potassium phosphate buffer, pH 7.4, and P450 2C9 secondary buffer contained 1 mM NADPH, 100 μM 7EFC, 40 μg/ml BSA in 50 mM MgCl₂, pH 7.4). P450 3A4 assays were incubated for 15 min and P450 2C9 assays were incubated for 10 min; both reactions were terminated with stop solution containing 20% 0.5 M Tris/80% acetonitrile. The BFC O-debenzylation/7EFC O-deethylation activities were then measured at room temperature on an SLM-Aminco model SPF-5000 spectrophotometer with an excitation at 410 nm and emission at 510 nm.

Testosterone Metabolism by Inactivated P450 3A4. The testosterone hydroxylase assay was used as an alternative substrate (in addition to BFC) for purified recombinant P450 3A4 activity. P450 3A4 (1.5 pmol) was reconstituted with NADPH-reductase (3 nmol), cholate (200 μg), and lipid mix (20 μg) as described above. The reconstitution mixture was preincubated at room temperature for 15 min and then diluted to a final volume of 2.5 ml with 50 mM Hepes buffer (pH 7.5), containing catalase (500 U), GSH (2 mM) and MgCl₂ (30 mM), NADP (1 mM), and glucose 6-phosphate (5 mM). Aliquots of the mixture then received increasing amounts of silybin (25–250 μM) in DMSO or DMSO alone (in control sample). The reactions were initiated by the addition of glucose-6-phosphate dehydrogenase (0.5 unit/ml). Aliquots (100 pmol of P450 3A4) of the reaction mixture were transferred into the secondary reaction mixture containing 50 mM Hepes buffer (pH 7.4), 20 mM testosteronone, and 1 mM NADPH. The secondary reaction was allowed to proceed for 15 min, stopped with 1 ml of ethyl acetate, and vortexed. The organic phase was removed after centrifugation and the sample was re-extracted with an additional 1 ml of ethyl acetate. The organic phases were combined and evaporated under nitrogen. The dried extract was resuspended in 100 μl of 65% methanol, and 90 μl were injected onto a C18 reverse-phase column (Microsorb 100 Å, 4.9 × 250 mm; Varian, Inc., Walnut Creek, CA) eluted with 65% methanol. Metabolites were eluted with 65% methanol at a flow rate of 1 ml/min and detected by their absorbance at 254 nm using a Waters 600E-HPLC coupled to a Photodiode Array Detector 996 controlled by Millennium software (Waters, Milford, MA).

P450 3A4/2C9 Reduced CO Spectra. Reconstitution of the enzymes with or without the inhibitor was carried out as above. Silybin and NADPH were absent in control reactions, whereas exposed samples contained silybin but no NADPH, and inactive samples contained both NADPH and the silybin. At 0 and 20 min, aliquots of the samples were removed and tested for enzymatic activity as described above. At the same time points, 100-μl samples containing 100 pmol of enzyme were removed and added to a quench buffer consisting of 50 mM potassium phosphate (pH 7.7), 40% glycerol, and 0.6% Tergitol NP-40. These samples were bubbled with CO for 60 s, and the sodium dithionite-reduced CO spectra were recorded between 400 nm and 500 nm on a DW2 UV-visible spectrophotometer equipped with an OLIS operating system (On Line Instruments Systems, Bogart, GA).

Pyridine Hemochromogen Assay. The total heme content of the P450 3A4 samples was also determined by the pyridine hemochromogen method (Koop, 1990).

HPLC Analysis for P450 Heme. For HPLC analysis, aliquots containing 100 pmol of P450 3A4 or P450 2C9 derived from control, exposed, or

Fig. 1. Structure of silybin.
inactivated samples were injected onto a C4 column (4.9 × 250 mm; Vydoc, Hesperia, CA) equilibrated with 30% acetonitrile and 0.1% trifluoroacetic acid. The sample components were resolved by increasing the percentage of acetonitrile to 90% over 30 min at a flow rate of 1 ml/min. The individual components were analyzed using a diode array detector, and the areas under the heme peaks, monitored at 405 nm, were integrated and compared individually.

Irreversibility of P450 Inactivation. P450 3A4 or 2C9 was reconstituted and incubated with silybin in the absence or presence of NADPH as described above. Aliquots of the reaction mixtures were removed and assayed immediately for residual activity. The remaining reaction mixtures were dialyzed separately at 4°C for 24 h against 2 × 500 ml of 30 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.7% cholate for P450 3A4. The dialyzed samples were then reconstituted with 20 μl of lipid for 30 min on ice.

