

The Effects of Milk Thistle (*Silybum marianum*) on Human Cytochrome P450 Activity

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ABSTRACT

Milk thistle (*Silybum marianum*) extracts are widely used as a complementary and alternative treatment of various hepatic conditions and a host of other diseases/disorders. The active constituents of milk thistle supplements are believed to be the flavonolignans contained within the extracts. In vitro studies have suggested that some milk thistle components may significantly inhibit specific cytochrome P450 (P450) enzymes. However, determining the potential for clinically significant drug interactions with milk thistle products has been complicated by inconsistencies between in vitro and in vivo study results. The aim of the present study was to determine the effect of a standardized milk thistle supplement on major P450 drug-metabolizing enzymes after a 14-day exposure period. CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 activities

were measured by simultaneously administering the four probe drugs, caffeine, tolbutamide, dextromethorphan, and midazolam, to nine healthy volunteers before and after exposure to a standardized milk thistle extract given thrice daily for 14 days. The three most abundant flavonolignans found in plasma, following exposure to milk thistle extracts, were silybin A, silybin B, and isosilybin B. The concentrations of these three major constituents were individually measured in study subjects as potential perpetrators. The peak concentrations and areas under the time-concentration curves of the four probe drugs were determined with the milk thistle administration. Exposure to milk thistle extract produced no significant influence on CYP1A2, CYP2C9, CYP2D6, or CYP3A4/5 activities.

Introduction

Milk thistle (*Silybum marianum*) extracts are one of the most commonly used botanical supplements in the world today. In 2012, US botanical supplement sales were an estimated \$5.6 billion USD, among which milk thistle extracts ranked sixth in total sales (Lindstrom et al., 2013). The crude extract obtained from milk thistle seeds, termed silymarin, contains a complex mixture of the seven flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B, and silydianin) and one flavonoid, which together account for 65–80% of the total extract composition (Kroll et al., 2007). Typically, following the oral administration of a milk thistle extract, the concentrations of silybin A, silybin B, and isosilybin B are found in much higher concentrations in the systemic circulation relative to isosilybin A, silychristin A and B, and silydianin (Brinda et al., 2012). Silymarin is to be distinguished from silibinin,

which specifically refers to a semipurified extract representing an approximately 1:1 mixture of silybin A and silybin B (Kroll et al., 2007). Milk thistle extracts are purported to be useful in the treatment of liver and gallbladder ailments, including alcoholic liver disease, acute and chronic viral hepatitis, and toxin-induced liver diseases (Choi et al., 2011; National Toxicology Program, 2011; Shi and Klotz, 2012). In the United States and Europe, up to 65% of patients with liver disease may use botanical preparations (Loguercio and Festi, 2011). Beyond reported hepatoprotectant effects, milk thistle extracts have also been shown to produce generalized antioxidant effects and potential antitumor, anti-inflammatory, antifibrotic, and antihyperglycemic actions (Loguercio and Festi, 2011; Shi and Klotz, 2012). As a consequence of the widespread use of milk thistle extracts to treat an array of conditions, the potential to be combined with conventional medications and possibility for drug-drug interactions exists.

Cytochrome P450s (P450s) are prominent phase I enzymes that catalyze the oxidative metabolism of a wide array of molecules, including drugs, chemical carcinogens, steroids, and fatty acids (Guengerich, 2001). CYP1A2, 2C9, 2D6, and 3A4/5 are the major isoenzymes present in the human liver and are believed to participate in the metabolism of over 70% of marketed drugs (Zanger and Schwab, 2013). Thus, any significant alteration in P450 activities can lead to altered metabolism and clearance of many of these drugs.

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ABBREVIATIONS: AUC, area under time-concentration curve; C_{max} , peak concentration; CAF, caffeine; CBC, complete blood count; CI, confidence interval; DEX, dextromethorphan; DOR, dextropropranolol; HCV, hepatitis C virus; HPLC, high performance liquid chromatography; HPLC-MS/MS, high performance liquid chromatography–tandem mass spectrometry; IS, internal standard; LLOQ, lower limit of quantification; MDZ, midazolam; NAFLD, nonalcoholic fatty liver disease; OHMDZ, 1'-hydrozimidazolam; P450, cytochrome P450; TOL, tolbutamide.

