

A Systematic Approach to Evaluate Herb-Drug Interaction Mechanisms:
Investigation of Milk Thistle Extracts and Eight Isolated Constituents as CYP3A Inhibitors

Scott J. Brantley, Tyler N. Graf, Nicholas H. Oberlies, and Mary F. Paine

Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of
Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC (S.J.B., M.F.P.);
Department of Chemistry and Biochemistry, The University of North Carolina at Greensboro,
Greensboro, NC (T.N.G., N.H.O.)

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Corresponding author:

Mary F. Paine, RPh, PhD
2320 Kerr Hall, CB #7569
UNC Eshelman School of Pharmacy
The University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7569
Telephone: 919-966-9984
FAX: 919-962-0644
E-mail: mpaine@unc.edu

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Abbreviations: CYP3A, cytochrome P450 3A; DHB, 6',7'-dihydroxybergamottin; DMPO, 5,5-dimethyl-1-pyrroline-n-oxide; HIMs, human intestinal microsomes; HLMs, human liver microsomes; MBI, mechanism-based inhibition; rCYP3A4, recombinant CYP3A4; SOD, superoxide dismutase.

Abstract

Despite increasing recognition of potential untoward interactions between herbal products and conventional medications, a standard system for prospective assessment of these interactions remains elusive. This information gap was addressed by evaluating the drug interaction liability of the model herbal product milk thistle (*Silybum marianum*) with the CYP3A probe substrate midazolam. The inhibitory effects of commercially available milk thistle extracts and isolated constituents on midazolam 1'-hydroxylation were screened using human liver and intestinal microsomes. Relative to vehicle, the extract silymarin and constituents silybin A, isosilybin A, isosilybin B, and silychristin at 100 μM demonstrated >50% inhibition of CYP3A activity with at least one microsomal preparation, prompting IC_{50} determination. The IC_{50} s for isosilybin B and silychristin were ~60, and 90 μM , respectively, whereas those for the remaining constituents were >100 μM . Extracts and constituents that contained the 1,4-dioxane moiety demonstrated a >1.5-fold shift in IC_{50} when tested as potential mechanism-based inhibitors. The semi-purified extract, silibinin, and the two associated constituents (silybin A, silybin B) demonstrated mechanism-based inhibition of recombinant CYP3A4 (K_i ~100 μM ; k_{inact} ~0.20 min^{-1}) but not microsomal CYP3A activity. The maximum predicted increase in midazolam AUC using the static mechanistic equation and recombinant CYP3A4 data was 1.75-fold, which may necessitate clinical assessment. Evaluation of the interaction liability of single herbal product constituents, in addition to commercially available extracts, will enable elucidation of mechanisms underlying potential clinically significant herb-drug interactions. Application of this framework to other herbal products would permit predictions of herb-drug interactions and assist in prioritizing clinical evaluation.

Introduction

An estimated 20% of adults in the US acknowledge taking herbal products (Bent, 2008), with nearly 70% failing to inform their health care provider (Gardiner et al., 2006; Kennedy et al., 2008). An herbal product that inhibits one or more drug metabolizing enzymes can perpetrate untoward interactions with conventional medications (Hu et al., 2005; Izzo and Ernst, 2009) (Gurley et al., 2012a). Prominent among these enzymes are the cytochromes P450 (P450s). Inhibition of P450 activity by constituents of the herbal product can reduce “victim” drug clearance, leading to higher systemic drug concentrations and potential adverse effects and toxicity. Dietary substances, including herbal products, are not regulated in the same manner as drugs. Consequently, herb-drug interaction liability is not evaluated routinely prior to marketing. This information gap prevents both clinicians and consumers from making informed decisions about the risk of adding herbal products to pharmacotherapeutic regimens.

Despite increasing recognition of potential untoward herb-drug interactions, a standard system for prospective assessment of the drug interaction liability of herbal products remains elusive. In addition to inconsistent experimental methods, variability in phytochemical content further confound interaction evaluations (Gurley, 2012b). As such, knowledge of mechanisms underlying these interactions is scarce. One approach to developing a system to assess herb-drug interaction potential is to examine individual constituents from a well-characterized herbal product using traditional drug-drug interaction (DDI) predictive tools. Milk thistle is a top-10 selling herbal product in the US (Blumenthal et al., 2012) used predominately to self-treat hepatic disorders, particularly hepatitis C (Kroll et al., 2007; Post-White et al., 2007; Seeff et al., 2008). Milk thistle represents an ideal model herbal product due to the following key properties: high sales numbers (12.8 million dollars in 2011) indicate that a large number of consumers are at risk for milk thistle-drug interactions (Blumenthal et al., 2012); individual constituents have been identified, isolated, and scaled-up to quantities sufficient for *in vitro* DDI evaluation (Kim et al., 2003; Graf et al., 2007; Monti et al., 2010; Sy-Cordero et al., 2012); and *in vitro-in vivo*

extrapolation has been inconsistent (Beckmann-Knopp et al., 2000; Zuber et al., 2002; Gurley et al., 2004; Gurley et al., 2006; Han et al., 2009).

Commercial preparations of milk thistle [*Silybum marianum* (L.) Gaertn.] include the crude extract silymarin, consisting of at least seven flavonolignans (Fig. 1), the flavonoid taxifolin, and fatty acids, and the semi-purified extract silibinin, consisting of the most prevalent flavonolignans (silybin A and silybin B). The relative composition of milk thistle constituents varies substantially between and among preparations (Davis-Searles et al., 2005; Wen et al., 2008). These extracts have been shown to inhibit the activity of several P450s in vitro, both reversibly (e.g., CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A) (Beckmann-Knopp et al., 2000; Zuber et al., 2002; Etheridge et al., 2007; Doehmer et al., 2008; Brantley et al., 2010) and irreversibly (e.g., CYP2C9, CYP3A4) (Sridar et al., 2004). However, these in vitro observations generally have not manifested clinically. For example, two healthy volunteer studies demonstrated no interaction between the CYP3A probe substrate midazolam and one silymarin product (Gurley et al., 2004; Gurley et al., 2006), whereas another study demonstrated a different silymarin product to increase significantly the systemic exposure to the CYP2C9/3A substrate losartan (Han et al., 2009). This discrepancy may be attributed to large variation in relative composition between herbal products. Rigorous characterization of P450 inhibition properties of individual constituents, as well as the commercial extracts, may assist explanation of these in vitro-in vivo disconnects (Paine and Oberlies, 2007; Won et al., 2012).

CYP3A metabolizes over 30% of clinically used drugs (Zanger and Schwab, 2013) and is expressed in both the intestine and liver (Thummel et al., 1996). Following oral administration of milk thistle, intestinal CYP3A likely will be exposed to higher concentrations of the constituents compared to hepatic CYP3A. This difference was demonstrated by the nearly 60-fold higher mean (\pm SD) concentration in intestinal relative to hepatic tissue in cancer patients administered 1.4 g/day silibinin (140 ± 170 vs. 2.5 ± 2.4 μ M) (Hoh et al., 2006). Such high intestinal concentrations could reduce markedly the intestinal first-pass metabolism of

susceptible CYP3A substrates, thereby increasing systemic drug exposure. Using milk thistle as a model herbal product, the objectives of this study were to (1) assess interaction liability of individual constituents towards CYP3A activity, (2) prioritize constituents for further evaluation, and (3) develop a framework to elucidate mechanisms underlying herb-drug interactions. Such mechanistic insight ultimately may assist clinicians and consumers to gauge the impact of adding herbal products to pharmacotherapeutic regimens.