Enzymatic activity was assessed in the presence or absence of fresh reductase with BFC or 7EFC for P450 3A4 or 2C9, respectively.

Effect of Silybin on the Activities of Other P450s. The ability of silybin to inactivate purified reconstituted P450 2B6, 2E1, or 2D6 was determined by measuring 7EFC O-dealkylation activity as previously described (Yanev et al., 1999).

HFC Glucuronidation Assay. Unless otherwise stated, 0.05 mg/ml recombiant UGTs were mixed with assay buffer containing 50 mM phosphate buffer (pH 7.1), 1 mM MgCl2, and alamethacin (50 μM) and placed on ice for 15 min. For determination of kinetic parameters, increasing concentrations of HFC in 0.5 μl of DMSO were added to a final incubation volume of 100 μl and preincubated at 37°C for 3 min. Reactions were initiated by the addition of UDPGA (final concentration of 5 mM). Blank incubations were performed without UDPGA. After 30 min, the reactions were stopped with 20 μl of 1.5 N perchloric acid. To determine inhibition of HFC glucuronidation, 20% EGFU H, 1A6, 1A9, 2B7, or 2B15 (0.1 mg/ml) was incubated with substrate at its respective apparent Km concentration, together with increasing concentrations of silybin (in 0.5 μl of DMSO) as described above. Initial experiments indicated that production form was linear with protein concentration up to 0.2 mg/ml for 45 min at 37°C. Protein was precipitated on ice for 30 min following centrifugation after stopping the reaction with 20 μl of 1.5 N perchloric acid. An aliquot of the supernatant (80 μl) was injected onto a C18 HPLC column (Luna 3 μm, 4.6 × 100 mm; Phenomenex, Torrance, CA) for determination of HFC glucuronide. The HPLC system consisted of a Waters 486 ultraviolet detector, Waters 501 HPLC pumps, Waters 600E controller, and Waters 717 temperature-regulated autosampler. The mobile phase was water/0.05% trifluoroacetic acid (A) and acetonitrile/0.05% trifluoroacetic acid (B) at a flow rate of 1 ml/min. Initial conditions were 20% B followed by a linear increase to 80% B in 11 min, followed by 80% B for 1 min before returning to initial conditions. HFC glucuronide was monitored at 325 nm and quantified based on a standard curve generated between 5 and 5000 pmol using authentic HFC.

Enzyme Kinetic Analysis. Rate constants for the inactivation of the P450 enzymes were calculated from the initial slopes of the linear regression lines of the semilogarithmic plots of the residual enzyme activity versus the preincubation time. The inverse of the rates of inactivation, 1/Vmax, was plotted against the reciprocal of the silybin concentration (1/Km) for the inactivation, and the rate constants at saturating concentrations of the inactivator (kmax) were determined from the intercepts on the abscissa and the ordinate, respectively. In the case of UGT enzyme reactions, substrate concentration (S) and velocity (V) data were fitted to the appropriate enzyme kinetic model by nonlinear least-squares regression analysis (Sigma Plot; SPSS Inc., Chicago, IL) (Bjornsson et al., 2003) to derive the enzyme kinetic parameters Vmax (maximal velocity) and Km (substrate concentration at half-maximal velocity).

Both the Michaelis-Menten model (eq. 1) and substrate activation model (eq. 2), which incorporates the exponent (n), were used:

\[ V = S \times \frac{V_{\text{max}}}{S + K_m} \]  
\[ V = V_{\text{max}} \left( \frac{S^n}{S^n + K_m^n} \right) \]

The best fit was based on a number of criteria, including visual inspection of the data plots (Michaelis-Menten and Eadie-Hofstee), distribution of the residuals, size of the sum of the squared residuals, and the standard error of the estimates. The IC50 estimate for silybin inhibition of HFC glucuronidation was determined by nonlinear curve fitting with GraphPad Prism (GraphPad Software Inc., San Diego, CA) and was defined as the concentration of inhibitor required to inhibit control glucuronidation reactions by 50%.