Because milk thistle extracts are commonly used, concerns over potential drug interactions exist. Several *in vitro* studies have suggested that silymarin extracts and various individual constituents inhibit CYP2D6, CYP2E1, CYP3A4, CYP2C9, and CYP2C8 (Beckmann-Knopp et al., 2000; Venkataramanan et al., 2000; Zuber et al., 2002; Sridar et al., 2004). Concentration-dependent inhibition of CYP2D6, CYP2E1, and CYP3A4 by silymarin and silybin and mechanism-based inactivation of CYP3A4 and CYP2C9 by silybins and silymarin extracts were reported (Venkataramanan et al., 2000; Zuber et al., 2002; Sridar et al., 2004). Despite the apparent ability of milk thistle extracts to produce significant inhibition of one or more P450 enzymes, as reported in several published *in vitro* studies, *in vivo* human data have been unable to replicate *in vitro* predictions (Leber and Knauff, 1976; Piscitelli et al., 2002; DiCenzo et al., 2003; Gurley et al., 2004, 2006a,b, 2008; Mills et al., 2005; van Erp et al., 2005; Fuhr et al., 2007; Rao et al., 2007; Deng et al., 2008). In a recent report, an *in vitro* study utilizing human liver microsomes evaluated a number of individual flavonolignans as potential P450 inhibitors and identified silybin B as the most potent inhibitor of CYP2C9 with an IC_{50} value of 8.2 μ M, followed by silybin A at 18 μ M (Brantley et al., 2010). Isosilybin A and isosilybin B were noted to be much weaker CYP2C9 inhibitors with IC_{50} values of 74 μ M and >100 μ M, respectively.

Brantley et al. (2010) noted that there is precedent for some silymarin extracts formulated to provide significantly enhanced bioavailability (i.e., silibinin-phosphatidylcholine complex) to produce systemic concentrations of silibinin (i.e., combined silybin A and silybin B) between 5 and 75 μ M (Flaig et al., 2007; Brantley et al., 2010). However, a previous dose escalation study assessing the pharmacokinetics of the formulation used in the present study produced silybin B plasma concentrations over an order of magnitude less than even the lowest concentration reported in the study by Flaig et al. (2007) (Zhu et al., 2013).

The objective of the present study was to address these inconsistencies by simultaneously determining the effect of milk thistle exposure on the activities of the major drug-metabolizing enzymes, CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5, via administration of a validated probe drug combination.

Materials and Methods

Study Supplement

A standardized milk thistle extract (Legalon 140 capsules; MADAUS GmbH, Cologne, Germany) was used in this study. Each capsule contains 175 mg dried extract of milk thistle achenes, or 140 mg silymarin, a complex mixture of phytoconstituents including flavonolignans silybin A and silybin B, isosilybin A, isosilybin B, silychristin A, silychristin B, and silydianin (Kroll et al., 2007; Javed et al., 2011). We independently analyzed the capsules to confirm the contents of the biologically active constituents with a stereoselective high performance liquid chromatographic (HPLC)-tandem mass spectrometric (HPLC-MS/MS) assay established in our laboratory (Brinda et al., 2012) and applied to a normal volunteer pharmacokinetic study of the same extract (Zhu et al., 2013). The results indicated that each Legalon 140 capsule (product lot number B0601214) contained the following major active components: silybin A (21.2 mg), silybin B (29.5 mg), isosilybin A (11.4 mg), isosilybin B (8.2 mg), silychristin (31.5 mg), silydianin (36.4 mg), and taxifolin (5.9 mg).

Subjects

After the provision of written informed consent approved by the Medical University of South Carolina's Office of Research Integrity (Charleston, SC), 12 healthy volunteers participated in this fixed-order, open-label study. All study participants were determined to be healthy by medical history and physical examination performed by the study physician. Furthermore, a satisfactory evaluation of baseline serum chemistries, complete blood counts (CBC), 12-lead electrocardiogram, and urinalysis was used to establish health status. Additionally, a urine drug screen, nicotine/cotinine screen, and urine

pregnancy test (females) were obtained in each subject and preceded study participation. All participants were nonsmokers not taking prescription or over-the-counter medications or botanical or dietary supplements (inclusive of vitamins). The participants were requested to abstain from grapefruit juice, caffeine (CAF)-containing beverages, and ethanol 2 weeks prior to and during the study period. All participants were genotyped, so that CYP2D6 poor metabolizers were to be excluded from this study.