Materials and Methods

Chemicals and reagents. Human liver (pooled from 50 donors, mixed gender) and intestinal (pooled from 18 donors, mixed gender) microsomes (HLMs, HIMs) were purchased from Xenotech, LLC (Lenexa, KS). Baculovirus insect cell-expressed CYP3A4 (rCYP3A4), supplemented with cDNA-expressed reductase and cytochrome b₅, was purchased from BD Biosciences (San Jose, CA). Midazolam, 1'-hydroxymidazolam, alprazolam, silibinin, ketoconazole, NADPH, glutathione, superoxide dismutase (SOD), catalase, 5,5-dimethyl-1-pyrroline-n-oxide (DMPO), acetonitrile, DMSO, HPLC-grade water, methanol, ammonium acetate, and 1-propanol were purchased from Sigma-Aldrich (St. Louis, MO). 6',7'-Dihydroxybergamottin (DHB) was purchased from Cayman Chemical (Ann Arbor, MI). Silymarin was obtained from Euromed S.A. (Barcelona, Spain). This product has been shown to consist of 16% silybin A, 24% silybin B, 6.4 % isosilybin A, 4.4% isosilybin B, 17% silydianin, 12% silychristin, 2.2% isosilychristin, and 1.6% taxifolin; the remainder consists of uncharacterized polyphenols and aliphatic fatty acids (Davis-Searles et al., 2005). Silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin were purified as described previously (Graf et al 2007); all milk thistle constituents were >97% pure as determined by high-performance liquid chromatography.

Evaluation of the stability of individual milk thistle constituents. *Stability in potassium phosphate buffer.* Each constituent was dissolved in DMSO to yield a 100 mM solution. Each solution was diluted in potassium phosphate buffer (0.1 M, pH 7.4), supplemented with MgCl₂ (3.3 mM), to yield a final concentration of 100 μM. Solutions were placed immediately into a heated (37°C) auto-sampler, and aliquots (0.3 μL) were collected serially from 0-1440 min and analyzed via UPLC-UV (described below). *Metabolic lability of selected constituents.* Silybin A, silybin B, isosilybin A, and isosilybin B were added to potassium phosphate buffer as described above with the addition of HLMs (0.05 mg/mL). Incubation mixtures were placed on a dry heat block and equilibrated for 5 min. Reactions were

initiated with the addition of NADPH to yield a final concentration of 1 mM. Aliquots (100 μ L) were removed from 0-60 min, and reactions were terminated with 2 volumes of ice-cold acetonitrile. Each constituent was quantified by UPLC-UV (described below).

Analysis of incubations for milk thistle constituents. Milk thistle constituents were quantified with an Acquity UPLC system (Empower 2 software; Waters Corp., Milford, MA) at a flow rate of 0.80 (stability study) or 0.75 (lability study) mL/min using an Acquity binary solvent manager and an Acquity HSS-T3 column (1.8 μ m, 2.1 \times 50 mm; Waters Corp.). The elution gradient for the stability study ranged from 10:90 to 40:60 methanol:water over 0.45 minutes, then from 40:60 to 55:45 over 1.5 minutes. The elution gradient for the lability study ranged from 40:60 to 55:45 methanol:water over 2 minutes. Signals were monitored at UV 288 nm using an Acquity PDA detector, and the volume injected was 0.3 (stability study) or 7.5 (lability study) μ L by an Acquity sample manager.

Evaluation of milk thistle constituents as inhibitors of CYP3A activity. The inhibitory effects of silymarin, silibinin, and individual constituents on midazolam 1'-hydroxylation activity were evaluated using HLMs, HIMs, and rCYP3A4. Midazolam and ketoconazole were dissolved in methanol to yield 10 mM and 1 mM solutions, respectively. Milk thistle constituents were dissolved in DMSO to yield a 200 mM solution. NADPH was prepared fresh in potassium phosphate buffer to yield a 4 mM solution. Under all experimental conditions, the amount of 1'-hydroxymidazolam formed was linear with respect to incubation time and concentration of microsomal or rCYP3A4 protein (data not shown). *Initial testing.* Incubation mixtures consisted of HLMs or HIMs (0.05 mg/mL protein), midazolam (4 μ M), milk thistle constituent/extract (1, 10, or 100 μ M), and potassium phosphate buffer. Control mixtures contained 0.1% (v/v) DMSO in place of milk thistle constituent/extract. As a positive control for CYP3A inhibition, mixtures contained ketoconazole (1 μ M) in place of milk thistle constituent/extract. The mixtures were equilibrated in a dry heat block at 37°C for 5 min before initiating the reactions with NADPH (1 mM final concentration) to yield a final volume of 200 μ L. After 2 (HLMs) or 4 (HIMs) min,

reactions were terminated with 2 volumes of ice-cold acetonitrile containing 20 $\mu\text{g/mL}$ alprazolam as the internal standard. After centrifugation (1350 \times g for 10 min at 4°C), supernatants were analyzed for 1'-hydroxymidazolam by HPLC-MS/MS as described previously (Wang et al., 2007; Ngo et al., 2009). Individual constituents demonstrating $\geq 50\%$ inhibition in either microsomal preparation were selected for further evaluation. *Screening of constituents as reversible inhibitors of CYP3A activity in HLMs and HIMs.* Incubation mixtures were prepared as described above, only milk thistle constituent/extract concentrations ranged from 0-200 μM . Reaction mixtures were processed and analyzed for 1'-hydroxymidazolam as described above. *Screening of constituents as metabolism-dependent inhibitors of CYP3A activity in HLMs, HIMs, and rCYP3A4.* IC₅₀ shift experiments (Obach et al., 2007) were used to screen milk thistle constituents and extracts as potential mechanism-based inhibitors of CYP3A activity. Primary incubation mixtures consisting of HLMs, HIMs (0.25 mg/mL), or rCYP3A4 (20 pmol/mL), milk thistle constituent/extract (0-1000 μM), and potassium phosphate buffer were equilibrated at 37°C for 5 min before initiating reactions with NADPH (1 mM). Control reactions were identical except NADPH was replaced with potassium phosphate buffer. After 15 min, an aliquot (40 μL) was removed and diluted 5-fold into a secondary incubation mixture containing midazolam (4 μM) and NADPH (1 mM). After 2 (HLMs) or 4 (HIMs and rCYP3A4) min, the secondary reactions were terminated and processed as described above. Individual constituents demonstrating a >1.5 -fold shift in IC₅₀ were selected for further evaluation. *Effect of nucleophilic trapping agents and reactive oxygen species scavengers on CYP3A inactivation in HLMs.* Primary mixtures consisted of HLMs (0.25 mg/mL), silybin A (0, 30, or 100 μM), potassium phosphate buffer, and trapping agent [DMPO (1 mM), SOD (1,000 U/mL), glutathione (2 mM), or catalase (5,000 U/mL)]. The mixtures were equilibrated at 37°C for 5 min before initiating reactions with NADPH (1 mM). Control reactions were identical except NADPH was replaced with potassium phosphate buffer. Reactions proceeded as described for the IC₅₀ shift experiment. *Determination of mechanism-based inhibition kinetics.* Time- and concentration-

dependent inhibition of CYP3A4 was assessed as described previously (Paine et al., 2004). Briefly, primary incubation mixtures consisting of HLMs, HIMs (2.5 mg/mL), or rCYP3A4 (0.2 nmol/mL), milk thistle constituent/extract (0-200 μ M), and potassium phosphate buffer were equilibrated at 37°C for 5 min before initiating the primary reactions with NADPH. As a positive control for mechanism-based inhibition, incubation mixtures contained DHB (5 μ M) in place of milk thistle constituent/extract (Paine et al., 2004). At designated times (0-15 min), an aliquot (4 μ L) was removed and diluted 50-fold into a secondary reaction mixture containing midazolam (8 μ M) and NADPH (1 mM). The secondary reactions were terminated and processed as described for the reversible inhibition experiments.