Results

Time- and Concentration-Dependent Inactivation of the O-Dibenzylation Activity of P450 3A4 by Silybin. In the presence of silybin and NADPH, a time- and concentration-dependent inactivation of the BFC O-debenzylation activity of P450 3A4 was observed (Fig. 2). Pseudofirst-order inactivation kinetics were observed for concentrations of silybin between 25 and 250 μM. The kinetic constants describing the inactivation of P450 3A4 by silybin were determined from the inset in Fig. 2. The concentration required for half-maximal inactivation (K1/2) at 37°C was found to be 32 μM. The kinact, the maximal rate of inactivation at a saturating concentration of silybin, was 0.06 min−1 and the t1/2 for inactivation was 14 min.

Time- and Concentration-Dependent Inactivation of Testosterone Metabolism of P450 3A4 by Silybin. β-hydroxytestosterone was the major metabolite detected when testosterone was incubated with purified reconstituted P450 3A4 in a reconstituted system in the presence of NADPH. Silybin inhibited the metabolism of testosterone by P450 3A4 in a time-, concentration-, and NADPH-dependent manner (Fig. 3). The inactivation exhibited pseudofirst-order kinetics with a K1/2 of 132 μM, a kmax of 0.08 min−1, and a t1/2 of 9 min.

Effect of Silybin Inactivation on the P450 3A4 Heme. Comparable losses in the enzymatic activity (62%) and the reduced CO spectrum (54%) were observed in P450 3A4 samples incubated in the presence of silybin and NADPH (Table 1). In control samples incubated with silybin but without NADPH, the activity loss was approximately 29% with no loss in the reduced CO spectrum, whereas control samples that received neither NADPH nor silybin exhibited no loss in the enzymatic activity or reduced CO spectrum. This suggests that the carryover of silybin into the secondary reaction mixture may have resulted in some inhibition of the P450 in the BFC reaction mixture. The concentration of silybin in the secondary reaction mixture was 72 μM. Reverse-phase HPLC analysis of P450 3A4 revealed a loss of about 39% of the P450 3A4 heme in the inactivated samples (Table 1). No significant decrease in the area under the heme peak at 405 nm was observed when control samples were incubated without silybin or with silybin in the absence of NADPH. Similarly, a 43% loss in the pyridine hemochrome spectrum was observed when P450 3A4 was incubated with silybin in the presence of NADPH when compared with control samples (Table 1).

Time- and Concentration-Dependent Inactivation of the 7EFC Activity of P450 2C9. As shown in Fig. 4, with P450 2C9 the silybin-mediated inactivation of the 7EFC O-deethylation activity was also time-, concentration-, and NADPH-dependent. Approximately 35% activity loss was seen in 15 min when 50 μM silybin was used. The inactivation exhibited pseudofirst-order kinetics. The double-reciprocal plot of the values of the initial rate constants versus the silybin concentrations gave a K1/2 of 5 μM, a kmax of 0.14 min−1, and a t1/2 of 7 min.

Effect of Silybin Inactivation on the P450 2C9 Heme. The decrease in the 7EFC O-deethylation activity of P450 2C9 in the reconstituted system (34%) incubated with 50 μM silybin and NADPH occurred with a concurrent loss of 34% in the reduced CO spectrum (Table 2). No significant loss of activity or in the reduced CO spectrum was observed in control samples without either silybin or NADPH. Samples incubated with silybin but without NADPH showed minimal losses in activity. However, some loss (12%) in the reduced CO spectrum was observed. The amount of heme loss as a
result of inactivation was determined by HPLC to be 23% (Table 2). Consistent with the decrease in the CO spectrum and the HPLC-detectable heme, a 20% loss in the pyridine hemochrome spectrum was seen in samples inactivated with silybin as compared with the control samples incubated with silybin in the absence of NADPH.

Irreversibility of the Inactivation of P450 3A4 and 2C9 by Silybin. Table 3 shows the activities of noninactivated and silybin-inactivated P450 3A4 or 2C9 samples following dialysis at 4°C to remove free or reversibly bound silybin. Extensive dialysis failed to restore the activity of the silybin-inactivated samples to levels seen in the control samples. Addition of fresh reductase back to the inactivated samples also did not result in a major recovery in the P450 3A4 and 2C9 activities. Since silybin is a rather hydrophobic molecule, dialysis may not have completely removed all free or loosely bound inactivator. Therefore, spin column gel filtration was used as well. With this method, the activity of the inactivated samples also could not be regained after passing the samples through a G-50 gel filtration spin column (data not shown). These results suggest that the inactivation of P450s 3A4 and 2C9 is primarily due to covalent modification of the P450s and that it is irreversible under these conditions and is not due to modification of the NADPH-reductase. Addition of glutathione (2.5 mM) during the inactivation reaction did not prevent or decrease the loss in activity, suggesting that the silybin reactive intermediate did not leave the active site to react with external residues on the P450s or with reductase (data not shown).