Study Design and Drug Administration

Phase I. After an overnight fast, participants were admitted to the Medical University of South Carolina General Clinical Research Center for a baseline assessment of P450 activity. In the morning, an indwelling venous catheter was placed in each subject's arm to facilitate serial blood sampling. At 8 AM, subjects were administered the previously validated probe drug combination, which consisted of single oral doses of the following: 10 mg midazolam (MDZ; Versed Syrup; Roche Laboratories, Nutley, NJ) for CYP3A4/5 assessment; 200 mg CAF (Vivarin; GlaxoSmithKline, Research Triangle Park, NC) for CYP1A2 assessment; 500 mg tolbutamide (TOL; Orinase; Pharmacia and Upjohn, Kalamazoo, MI) for CYP2C9 assessment; and 30 mg dextromethorphan (DEX; Robitussin Maximum Strength Syrup; Wyeth, Madison, NJ) for CYP2D6 assessment (Wang et al., 2001; Wohlfarth et al., 2012). Drug administration was followed by 240 ml tap water. To reduce any potential variability in drug absorption due to food, the subjects remained in a fasted state for 4 hours following probe drug administration. Standard meals were then served by the registered dietician at the General Clinical Research Center and did not include grapefruit-containing products or CAF. A total of 10 blood samples (10 ml each) was obtained over a 12-hour period. Time points of blood collection were immediately before drug administration (0 hour), and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, and 12 hours after which they were discharged from the unit following medical clearance. The subjects then returned to the General Clinical Research Center for collection of single blood samples through a separate venipuncture at time points of 24, 36, and 48 hours. All blood was collected in 10-ml heparinized blood collection tubes (Vacutainer; BD Biosciences, Franklin Lakes, NJ) and stored on ice until centrifugation, and plasma was stored at -70°C until analysis. Phase I served as the baseline measurement of CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 activity.

Phase II. Following a minimum 7-day washout period, subjects were provided a 14-day supply of the milk thistle extract with instructions to take one capsule three times daily at 8 AM, 1 PM, and 8 PM. Milk thistle capsules were dispensed in the original Legalon unit dose blister packs.

Phase III. After 14 days of thrice daily milk thistle extract, participants were readmitted to the General Clinical Research Center for a postexposure assessment. Following the placement of intravenous catheters, subjects were again dosed with the probe drug cocktail, as described in "Phase I," with identical blood collection times. In addition, all participants consumed an identical meal at the same times as in "Phase I." Finally, one milk thistle capsule was given concomitantly with the probe drugs and again at 1 PM and 8 PM prior to discharge from the unit. Additionally, participants continued the milk thistle extract three times daily for 2 additional days thereafter during the extended sampling period.

Follow-Up. Subjects returned 7 days following the final blood draw to have follow-up blood chemistries and CBC performed to provide an additional assurance of subject safety.

Chemicals and Reagents

Milk thistle capsules (Legalon: 175 mg dried extract equivalent to 140 mg silymarin) were donated by MADAUS GmbH. Analytical standards for CAF, DEX, and dextrophan (DOR) were obtained from Sigma-Aldrich (St. Louis, MO). TOL was purchased from Fluka Chemical (Milwaukee, WI). MDZ, MDZ- d_4 , 1'-hydroxymidazolam (OHMDZ), and OHMDZ- d_4 were purchased from Cerilliant (Round Rock, TX). CAF- d_9 , DEX- d_3 , and dextrophan- d_3 were obtained from Toronto Research Chemicals (Toronto, ON, Canada), and TOL- d_9 was from TLC PharmaChem (Concord, ON, Canada).

Analytical Methods

The plasma concentrations of all four probe drugs were measured using HPLC-MS/MS on a system consisting of a Surveyor HPLC autosampler, Surveyor MS quaternary pump, and TSQ Quantum Discovery triple-quadrupole

mass spectrometer (Thermo Scientific, San Jose, CA). In addition to the four probe drugs, the major metabolite of DEX, DOR, and the major metabolite of MDZ, OHMDZ, were analyzed to better characterize CYP2D6 and CYP3A4/5 activities, respectively.