Data analysis. *Determination of apparent IC_{50} .* The apparent IC_{50} of milk thistle constituent/extract was recovered according to previously published methods (Brantley et al., 2010). Initial estimates of IC_{50} s were determined by visual inspection of the velocity of 1'-hydroxymidazolam formation versus the natural logarithm of milk thistle constituent/extract concentration data. Final parameter estimates were recovered by fitting equation 1 or 2 to untransformed data using WinNonlin (version 5.3; Pharsight, Mountain View, CA):

$$v = \frac{v_0}{1 + \left(\frac{I}{IC_{50}} \right)} \quad (\text{Eq. 1})$$

$$v = \frac{v_0}{1 + \left(\frac{I}{IC_{50}} \right)^h} \quad (\text{Eq. 2})$$

where v denotes the velocity of 1'-hydroxymidazolam formation, v_0 denotes the initial velocity of 1'-hydroxymidazolam formation, I denotes the concentration of milk thistle constituent/extract, and h denotes the Hill coefficient. The best-fit equation was assessed from visual inspection of the observed versus predicted data, randomness of the residuals, Akaike information criteria, and SEs of the parameter estimates.

Determination of K_i and k_{inact} . Mechanism-based inhibition (MBI) parameters were recovered according to previously published methods (Paine et al., 2004; Obach et al., 2007). Briefly, the natural logarithm of the percentage of CYP3A activity remaining was plotted as a function of primary reaction time. The apparent inactivation rate constant ($k_{inact,app}$) associated with each inhibitor concentration was determined from the slope of the initial mono-exponential decline in activity. Initial estimates of K_i and k_{inact} were obtained from a Kitz-Wilson plot. Final parameter estimates were obtained by nonlinear least-squares regression using equation 3:

$$k_{inact,app} = k_{zero} + \frac{k_{inact} * I}{K_i + I} \quad (\text{Eq. 3})$$

where k_{zero} denotes the rate of CYP3A inactivation in the absence of inhibitor. The efficiency of inactivation was calculated as the ratio of k_{inact} to K_i .

Milk thistle-midazolam drug interaction prediction. The interaction between milk thistle constituents and midazolam was predicted using the intestinal portion of the static mechanistic MBI equation detailed previously (Obach et al., 2007; US FDA, 2012):

$$\frac{AUC_i}{AUC} = \frac{1}{F_g + \left((1-F_g) * \frac{1}{1 + \frac{(k_{inact} * I_g)}{k_{deg} * (I_g + K_i)}} \right)} \quad (\text{Eq. 4})$$

where AUC_i/AUC denotes the predicted ratio of in vivo exposure of a victim drug, I_g denotes the inhibitor concentration in the enterocyte (140 μM) after a silibinin dose of 1.4 g (Hoh et al., 2006), k_{deg} denotes the rate of intestinal CYP3A4 degradation (0.00481 min^{-1}) (Obach et al., 2007), and F_g denotes the fraction of the dose of midazolam escaping first-pass extraction in the intestine (0.57) (Paine et al., 1996).

Statistical analysis. Data are presented as means \pm SDs of triplicate determinations unless indicated otherwise. Enzyme kinetic parameters are presented as the estimates \pm SEs. All statistical comparisons were made according to previously published methods (Brantley et

al., 2010). In brief, concentration-dependent inhibition of single constituents/extracts was evaluated by a one-way ANOVA; post-hoc comparisons were made using Tukey's test when an overall significance resulted. Statistical differences between calculated IC_{50} values were evaluated by a Student's *t*-test of two independent samples. A $p < 0.05$ was considered significant for all statistical tests.

Results

Selected milk thistle constituents are metabolically stable in HLMs for up to 15 min. All tested constituents were stable (>95% remaining) for 1 h at 37°C in potassium phosphate buffer (data not shown). Metabolic stability experiments determined the optimal primary incubation time such that inhibitor depletion would be $\leq 20\%$. All constituents at 10 μM demonstrated $\geq 50\%$ oxidative depletion by 60 min (Fig. 2). Isosilybin A was the least stable, showing 40% depletion by 10 min, whereas the remaining constituents were depleted by <10%. At 100 μM , silybin A was slightly more stable than the other constituents, (~40% vs. 50% depletion by 60 min). Isosilybin A at 100 μM was again the least stable, showing 30% depletion by 10 min, whereas the remaining constituents were depleted by <10%. Based on these observations, the primary incubation time of the IC_{50} shift and mechanism-based inhibition experiments involving selected constituents, with the exception of isosilybin A, was limited to 15 min.

Milk thistle constituents differentially inhibit CYP3A-mediated midazolam 1'-hydroxylation. *Initial testing.* All flavonolignans and the two extracts (silibinin, silymarin) inhibited midazolam 1'-hydroxylation in a concentration-dependent manner (10 vs. 100 μM) in HLMs (Fig. 3A) and, with the exception of isosilychristin, in HIMs (Fig. 3B). The sole flavonoid, taxifolin, showed no concentration-dependent inhibition with either preparation. Only silybin B (HLMs), silychristin (HIMs), and silydianin (HIMs) showed concentration-dependent inhibition from 1 to 10 μM (1 μM data not shown). With HLMs, silybin A at 100 μM was the most potent, followed by silymarin, isosilybin B, and isosilybin A (Fig. 3A); with HIMs, silymarin at 100 μM was the most potent, followed by isosilybin A, isosilybin B, and silychristin (Fig. 3B). Ketoconazole inhibited CYP3A activity in both microsomal preparations by at least 95% (data not shown). Based on >50% inhibition at 100 μM in at least one microsomal preparation, silybin A, isosilybin A, isosilybin B, and silychristin were selected for IC_{50} determination. Additionally, based on the high concentrations (>140 μM) observed in enterocytes following silibinin

administration to human subjects (Hoh et al 2006), silybin B was selected for further evaluation. *Reversible inhibition.* With both HLMs and HIMs, isosilybin B was the most potent constituent, followed by silychristin, silybin B, and silybin A (Table 1). Because isosilybin A was not soluble at concentrations >100 μ M, a complete IC_{50} curve could not be recovered. *IC_{50} shift.* The IC_{50} for silybin A, silybin B, and silibinin was 73-98% lower in the presence compared to the absence of NADPH in the primary incubation (Table 2). rCYP3A4 was more sensitive to inhibition than HLMs and HIMs (Table 2, Fig. 4). The IC_{50} for silychristin did not decrease with either HLMs or HIMs (Table 2), precluding further evaluation of this constituent. Reactive species scavengers did not ameliorate the NADPH-dependent increase in potency of silybin A towards CYP3A activity (Fig. 5).