Effect of Silybin on the Enzymatic Activities of P450s 2B6, 2D6, and 2E1. The effect of silybin on the activities of several other P450 isoforms was also examined. Incubations with silybin at concentrations up to 100 μM in the presence of NADPH indicated no metabolism-dependent inactivation of P450 2B6, 2D6, or 2E1 (data not shown).

Inhibition of UGT Activities. The kinetic parameters for HFC glucuronidation catalyzed by the major human hepatic UGTs are listed in Table 4. The kinetics of UGT1A6-catalyzed and UGT2B7-catalyzed HFC glucuronidation were sufficiently described by the Michaelis-Menten equation. HFC glucuronidation catalyzed by UGTs 1A1, 1A9, and 2B15 exhibited autoactivation kinetics and were better described with the Hill equation incorporating the Hill coefficient (n), ranging between 1.2 and 1.9, which gives an indication of the degree of sigmoidicity of the curve. Interestingly, silybin inhibited all of the major hepatic UGTs examined, with IC50 values below 100 μM. Silybin was more selective at inhibiting UGT1A enzymes compared with UGT2B isozymes and was shown to be a very potent inhibitor of UGT1A1 with an IC50 of approximately 1 μM. Silybin was approximately 14- and 20-fold more selective at inhibiting UGT1A1, compared with UGTs 1A9 and 1A6, respectively.
Discussion

Flavonoids are present ubiquitously in plants and are consumed when vegetables, fruits, and beverages such as tea and red wine are ingested (Hertog et al., 1993). Flavonoids have been shown to play a prominent role in cancer prevention (Bravo, 1998) and to reduce the risk of cardiovascular diseases (Dwyer, 1995). Inhibition of P450 3A4 by flavonoids has been shown (Fuhr, 1998). In addition, in vitro studies have focused on the effect that flavonoids with hydroxyl groups have on the activities of P450 enzymes. Flavonoids with hydroxyl groups appear to inhibit P450-dependent reactions, whereas flavonoids without hydroxyl groups seem to stimulate P450 activity (Siess et al., 1995). Silybin, a major flavonoid constituent of milk thistle extract, has been shown to have hepatoprotective properties as well as anticarcinogenic and antiproliferative effects in several in vitro and animal models (Valenzuela et al., 1985; Ferenci et al., 1989; Mourelle et al., 1989; Muriel et al., 1992; Bravo, 1998; Flora et al., 1998). Silybin contains five hydroxyl groups, and this may be related to its ability to cause inhibition of some P450 isoforms.

The current study demonstrates that silybin is a mechanism-based inactivator for P450s 3A4 and 2C9 according to the following observations: 1) inactivation reactions were time-, concentration-, and NADPH-dependent; 2) loss in activity was pseudofirst-order; and 3) loss in enzyme activity was irreversible after extensive dialysis. The percentage loss in enzyme activity of P450 3A4 in a reconstituted system when incubated with silybin in the presence of NADPH resulted in a similar percentage loss in the P450 reduced CO spectrum. With P450 2C9, the extent of activity loss was also accompanied by a concurrent loss in the reduced CO spectrum. These observations suggested that the metabolism of silybin resulted in the formation of...
reactive intermediate that alkylated the heme moiety. Additional evidence supporting this notion came from HPLC analysis of samples incubated with silybin in the presence or absence of NADPH. The percentage loss in the area under the heme peak observed by HPLC at 405 nm correlated with the percentage loss in the reduced CO spectrum, suggesting that the metabolism of silybin resulted in the formation of a reactive intermediate that bound to the 3A4 heme moiety. Further evidence for a loss in heme was observed using the pyridine hemochrome method, where the percentage loss in the pyridine hemochrome again correlated with the heme loss seen with the HPLC assay or the reduced CO spectrum. It was, therefore, not surprising to find no recovery in the enzymatic activity when silybin-inactivated P450 3A4 or 2C9 samples were extensively dialyzed. Addition of fresh