MDZ and OHMDZ. Plasma MDZ and OHMDZ concentrations were quantified simultaneously after liquid-liquid extraction by electrospray-positive liquid chromatography tandem mass spectrometry, as described previously (Nolin et al., 2009). The lower limit of quantification (LLOQ) for MDZ and OHMDZ was 0.2 ng/ml, and the accuracy and precision were within 7%.

Caffeine. The plasma concentration of CAF was determined after liquid-liquid extraction by electrospray-positive liquid chromatography tandem mass spectrometry, as described previously (Karunathilake et al., 2012). The LLOQ was 50 ng/ml, and the accuracy and precision were within 15%.

Tolbutamide. The plasma concentration of TOL was determined by liquid chromatography tandem mass spectrometry. Briefly, plasma was processed by protein precipitation. In 2 ml centrifuge tubes, 50 μ l plasma, 80 μ l 1.5 μ g/ml internal standard (IS), and 200 μ l acetonitrile were combined and vortex-mixed. After the addition of 100 μ l water, the tubes were centrifuged at 20,817g for 10 minutes. The column used for the HPLC gradient separation was a Synergi 4 μ MAX-RP 80A (Torrence, CA; 75 \times 2 mm, 4 μ m). The mobile phases were 1 mM ammonium formate in water with 0.2% formic acid and 1 mM ammonium formate in acetonitrile with 0.2% formic acid. The gradient started at 40:60 (1 mM ammonium formate in water with 0.2% formic acid:1 mM ammonium formate in acetonitrile with 0.2% formic acid) and changed linearly to 5:95 at 0.5 minute, was held for 2.5 minutes, and then changed back to 40:60 at 3 minutes. The m/z transitions monitored in the positive ionization mode were 271 > 144 (collision energy: 23 V) for TOL and 280 > 155 (23 V) for the IS. The total run time for a sample was 5.5 minutes, and the retention time for both TOL and the IS was 2.95 minutes. The LLOQ was 0.25 μ g/ml, and the accuracy and precision were within 13%.

DEX and DOR. Plasma (300 μ l) was combined with glycine buffer (300 μ l) and deuterated internal standards (10 μ l) and vortex-mixed briefly before the addition of ethyl ether (1 ml). Tubes were capped and vortex-mixed vigorously for 2 minutes and centrifuged at 20,817g for 5 minutes. The upper ether layer was transferred to a clean glass culture tube and evaporated under a stream of nitrogen at 45°C. The residue was reconstituted with 200 μ l acetonitrile:water (1:1) containing 0.1% formic acid and vortex-mixed for 1 minute. Samples were transferred to autosampler vials, and 5 μ l was injected into the system. The autosampler temperature was maintained at 10°C. The column used for the HPLC separation was a Discovery HS F5 (50 \times 2.1 mm, 3 μ m; St. Louis, MO). The m/z transitions monitored in the positive ionization mode were 272 > 171 (collision energy: 37 V) for DEX, 275 > 174 (37 V) for the DEX IS, 258 > 157 (38 V) for DOR, and 261 > 175.0 (38 V) for the DOR IS. The LLOQ for both DEX and DOR was 0.05 ng/ml, and the accuracy and precision were within 13.4%.

Analysis of Silymarin Flavonolignans. The simultaneous analysis of the major free (i.e., nonconjugated) flavonolignans silybin A, silybin B, and isosilybin B was achieved utilizing a previously published and validated HPLC-MS/MS assay. It is described in detail elsewhere (Brinda et al., 2012). The lower limit of quantification for the assay was 2 ng/ml for each flavonolignan. Calibration curves were linear over the range of 2–100 ng/ml for all analytes. The intra- and interday accuracies were 91.0–106.5% and 95.1–111.9%, respectively. The intra- and interday precision was within 10.5%.

Genotyping. The carrier status of the deficient alleles for *CYP2D6* gene was identified by allele-specific polymerase chain reaction based on the previously reported method (Heim and Meyer, 1990). It involves amplification of segments of the locus for *CYP2D6* on chromosome 22 using primers that match the common mutant genes. Leukocyte deoxyribonucleic acid was extracted from freshly collected serum samples of volunteers using a spin column extraction kit (QIAmp blood kit; Qiagen, Valencia, CA). Taq polymerase, deoxynucleotide triphosphates, buffers, and standards were obtained from PerkinElmer (Norwalk, CT). A PerkinElmer thermocycler (model GeneAmp PCR system 2400) was employed for the reaction.