Silybin A, silybin B, and silibinin demonstrate mechanism-based inhibition of rCYP3A4 but not microsomal CYP3A activity. Mechanism-based inhibition of CYP3A activity by silybin A, silybin B, and silibinin was evaluated to derive relevant kinetic parameters. No tested constituent or extract demonstrated time- and concentration-dependent inhibition of CYP3A activity with either HLMs or HIMs (data not shown). Unlike with microsomes, rCYP3A4 demonstrated both time- and concentration-dependent inhibition (Fig. 6). Because the rate of rCYP3A4 inactivation for silybin B at 200 μ M deviated from linearity (Fig. 6B), these data were excluded from further analysis. The kinetics were similar between silybin A and silybin B; the kinetics of the 1:1 mixture of silybin A and silybin B (silibinin) were similar to those recovered for the single constituents (Table 3). Using the rCYP3A4 inactivation parameters recovered for silibinin and assuming the enterocyte concentration of silibinin approximates that measured in intestinal tissue (Hoh et al., 2006), a 1.75-fold increase in midazolam AUC was predicted by the static mechanistic equation (Obach et al., 2007; US FDA, 2012).

Discussion

Herb-drug interactions are a growing concern in clinical practice as consumers turn increasingly to herbal products as a means to self-treat various conditions. Despite increasing recognition of these potential untoward interactions, a standard system for prospective assessment of the drug interaction liability of herbal products remains elusive. Milk thistle was selected as a model herbal product based on market share, well-characterized nature, and clinical relevance. Milk thistle extracts have been reported to inhibit P450 activity in vitro (Beckmann-Knopp et al., 2000; Zuber et al., 2002; Doehmer et al., 2008); however, clinical interaction liability has been inconsistent, particularly with CYP3A-mediated interactions (Gurley et al., 2004; Gurley et al., 2006; Han et al., 2009). Taken together, inhibition of CYP3A by single constituents was evaluated to address these in vitro-in vivo disconnects, as well as to develop a framework to elucidate potential mechanisms underlying herb-drug interactions.

As demonstrated previously with CYP2C9 activity (*i.e.*, (S)-warfarin 7-hydroxylation) (Brantley et al., 2010), milk thistle constituents differentially inhibited CYP3A-mediated midazolam 1'-hydroxylation (Fig. 3). The crude extract silymarin was consistently one of the most potent inhibitors of CYP3A activity in microsomal preparations. Among single constituents, the relatively less abundant isosilybin A and isosilybin B (Davis-Searles et al., 2005; Wen et al., 2008) were two of the more potent inhibitors in both HLMs and HIMs (Fig. 3). Inhibitory kinetic parameters were recovered for isosilybin B, whereas metabolic lability (Fig. 2) and insolubility above 100 μ M precluded recovery of these parameters for isosilybin A. Inhibition of midazolam 1'-hydroxylation in HLMs by silymarin at 100 μ M (47%) was comparable to that by a different silymarin product at approximately 50 μ M (43%) in a previous study (Etheridge et al., 2007). With microsomal preparations, silibinin was a slightly more potent inhibitor of both nifedipine dehydrogenation (IC_{50} , 27-60 μ M) (Zuber et al., 2002) and testosterone 6 β -hydroxylation (IC_{50} , 50 μ M) (Jancova et al., 2007) compared to midazolam 1'-hydroxylation (IC_{50} , 67-120 μ M). Although these comparisons of IC_{50} values have inherent limitations, the apparent substrate-

dependent inhibition is consistent with multiple CYP3A4 substrate binding domains (Schrag and Wienkers, 2001; Galetin et al., 2003). Since midazolam was the only substrate tested in the current work, the reversible inhibition kinetics of individual constituents may not correlate with other CYP3A subclasses. In addition, the extent of inhibition by silymarin at 100 μM was greater than expected assuming additive inhibition by flavonolignans in aggregate (Fig. 3), suggesting the potential for synergistic inhibition of CYP3A by milk thistle flavonolignans. Further evaluation is needed to confirm this putative synergism.

Except for silychristin, all tested constituents and both extracts demonstrated potential MBI of CYP3A activity with HLMs, HIMs, and rCYP3A4 (Table 2), as evidenced by a >1.5-fold shift in IC_{50} (Berry and Zhao, 2008). However, the kinetic parameters associated with MBI could only be recovered using rCYP3A4. The K_i for silibinin in the current work (110 μM) was within, whereas the k_{inact} (0.20 min^{-1}) was roughly three times faster than, corresponding parameters reported using the CYP3A substrates 7-benzyloxy-4-(trifluoromethyl) coumarin (32 μM and 0.06 min^{-1}) and testosterone (132 μM and 0.08 min^{-1}) (Sridar et al., 2004). The difference in k_{inact} may reflect different enzyme sources (recombinant vs. reconstituted CYP3A4). Loss of rCYP3A4 activity was rapid, occurring within the 2-4 min incubation times for the reversible inhibition experiments. As such, some MBI would be expected under these conditions; however, the presence of midazolam should mitigate enzyme inactivation through substrate protection (Silverman and Daniel, 1995).

Unlike with rCYP3A4 (current work) and reconstituted CYP3A4 (Sridar et al., 2004), MBI was not evident with either microsomal preparation. P450-mediated metabolism can generate superoxide and/or hydrogen peroxide, potentially leading to enzyme autoinactivation (Kalgutkar et al., 2007). Isolated enzyme systems are believed to be more sensitive to reactive oxygen species, manifesting as MBI-like behavior; however, inclusion of reactive species scavengers would abrogate this process (Kalgutkar et al., 2007). The likely presence of CYP3A5 in the microsomal preparations also may have compensated for the loss of CYP3A4 activity. Unlike

the IC₅₀ shift experiment, saturating substrate concentrations were used for the MBI experiment, allowing potential compensation by CYP3A5 if this enzyme is less susceptible to MBI (McConn et al., 2004).

The major oxidative metabolite of silibinin is reported to be an O-demethylated product produced by CYP2C8 and CYP3A4 (Jancova et al., 2007). Further oxidation of the resulting catechol could create a reactive 1,2-benzoquinone moiety. However, MBI by this mode was unlikely, as MBI was not observed with compounds containing either the catechol (taxifolin) (data not shown) or the 2-methoxyphenol (silychristin) moiety (Fig. 1, Table 2). The only constituents that tested positive for MBI contained a 1,4-dioxane moiety (Fig. 1). Oxidation of this region may create reactive oxygen species capable of inactivating CYP3A4. Since activity loss was not abrogated by the trapping agents, the inactivating species exerted their effect before leaving the CYP3A4 active site. If this proposed mode of MBI is verified, this chemical moiety may be considered an addition to the list of structural alerts (Kalgutkar et al., 2007).

Following typical “doses” (<600 mg/day) of milk thistle preparations, individual constituents achieve relatively low peak systemic concentrations (C_{\max} <1 μ M) and are cleared rapidly from the systemic circulation ($t_{1/2}$ <4 h) (Wen et al., 2008). Accordingly, the interaction potential of milk thistle due to reversible inhibition likely is limited to inhibition of first-pass clearance of sensitive substrates. The clinical interaction potential for mechanism-based inhibitors is higher than that for reversible inhibitors, since restoration of P450 activity is dependent upon *de novo* protein synthesis, rather than removal of the perpetrator compound(s) (Watanabe et al., 2007). For example, MBI has been hypothesized as the only means by which fruit juices can elicit clinically significant interactions with CYP3A substrates (Hanley et al.). Whereas the maximum rates of rCYP3A4 inactivation by silybin A and silybin B were roughly half those reported for a major mechanism-based inhibitor in grapefruit juice, DHB (~0.20 vs. 0.41 min⁻¹) (Paine et al., 2004), the K_i s for silybin A and silybin B were approximately one hundred times greater than those for DHB (~100 vs. 1.1 μ M). Thus, at equivalent exposures,

DHB will inactivate CYP3A4 much more efficiently than the milk thistle constituents (370 vs. ~2 $\mu\text{l}/\text{min}/\text{pmol}$).