![Graph showing time- and concentration-dependent decreases in the 7EFC O-deethylation activity of P450 2C9 in a reconstituted system.](image)

**TABLE 2**  
Effect of silybin on the P450 2C9 7EFC O-deethylation activity, reduced CO spectrum, and percentage of heme remaining by HPLC and pyridine hemochrome

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Activity Remaining</th>
<th>P450 Remaining*</th>
<th>Heme Remaining (HPLC)*</th>
<th>Heme Remaining (Pyridine Hemochrome)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Silybin, − NADPH</td>
<td>94 ± 5</td>
<td>88 ± 2</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>+ Silybin, + NADPH</td>
<td>66 ± 8</td>
<td>66 ± 3</td>
<td>77 ± 2</td>
<td>80 ± 3</td>
</tr>
</tbody>
</table>

N.D., not determined.

*P450 remaining was determined from the reduced CO binding spectrum. The control sample value was set to 100%. The 100% values for activity, P450, and heme by HPLC and pyridine hemochrome were 1.7 ± 0.7 nmol/nmol/min, 94 ± 4 pmol, 100 pmol, and 190 ± 22 pmol, respectively.

The amount of heme remaining was calculated after integrating the area under the heme peak at 405 nm from the HPLC elution profile. The area obtained for the control sample was set to 100%.

The pyridine hemochrome spectrum was obtained as described by Koop (1990).

**TABLE 3**  
Irreversibility of P450 3A4 and 2C9 inactivation by silybin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity Remaining (Percentage of Control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4 before dialysis</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>3A4 after dialysis</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>3A4 after dialysis + reductase</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>2C9 before dialysis</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>2C9 after dialysis</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>2C9 after dialysis + reductase</td>
<td>56 ± 1</td>
</tr>
</tbody>
</table>

* Each data point shown represents the average and standard deviation expressed as a percentage of enzyme incubated in the absence of NADPH from three separate experiments. The 100% values for the activity of P450 3A4 were 92 ± 2.4 and 80 ± 4.3 pmol before and after dialysis, respectively. For P450 2C9 the 100% values were 88 ± 0.3 and 75 ± 3 pmol before and after dialysis, respectively.
reduced to the dialyzed control and inactivated samples also did not restore enzymatic activity.

Silybin also caused a time-, concentration-, and NADPH-dependent loss in P450 3A4 activity, as seen with testosterone as a substrate. However, the $K_i$ for the inactivation of testosterone metabolism was over 4-fold higher than that observed using BFC as substrate. The P450 3A4 active site is thought to be large, and there is evidence for multiple substrate binding sites within a single active site (Schrag and Wienkers, 2001). Different substrates apparently can coexist in the active site of P450 3A4, and BFC and testosterone are thought to bind to different active site domains (Lu et al., 2001). Some modulators may increase or decrease the activity of CYP3A4 for one substrate without affecting the activity for another one. Whether this effect is brought about by competition at the site or inhibition for substrate binding and for metabolism at a particular binding pocket in the active site or due to a conformational change in the active site remains to be elucidated experimentally.