Data and Statistical Analyses

Pharmacokinetic parameters [peak concentrations (C_{\max}) and areas under time-concentration curves (AUCs)] were obtained from noncompartmental

analyses by Kinetica 5.0 (Thermo Fisher Scientific, Waltham, MA). A linear-log trapezoidal method was used to calculate AUCs. Two-tailed paired t tests were conducted to detect difference in the pharmacokinetic parameters between pre- and postexposure P450 activities with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). For each pharmacokinetic parameter, a P value of 0.05 was used as the level of statistical significance. The phenotyping metrics were also evaluated using the standard bioequivalence approach in which the absence of a clinically relevant interaction was concluded if the 90% confidence interval (CI) was within the bounds of 0.7–1.43. This no-effect boundary was used instead of the bioequivalence boundary of 0.8–1.25 because the Food and Drug Administration considers that the latter is conservative for industry on drug interactions [Ring et al., 2005; Tomalik-Scharte et al., 2005; U.S. Department of Health and Human Services and Food and Drug Administration Center for Drug Evaluation and Research (CDER), 2012]. Probe drugs exhibit high between- and within-subject variability in pharmacokinetics, and the conservative limits of 0.8–1.25 could lead to a false-positive conclusion of a statistically significant difference (Chien et al., 2006). Based on a conservative estimate for within-subject correlation of 0.7 or higher for repeated measures, a sample size of eight subjects would provide at least 80% power to demonstrate that the 90% CI of the geometric mean ratio of the phenotyping metrics would fall within the no-effect range of 0.70–1.43.

Results

Subjects. Nine healthy volunteers (three men and six women) aged 22–31 years (mean \pm S.D., 26.5 \pm 2.7 years; weight, 67.7 \pm 11.9 kg) completed the entire protocol. Two participants were discontinued from the study due to protocol violations, and one participant withdrew for a personal reason. No subject experienced any unanticipated adverse event. No abnormalities were noted in the follow-up blood chemistries and CBC.

Plasma Concentrations of Unconjugated Flavonolignans. HPLC-MS/MS analysis of plasma concentrations of the most abundant flavonolignans, silybin A, silybin B, and isosilybin B, revealed all subjects had measurable concentrations during phase III of the study. The C_{\max} (mean \pm S.D.) for silybin A was 70.75 \pm 85.3 ng/ml (range 10.6–294.0); for silybin B it was 23.6 \pm 35.8 ng/ml (range 2.1–34.8); and for isosilybin B it was 15.3 \pm 25.2 ng/ml (range 2.1–81.6).

Pharmacokinetic Analysis. C_{\max} and AUC_{0–12h} data of CAF, TOL, DEX, and MDZ are shown in Table 1 to indicate the corresponding CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 activities. There were no significant differences in the pharmacokinetic parameters. The geometric mean ratios for each metric were similar, and the 90% CI for the ratios of the metrics was within the defined acceptance criteria.

To further investigate two parameters, TOL C_{\max} and MDZ C_{\max} , which were slightly outside of the Food and Drug Administration bioequivalence criteria (Table 1), the relationship between these parameters and the concentrations of the milk thistle constituents was examined. When each subject's silybin B concentrations were plotted against changes in TOL C_{\max} , significant correlation was observed ($P = 0.0247$, $r^2 = 0.537$; Fig. 1). However, no significant correlations were observed between TOL C_{\max} and other constituents' concentrations. MDZ C_{\max} was also not significantly correlated with any constituent's concentrations.

The metabolic ratios of DEX to DOR AUC_{0–12h} (area under the time-concentration curve analyzed from time 0 hour to 12 hours) were 0.416 \pm 0.211 pre-exposure versus 0.474 \pm 0.233 postexposure ($P = 0.0681$). The metabolic ratio of OHMDZ to MDZ area under the time-concentration curve analyzed from time 0 hour to infinity was 0.613 \pm 0.352 pre-exposure versus 0.611 \pm 0.390 postexposure ($P = 0.972$). The metabolic ratios of DEX/DOR and MDZ/OHMDZ AUCs assured respective CYP2D6- and CYP3A4/5-mediated metabolism was not significantly altered.