The high K_i and low systemic exposure of silybin A and silybin B following milk thistle administration indicate a low risk for an interaction between milk thistle at typical doses and midazolam. The much higher colorectal tissue concentrations of silibinin compared to peripheral blood concentrations (140 ± 170 vs. $3.0 \pm 2.3 \mu\text{M}$) following administration of 1.4 g/day to cancer patients (Hoh et al., 2006) further indicate that any clinically relevant MBI of CYP3A likely will be limited to the intestine. Enterocyte concentrations of silibinin this high would be expected to produce a 1.75-fold increase in systemic midazolam exposure based on the mechanistic static MBI equation (Obach et al., 2007; US FDA, 2012). Although the extent of MBI was not consistent between different enzyme preparations, predictions using parameters recovered from recombinant enzymes will provide more conservative estimates of interaction liability.

The large milk thistle doses required to achieve a relatively low risk of interaction with midazolam suggests that the clinical interaction liability of milk thistle may be limited to substrates sensitive to extensive intestinal first-pass extraction (e.g., simvastatin, felodipine) and/or to patient populations at risk for elevated milk thistle exposure (e.g., impaired hepatic function). Self-administration of milk thistle products is particularly popular among hepatitis C patients, in which an estimated 33% have used milk thistle as part of their therapeutic regimen (Seeff et al., 2008). Compared to healthy volunteers, patients with impaired hepatic function, including those with hepatitis C, demonstrated increased plasma exposure to milk thistle flavonolignans (Schrieber et al., 2008). Whereas the disease-related decrease in hepatic function alone could place these patients at increased risk for metabolic DDIs, intake of milk thistle products may provide an additional insult, increasing the risk for adverse events.

In summary, herb-drug interaction predictions are challenging due to the multitude of bioactive constituents typically composing herbal products. As such, identification of individual

perpetrator compound(s) is necessitated. The current work outlines a framework to facilitate prospective evaluation of herb-drug interaction potential using milk thistle as a model herbal product. Of the eight constituents tested, this approach identified two constituents, silybin A and silybin B, which may perpetrate interactions via MBI of intestinal CYP3A activity. Products enriched with these constituents, such as silibinin, may have increased herb-drug interaction liability compared to other milk thistle products. Intestinal concentrations of these constituents could achieve those near the K_i (~100 μ M), particularly with gram doses of silibinin that have been tested in patient populations (Flaig et al., 2007). Modeling and simulation incorporating these higher doses would help assess interaction liability of milk thistle products with CYP3A substrates sensitive to first-pass elimination. Refinement and application of this framework to other herbal products ultimately may assist clinicians and consumers to make informed decisions about the consequences of adding an herbal product to conventional pharmacotherapeutic regimens.

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Authorship Contributions

Participated in research design: Brantley, Graf, Oberlies, and Paine.

Conducted experiments: Brantley and Graf.

Contributed new reagents or analytic tools: Graf and Oberlies.

Performed data analysis: Brantley, Graf, Oberlies, and Paine.

Wrote or contributed to the writing of the manuscript: Brantley, Graf, Oberlies, and Paine.

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Footnotes

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- b. This work was presented in part as a poster: Brantley SJ, Graf TN, Oberlies NH, and Paine MF (2011) Identification of a single compound from the herbal supplement milk thistle as a candidate mechanism-based inhibitor of CYP3A activity. AAPS annual meeting and exposition; 2011 Oct 23-27; Washington, DC. American Association of Pharmaceutical Scientists, Washington DC.
- c. Reprint requests: Mary F. Paine, R.Ph., Ph.D.

2320 Kerr Hall, CB #7569

UNC Eshelman School of Pharmacy

University of North Carolina

Chapel Hill, NC 27599-7569

Office: (919) 966-9984

Fax: (919) 962-0644

Email: mpaine@unc.edu

Figure Legends

Fig. 1. Structures of flavonolignans and flavonoid (taxifolin) from milk thistle.

Fig. 2. Metabolic lability of selected milk thistle constituents in human liver microsomes. Microsomes (0.05 mg/mL) were incubated with silybin A (solid circle), silybin B (solid diamond), isosilybin A (open circle) or isosilybin B (open diamond) at 10 or 100 μ M in potassium phosphate buffer. Incubations were initiated with NADPH (1 mM) and quenched at designated times. Symbols and error bars denote means and SDs, respectively, of triplicate incubations.

Fig. 3. Inhibitory effects of flavonolignans on midazolam 1'-hydroxylation activity in human liver microsomes (A) and human intestinal microsomes (B). Microsomes (0.05 mg/mL) were incubated with midazolam (4 μ M) and flavonolignan (10 or 100 μ M; grey and black bars, respectively) for 2 (HLMs) or 4 (HIMs) min. Silymarin and silibinin "concentrations" were calculated by assuming a consistent molecular weight of 482 g/mol (Davis-Searles et al., 2005). Reactions were initiated with NADPH (1 mM). Midazolam 1'-hydroxylation activity in the presence of vehicle control [0.1% (v/v) DMSO] was 800 ± 40 pmol/min/mg or 280 ± 16 pmol/min/mg microsomal protein for HLMs and HIMs, respectively. Bars and error bars denote means and SDs, respectively, of triplicate incubations.

Fig. 4. IC₅₀ shift plot for silybin A, silybin B, and silibinin. Human liver microsomes (A), human intestinal microsomes (B), and recombinant CYP3A4 (C) were incubated with silybin A (circles), silybin B (diamonds) or silibinin (squares) (0.1-200 μ M) in the presence (open symbols) or absence (solid symbols) of NADPH (1 mM). The primary reaction mixture was diluted 5-fold to initiate the secondary reaction, which contained NADPH (1 mM) and midazolam (4 μ M). Midazolam 1'-hydroxylation activity in the presence of vehicle control [(0.1% (v/v) DMSO)] was 2000 ± 90 pmol/min/mg (HLMs), 350 ± 10 pmol/min/mg (HIMs), and 7.9 ± 1.0 pmol/min/pmol (rCYP3A4). Symbols and error bars denote means and SDs, respectively, of triplicate incubations. Open symbols denote observed data when NADPH was present in the primary incubation; solid symbols denote observed data when NADPH was absent in the primary

incubation. Curves denote nonlinear least-squares regression of observed data using WinNonlin (version 5.3).

Fig. 5. Effect of traditional reactive species scavengers. Human liver microsomes were incubated for 15 min with silybin A (100 μ M) and traditional reactive species scavengers in the presence or absence of NADPH. The primary reaction mixture was diluted 5-fold to initiate the secondary reaction, which contained NADPH (1 mM) and midazolam (4 μ M). Midazolam 1'-hydroxylation activity in the presence of vehicle control [(0.1% (v/v) DMSO)] was 1700 ± 46 pmol/min/mg microsomal protein. With the exception of glutathione and catalase (duplicate incubations), bars and error bars denote means and SDs, respectively, of triplicate incubations.