The lack of inhibitory effect of silybin on P450 2E1 confirms results of previous studies with chlorozoxazine as the substrate (Miguez et al., 1994; Beckmann-Knopp et al., 2000). Similarly, no metabolism-dependent increase or decrease in the enzymatic activities of P450s 2B6 and 2D6 was seen when these enzymes were incubated with silybin in a reconstituted system (data not shown). A report by Beckmann-Knopp et al. (2000) showed that silybin inhibits seven important human hepatic P450s, of which P450s 3A4 and 2C9 are inhibited more potently. The mechanism for P450 3A4 inhibition was described as mixed inhibition with competitive ($K_i = 105 \mu M$) and noncompetitive ($K_i = 11 \mu M$) inhibition constants, whereas P450 2C9 was inhibited in a competitive manner ($K_i = 19 \mu M$) (Beckmann-Knopp et al., 2000). The authors concluded that, although these inhibition constants were at least 20-fold above silybin plasma concentrations, drug interactions with substrates for these enzymes could not be excluded, mainly because the concentration of chemicals is difficult to estimate and biliary concentrations are often an order of magnitude above the in vitro inhibition constants. The results of the present study imply that this inhibition might be more physiologically relevant than previously thought. Several mechanism-based inactivators of P450 enzymes have been shown to result in clinically relevant drug-drug interactions, such as mibebradil, clarithromycin, and erythromycin, thus resulting in withdrawal of the drugs from the market (Bjornsson et al., 2003). We have shown that silybin is a mechanism-based inactivator of both P450 3A4 and 2C9 and that this interaction could lead to irreversible enzyme degradation. The inhibition of P450 3A4 by silybin involves several mechanisms. In addition to the mechanism-based inactivation of P450 3A4 shown in this study, our results also indicate a competitive inhibition as seen in the loss in enzyme activity at time 0 (Fig. 2). Beckmann-Knopp et al., (2000) described the inhibition of P450 3A4 as both competitive and noncompetitive using demitrofenidipine as substrate. The contribution of a mechanism-based component with higher affinity ($K_i = 32 \mu M$; 7EF C) than that in published competitive inhibition provides an explanation for the mixed inhibition previously reported. We also reported the metabolism-dependent inactivation of P450 2C9 ($K_i = 5 \mu M$) was 4-fold more potent than the reported competitive inhibition (Beckmann-Knopp et al., 2000), again indicating that although inhibition may appear to be competitive, this mechanism also involves destruction of enzyme. This finding is particularly important because mechanism-based enzyme inactivation is likely to increase the potential for drug-drug interactions, inasmuch as estimates of competitive inhibition may underestimate the true interaction that could occur in vivo in the presence of a time-dependent inhibitor (Bjornsson et al., 2003).

In addition to the metabolism-dependent inactivation of P450s 3A4 and 2C9, we have demonstrated the ability of silybin to inhibit the glucuronidation of HFC mediated by a number of important hepatic UGTs. Interestingly, silybin is a very potent and selective inhibitor of UGT1A1 (IC$_{50}$ = 1.4 $\mu M$). Although UGT1A6 and UGT1A9 were also inhibited, silybin was 14- to 20-fold more selective for UGT1A1. The in vivo significance of this phenomenon could be important but is presently unknown, since there are no published reports indicating a clinical interaction with bilirubin or glucuronidation of UGT1A1 substrates. UGT1A1 is the sole enzyme responsible for the glucuronidation of bilirubin and contributes to the glucuronidation of several drugs including estradiol, irinotecan, buprenorphine, and naltrexone (Liston et al., 2001). The mean peak plasma concentration of silybin after oral administration of a 700-mg dose of silymarin (containing approximately 254 mg of silybin) is 0.6 $\mu M$. Silybin undergoes primarily biliary excretion, and concentrations in the bile approximating 150 $\mu M$ have been reported (Weyhenmeyer et al., 1992). It is therefore possible that the reduced excretion of bilirubin glucuronide previously observed in alcoholics with liver cirrhosis (Salmi and Sarna, 1982) could be related to inhibition of hepatic UGT1A1 by silybin. It was recently reported that the glucuronidation of SN-38, the active metabolite of the anticancer drug irinotecan, is mainly mediated by UGT1A1 and that patients with Gilbert’s disease who have a common genetic polymorphism for UGT1A1 are more likely to present with adverse effects of irinotecan (Iyer et al., 1999). The IC$_{50}$ of silybin for UGT1A1 approximates plasma levels, and because biliary levels are much higher, an interaction of silybin with bilirubin or UGT1A1 substrates might be possible. The finding that silybin is also a modestly potent inhibitor of UGT1A6 and 1A9 is in agreement with previous findings in hepatocytes (Venkataramanan et al., 2000), and interactions with compounds that are glucuronidated by these isozymes are not excluded, although based on in vitro findings, an interaction with UGT1A1 substrates would be more likely, if at all.

In conclusion, our results demonstrate that silybin inhibits both phase I and phase II enzymes. This is particularly important because milk thistle is widely available as a herbal supplement or prescription drug and the principal agent in the milk thistle extract inactivates P450s 3A4 and 2C9. Silybin, furthermore, is a potent in vitro inhibitor of UGT1A1. It is therefore advised that care should be taken when milk thistle is used. Physicians should take special note of all coadministered supplements/medications to appropriately monitor therapeutic drug plasma levels or adverse events when prescribing agents primarily cleared by P450s 3A4 and 2C9, or UGT1A1, especially in the absence of clinically relevant information. Carefully controlled clinical studies are needed to define the clinical relevance, if any, of this finding.
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