TABLE 1
Summary of pharmacokinetic parameters and statistical tests (mean \pm S.D.)

Parameters	Pre-Exposure	Postexposure	<i>P</i> Value	Equivalency Test Geometric Mean Ratio (90% CI)
CAF AUC _{0-12h} (mcg/ml \times h)	35.1 \pm 11.9	35.3 \pm 13.0	0.878	0.99 (0.93, 1.05)
CAF <i>C</i> _{max} (mcg/ml)	4.80 \pm 1.22	4.93 \pm 1.16	0.614	1.04 (0.93, 1.17)
MDZ AUC _{inf} (ng/ml \times h)	97.9 \pm 43.5	98.6 \pm 37.9	0.893	1.03 (0.93, 1.13)
MDZ <i>C</i> _{max} (ng/ml)	33.2 \pm 12.7	31.6 \pm 14.4	0.703	0.93 (0.69, 1.24)
TOL AUC _{inf} (mcg/ml \times h)	745 \pm 177	815 \pm 251	0.197	1.07 (0.95, 1.20)
TOL <i>C</i> _{max} (mcg/ml)	54.5 \pm 5.9	63.6 \pm 15.4	0.074	1.15 (1.01, 1.30)
DEX AUC _{0-12h} (ng/ml \times h)	9.41 \pm 6.96	10.3 \pm 8.5	0.337	1.04 (0.91, 1.20)
DEX <i>C</i> _{max} (ng/ml)	1.37 \pm 1.02	1.05 \pm 0.35	0.766	1.02 (0.86, 1.20)

AUC_{0-12h}, area under time-concentration curve analyzed from time 0 hour to 12 hours; AUC_{inf}, area under time-concentration curve analyzed from time 0 hour to infinity.

Discussion

The results of this study show that chronic administration of a milk thistle supplement does not have a clinically relevant effect on the major P450 enzymes. According to the US Food and Drug Administration bioequivalence criteria, equivalent P450 activities can be suggested if the 90% CIs of the geometric mean ratios lie within the range of 0.80–1.25 [U.S. Department of Health and Human Services and Food and Drug Administration Center for Drug Evaluation and Research (CDER), 2012]. The present results demonstrated that the CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 activities were essentially unchanged from baseline measures after a 14-day milk thistle exposure. Our results suggest that standardized milk thistle extracts, administered at typical doses and dosing regimens, had no significant inhibitory or inductive effects on major hepatic P450 enzymes. Furthermore, systemic exposure to free concentrations of the major flavonolignans (silybin A, silybin B, and isosilybin B) was confirmed in this study via plasma concentration monitoring. The *C*_{max} values of the flavonolignans were consistent with those observed in a separate dose escalation pharmacokinetic assessment of this same milk thistle formulation recently completed in our laboratory (Zhu et al., 2013). We should also note that no participants experienced major side effects or abnormal blood chemistries or CBC after the milk thistle supplementation.

Parameters that were outside of the Food and Drug Administration bioequivalence criteria were TOL *C*_{max} and MDZ *C*_{max}. All other parameters fell within the defined range. The upper 90% CI of TOL *C*_{max} was 1.30, which was slightly outside the threshold (i.e., 1.25)

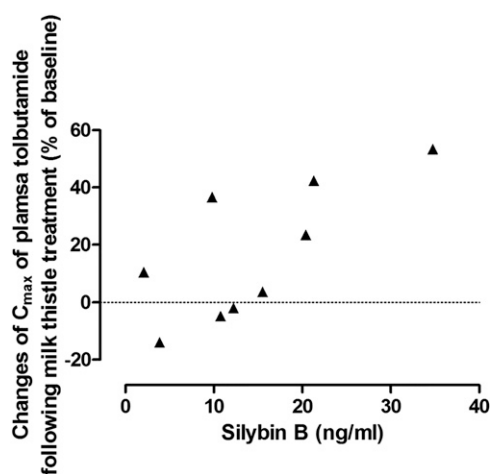


Fig. 1. Correlation between plasma silybin B concentrations and changes in TOL peak plasma concentrations after milk thistle exposure.