Fig. 6. Time- and concentration-dependent plot of CYP3A activity. Recombinant CYP3A4 was incubated with silybin A (A), silybin B (B), and silibinin (C) (0-200 μ M). The primary reaction mixture was diluted 50-fold to initiate the secondary reaction, which contained NADPH (1 mM) and midazolam (8 μ M), at designated times. Symbols denote means of duplicate incubations; all replicates deviated by <30%. Lines denote linear regression of the initial mono-exponential decline in activity. The inset depicts the rate of CYP3A4 inactivation as a function of inhibitor concentration. Symbols denote observed inactivation rates at each inhibitor concentration. Curves denote nonlinear least-squares regression of observed values using WinNonlin (version 5.3).

Table 1. Comparison of IC₅₀s for milk thistle constituents under reversible inhibition experimental design

Values represent the estimate \pm S.E. from nonlinear regression using WinNonlin (v5.3).

Enzyme Source	IC ₅₀ (μM)				
	Silybin A	Silybin B	Isosilybin A	Isosilybin B	Silychristin
HLMs ^a	180 \pm 16	150 \pm 7.6	N.D.	56 \pm 4.4	90 \pm 8.8
HIMs ^b	130 \pm 12	90 \pm 10	N.D.	60 \pm 4.8	93 \pm 8.7

N.D., not determined due to insolubility at concentrations > 100 μM

^aHuman liver microsomes

^bHuman intestinal microsomes

Table 2. Comparison of IC₅₀s for milk thistle constituents and extracts under IC₅₀ shift experimental design

Values represent the estimate ± S.E. from nonlinear regression using WinNonlin (v5.3)

Constituent/Extract	Enzyme Source								
	HLMs ^a			HIMs ^b			rCYP3A4 ^c		
	IC ₅₀ (μM)		Fold Change	IC ₅₀ (μM)		Fold Change	IC ₅₀ (μM)		Fold Change
	- NADPH ^d	+ NADPH ^e		- NADPH	+ NADPH		- NADPH	+ NADPH	
Silybin A	120 ± 4.8	28 ± 3.1	4.3	100 ± 6.9	23 ± 1.7	4.3	53 ± 2.0	1.6 ± 0.2	33
Silybin B	140 ± 5.7	19 ± 2.1	7.4	100 ± 3.8	27 ± 1.9	3.7	63 ± 3.2	1.4 ± 0.2	45
Silibinin	120 ± 5.7	14 ± 2.2	8.6	67 ± 7.9	15 ± 3.0	4.5	45 ± 2.8	1.2 ± 0.2	38
Silychristin	26 ± 3.3	52 ± 5.2	0.5	25 ± 4.2	72 ± 7.7	0.3	N.D.	N.D.	N.D.
Isosilybin B	78 ± 6.2	30 ± 3.3	2.6	54 ± 3.3	23 ± 2.3	2.3	N.D.	N.D.	N.D.
Silymarin	95 ± 12	28 ± 4.5	3.4	29 ± 2.7	7.0 ± 0.8	4.1	N.D.	N.D.	N.D.

N.D., not determined

^aHuman liver microsomes^bHuman intestinal microsomes^cBaculovirus insect cell-expressed CYP3A4^dNADPH was absent during the primary incubation^eNADPH was present during the primary incubation

Table 3. Inactivation kinetics of selected milk thistle constituents and extract

Values represent the estimate \pm S.E. from nonlinear regression using WinNonlin (v5.3)

Constituent/Extract	K_i (μM)	k_{inact} (min^{-1})	k_{inact} / K_i ($\mu\text{l/min/pmol}$)	$t_{1/2}$ (min)	k_{zero} (min^{-1})
Silybin A	100 ± 27	0.22 ± 0.02	2.2	3.2	0.025 ± 0.005
Silybin B	89 ± 60	0.15 ± 0.04	1.7	4.5	0.023 ± 0.008
Silibinin	110 ± 15	0.20 ± 0.01	1.8	3.5	0.016 ± 0.002

Figure 1

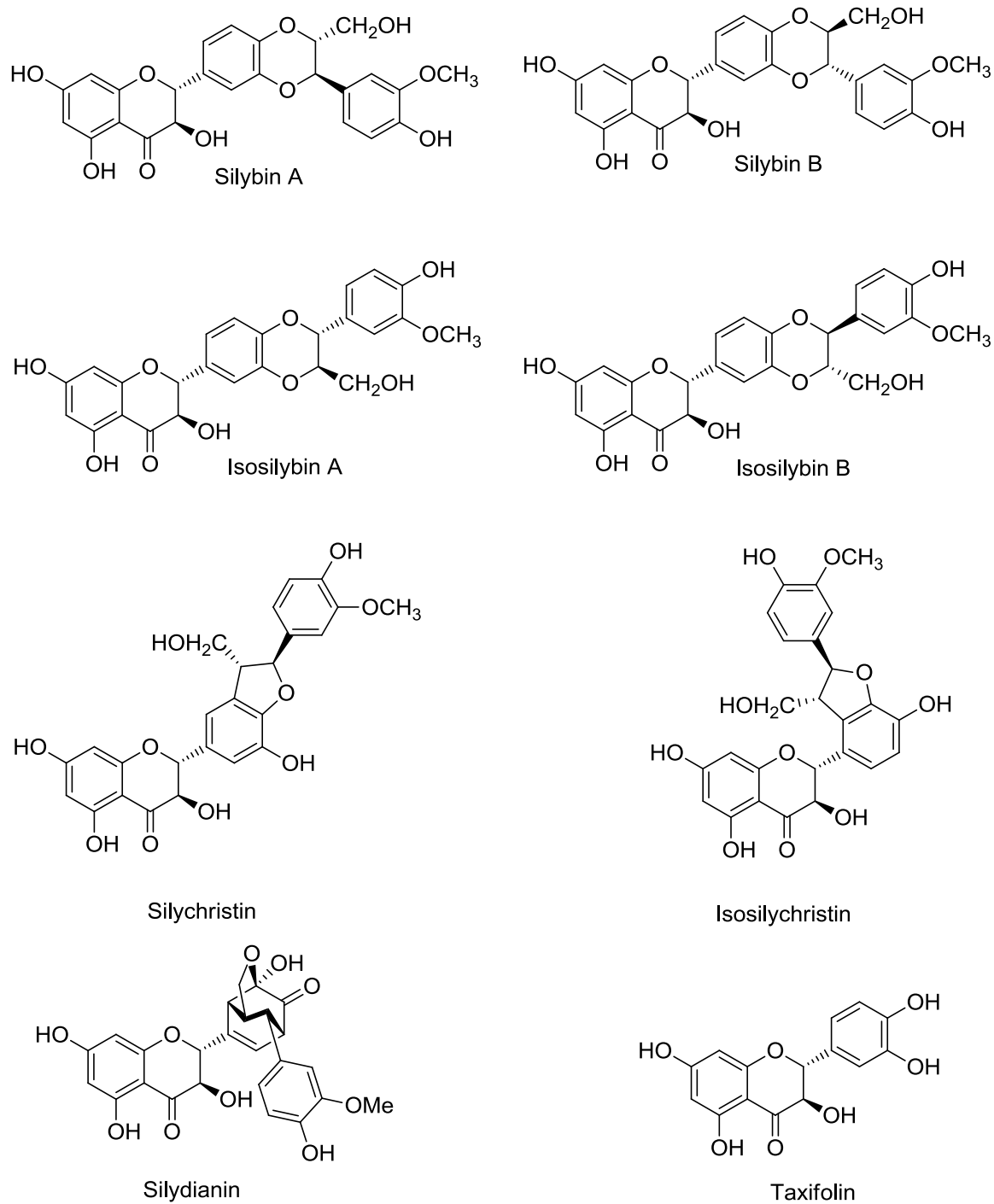


Figure 2

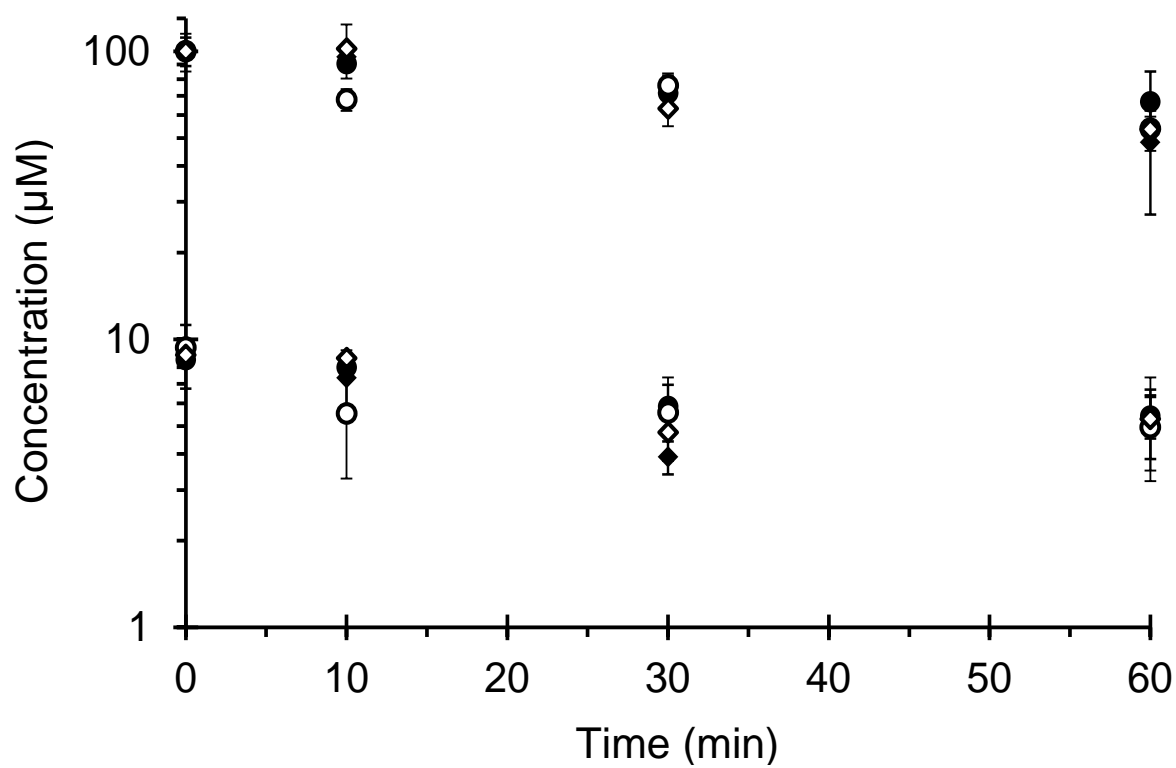


Figure 3

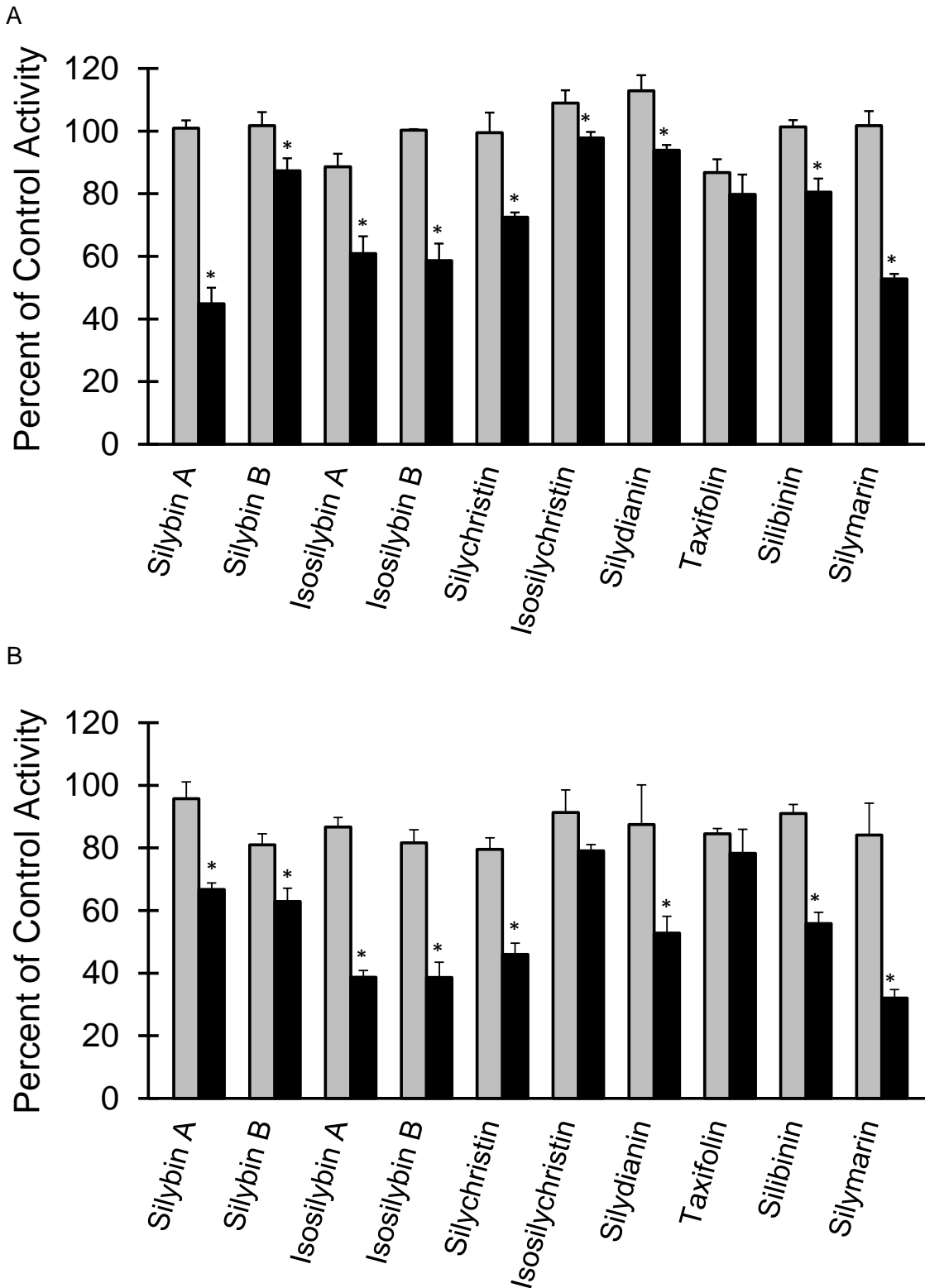