and did not signal a concern of major clinical significance. A number of in vitro studies have been conducted that have investigated the potential inhibitory effect of milk thistle extracts or individual flavonolignans on P450 isoenzyme activity (Beckmann-Knopp et al., 2000; Venkataraman et al., 2000; Zuber et al., 2002; Sridar et al., 2004). Among all evaluated P450 isoenzymes, CYP2C9 appears to be the P450 isoform most sensitive to inhibition by flavonolignans. In a human liver microsome incubation study, silybin B was determined to be the most potent flavonolignan for the inhibition of CYP2C9 with an IC₅₀ value of 8.2 μ M, followed by silybin A (Brantley et al., 2010). Isosilybin A and isosilybin B were much weaker CYP2C9 inhibitors with IC₅₀ values approximately 10-fold higher than those of silybin B. The pharmacokinetic assessments from our laboratory and others have shown that silybin A and silybin B are the most abundant flavonolignans in the plasma of subjects supplemented with milk thistle extracts (Zhu et al., 2013). To determine whether the increase of mean TOL *C*_{max} was caused by the exposure to silybin A and/or silybin B, we analyzed the correlation of the plasma concentrations of silybin diastereomers with the changes of TOL *C*_{max} relative to the baseline levels after milk thistle administration. The results indicated that neither silybin A nor combined silybin A and silybin B concentrations were correlated to the changes of TOL *C*_{max}. However, individual's silybin B concentrations appeared to be significantly correlated to the increases of TOL *C*_{max} ($P = 0.0247$, $r^2 = 0.537$; Fig. 1). This observation is in agreement with published in vitro studies, suggesting that silybin B is a more potent CYP2C9 inhibitor than other flavonolignans. However, it should be noted that a relatively low dose of milk thistle extracts (i.e., one capsule 175 mg three times daily) was used in the present study. In previous studies in prostate cancer patients, plasma concentrations of silibinin (i.e., combined silybin A and silybin B) were reported to range between 5 and 75 μ M (Flaig et al., 2007). For comparative purposes, in the present study, the mean silybin B *C*_{max} expressed in molar concentrations (mol. wt. 482.44) was only $0.049 \pm 0.074 \mu$ M (range 0.004–0.072 μ M). Clearly, these concentrations were far lower than those reported in a study of an alternative silymarin formulation with greatly enhanced bioavailability (Flaig et al., 2007) and also far below the IC₅₀ values from previously reported in vitro studies. This most likely explains the absence of P450 inhibition/induction detected in the present study. We speculate that an interaction between silybin B and CYP2C9 might become clinically significant when patients are treated with sufficiently high doses and/or formulations with enhanced bioavailability of silybin B (e.g., phosphatidylcholine complexes, phytosomes). We further recognize that no matter which milk thistle formulation is used, hepatic concentrations of silybin B and other flavonolignans are likely to be far higher than peripheral concentrations. Nevertheless, in this study, the plasma concentrations of the major flavonolignans, silybin A,

silybin B, and isosilybin B, which may be regarded as suspect constituents in terms of participating in drug-drug interactions, were measured and found to be at levels consistent with those attained during routine milk thistle dosing with this particular milk thistle formulation (Zhu et al., 2013).