Figure 4

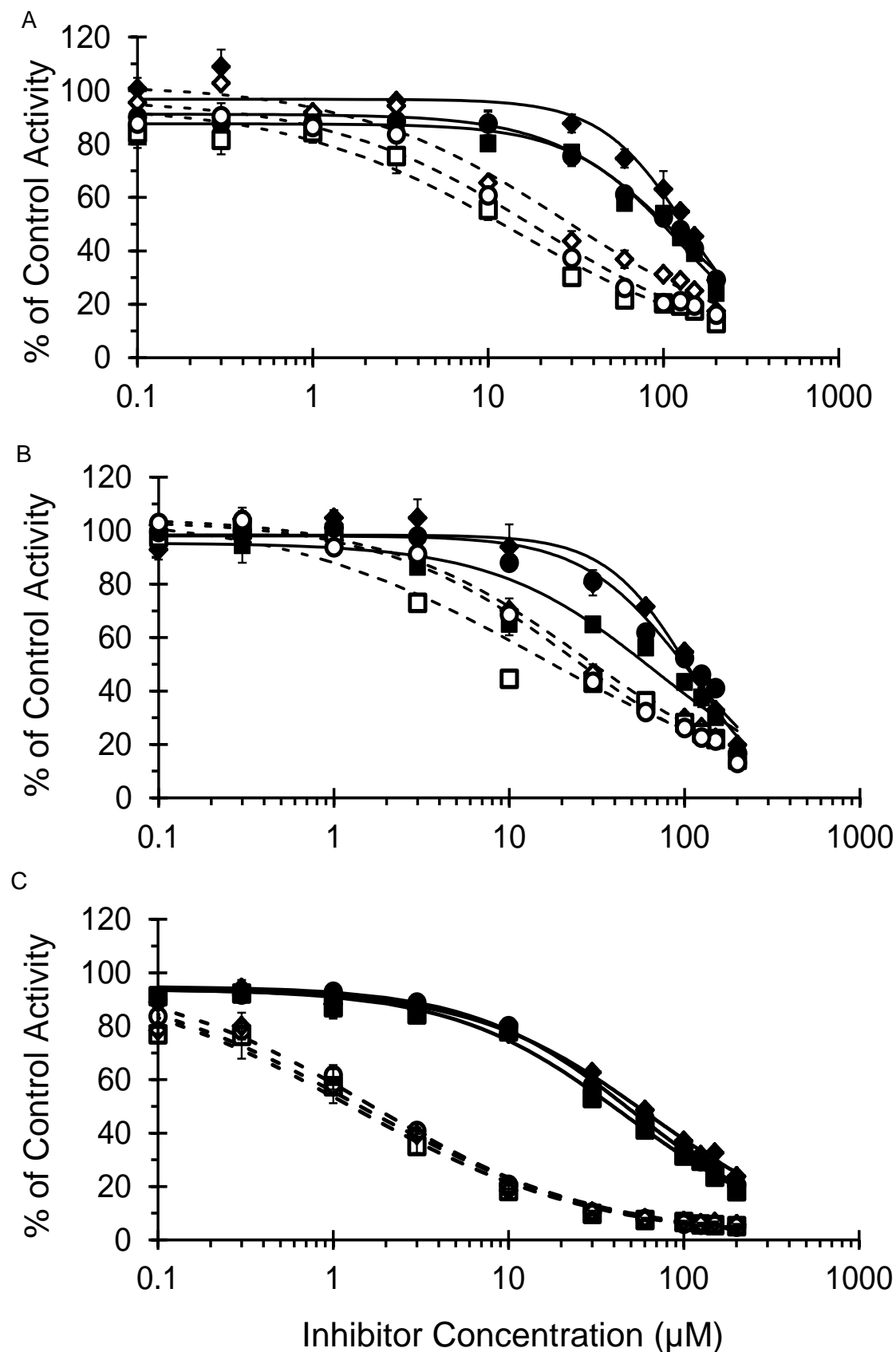


Figure 5

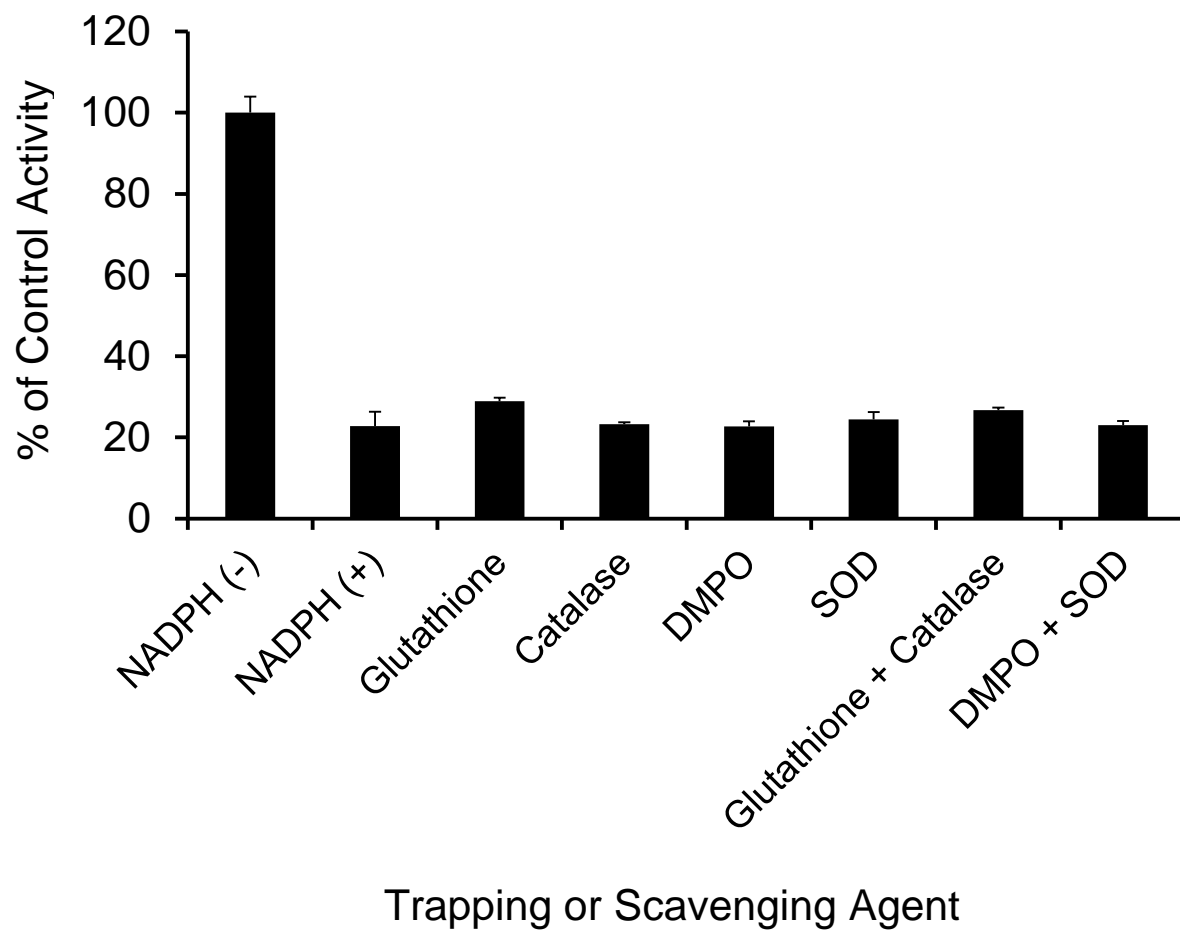


Figure 6