The present study suggests that clinically significant inhibitory drug interactions mediated by CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 are unlikely to be of clinical significance. Some discrepancy between our study and previous *in vitro* studies may be explained by the use of higher concentrations used to inhibit the P450 enzymes *in vitro* than physiologically attainable concentrations. One human study investigated the metabolism and disposition of metronidazole among 12 healthy volunteers and suggested induction of intestinal CYP3A4 and P-glycoprotein following a 9-day exposure to 140 mg/d silymarin (Rajnarayana et al., 2004). The reason for this contradictory result on CYP3A4 relative to our findings remains unclear because the daily silymarin dose of the study was lower than what was used in the present study. However, it may be explained by the difference in metabolism between the probe drug substrates used, that is, metronidazole versus the MDZ used in the present study. Several other healthy volunteer studies, which investigated the metabolism of indinavir, ranitidine, and digoxin, did not demonstrate significant effects of milk thistle on CYP3A4 or P-glycoprotein activities (Piscitelli et al., 2002; DiCenzo et al., 2003; Mills et al., 2005; Gurley et al., 2006b; Rao et al., 2007). Similarly, previous studies in healthy volunteers showed no significant effect of milk thistle on CYP1A2, CYP2D6, CYP2E1, or CYP3A4, which incorporated different probe drugs, including debrisoquine, chlorzoxazone, nifedipine, and rosuvastatin (Gurley et al., 2004, 2008; Fuhr et al., 2007; Deng et al., 2008). In addition, CYP3A4 activity was not influenced by milk thistle supplementation in cancer patients and human immunodeficiency virus-infected patients when the activity was measured via influence on irinotecan and darunavir metabolism, respectively, with higher silymarin doses than the present study dose (van Erp et al., 2005; Molto et al., 2012). Thus, our study results were in agreement with the findings of the majority of previous human studies that suggested an absence of significant P450 enzyme inhibition and induction by milk thistle constituents.

One potential limitation of our study was that the study was conducted in healthy volunteers, and there has been some suggestion from a previous study in patients with nonalcoholic fatty liver disease (NAFLD) that disposition of flavonolignans may be altered (Schrieber et al., 2011). Indeed, plasma concentrations of the flavonolignans, silybin A and silybin B, were higher in patients with NAFLD, compared with patients infected with hepatitis C virus (HCV) (Schrieber et al., 2011). Decreased conjugation of silybin B and more extensive enterohepatic cycling were observed in patients with NAFLD as well (Schrieber et al., 2011). Because the comparator was HCV-infected patients, it is difficult to make interpretations relative to healthy volunteers. However, an earlier study showed that, compared with healthy volunteers, exposures to total silymarin flavonolignans in HCV noncirrhosis, NAFLD, and HCV cirrhosis cohorts were considerably increased by 2.4-, 3.3-, and 4.7-fold, respectively (Schrieber et al., 2008). The altered disposition of milk thistle constituents in certain disease states could have more pronounced effects on P450 enzymes that we were not able to detect. Another potential limitation may be the dosing regimen that may represent the initial and perhaps lower end of clinical exposures. We investigated CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5 activities in this study, but effects of milk thistle on other metabolizing enzymes were not evaluated in this study. Because CYP2D6 poor metabolizers were not included in our study, extrapolation of the results requires caution to this population. Another factor that was not considered in

this current study was CYP2C9 genotype. Recently, the effect of milk thistle on the metabolism of the angiotensin II receptor antagonist, losartan, to its predominantly CYP2C9-dependent metabolite, E-3174, was evaluated in 12 healthy males by employing the same milk thistle regimen as the present study (Han et al., 2009). The metabolic ratio of E-3174 to losartan AUC was decreased in individuals with CYP2C9*1/*1 genotype, but not in those with CYP2C9*1/*3 genotype after the milk thistle exposure, suggesting potential genotype-dependent CYP2C9 impairment (Han et al., 2009). Because we did not genotype participants' CYP2C9 gene, the genotype may have contributed to the additional variability in TOL pharmacokinetics observed in Fig. 1. CYP2C9 metabolizer status may be another important aspect to be considered for future studies. In addition, this study investigated only four P450 enzymes; the activity of other P450 enzymes, such as CYP2A6, CYP2B6, CYP2C8, and CYP2C19, was not considered. Finally, the present assessment cannot necessarily be generalized to other milk thistle extract formulations. An extension of studies of the present type to formulations with significantly enhanced flavonolignan bioavailability may be an important area for future study. In summary, the results of the present study suggest little potential for significant drug interactions when milk thistle extracts are used concurrently with the majority of currently marketed drugs metabolized by CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5.

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Authorship Contributions

Participated in research design: Markowitz, Chavin, Bernstein.
Conducted experiments: Kawaguchi-Suzuki, Frye, Brinda, Markowitz, Zhu.
Contributed new reagents or analytic tools: Brinda, Frye, Markowitz, Zhu.
Performed data analysis: Kawaguchi-Suzuki, Frye, Markowitz, Zhu.
Wrote or contributed to the writing of the manuscript: Markowitz, Chavin, Bernstein, Kawaguchi-Suzuki, Frye, Brinda, Zhu.

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