Milk Thistle Constituents Inhibit Raloxifene Intestinal Glucuronidation: A Potential Clinically Relevant Natural Product–Drug Interaction

Brandon T. Gufford, Gang Chen, Ana G. Vergara, Philip Lazarus, Nicholas H. Oberlies, and Mary F. Paine

ABSTRACT

Women at high risk of developing breast cancer are prescribed selective estrogen response modulators, including raloxifene, as chemoprevention. Patients often seek complementary and alternative treatment modalities, including herbal products, to supplement prescribed medications. Milk thistle preparations, including silybin and silymarin, are top-selling herbal products that may be consumed by women taking raloxifene, which undergoes extensive first-pass glucuronidation in the intestine. Key constituents in milk thistle, flavonolignans, were previously shown to be potent inhibitors of intestinal UDP-glucuronosyl transferases (UGTs), with IC₅₀ values ranging from 65 to 120 μM; UGT1A1, 3.2–8.3 μM; UGT1A8, 19–73 μM; and UGT1A10, 65–120 μM) encompassed reported intestinal tissue concentrations (20–310 μM), prompting prediction of clinical interaction risk using a mechanistic static model. Silibinin and silymarin were predicted to increase raloxifene systemic exposure by 4- to 5-fold, indicating high interaction risk that merits further evaluation. This systematic investigation of the potential interaction between a widely used herbal product and chemopreventive agent underscores the importance of understanding natural product–drug interactions in the context of cancer prevention.

Introduction

Nearly one-quarter of a million women in the United States are newly diagnosed with breast cancer every year (Siegel et al., 2014). Women at increased risk for developing breast cancer due to family history, genetic markers, precancerous conditions, or other factors frequently are prescribed preventive medications (Visvanathan et al., 2013). Such medications, including selective estrogen response modulators (e.g., tamoxifen and raloxifene) and aromatase inhibitors (e.g., exemestane), reduce breast cancer development by up to 50% (Visvanathan et al., 2013). Despite the demonstrated success of pharmaceutical interventions, many patients (up to 33%) seek complementary and alternative treatment modalities, including herbal and other presumed medicinal natural products, to reduce side effects or complement efficacy of prescribed regimens (Gardiner et al., 2006; Davis et al., 2013; Lindstrom et al., 2014). Raloxifene is recommended to reduce the risk of estrogen receptor (ER)–positive invasive breast cancer in postmenopausal women (Visvanathan et al., 2013), a population in which nearly three of four individuals reportedly use herbal products (Gentry-Maharaj et al., 2015). Milk thistle (Silybum marianum) is a top-selling herbal product (Kroll et al., 2007; Davis et al., 2013; Lindstrom et al., 2014) that is used most commonly as a hepatoprotective (Polyak et al., 2013) and chemopreventive (Agarwal et al., 2006) agent. Milk thistle preparations include the crude extract, silybin, and the semipurified extract, silybin. Silymarin is composed of the flavonolignans silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin, the flavonoid taxifolin, and other uncharacterized polyphenols and fatty acids; silibinin is composed primarily of a 1:1 mixture of silybin A and silybin B (Davis-Searles et al., 2005; Kroll et al., 2007). Collectively, milk thistle extracts represent widely used herbal products that may be consumed by women taking raloxifene. Several milk thistle constituents, including silybin A and silybin B, are metabolized by members of the UDP-glucuronosyltransferase (UGT) superfamily of metabolizing enzymes, including UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9, and UGT1A10 (Jančová et al., 2011). In addition, several milk thistle constituents have been shown to inhibit human intestinal UGTs, including isoforms responsible for raloxifene clearance (Sridar

ABBREVIATIONS: ER, estrogen receptor; HEK293, human embryonic kidney 293; HIM, human intestinal microsome; HLM, human liver microsome; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; R4G, raloxifene 4′-glucuronide; R6G, raloxifene 6-glucuronide; UGT, UDP-glucuronosyl transferase; UHPLC, ultra high-performance liquid chromatography.
et al., 2004; Gufford et al., 2014a), raising concern for pharmacokinetic interactions mediated by modulation of these shared metabolic pathways.

Inhibition of intestinal drug metabolism is one pharmacokinetic mechanism underlying natural product-drug interactions. Mechanism-based inhibition of intestinal CYP3A4 by constituents in grapefruit juice is an extensively studied example that translates to clinically relevant based inhibition of intestinal CYP3A4 by constituents in grapefruit juice interactions (Won et al., 2010, 2012; Bailey et al., 2013). Raloxifene exhibits an extremely low oral bioavailability (<2%) due primarily to rapid presystemic glucuronidation in the intestine (Kemp et al., 2002; Dalvie et al., 2008; Cubitt et al., 2009). Glucuronidation is frequently described as a low-affinity, high-capacity metabolic process, resulting in relatively minimal impact on substrate exposure if perturbed (Williams et al., 2004; Mohamed and Frye, 2011). However, raloxifene is a high-affinity substrate (Km < 10 μM) compared with other UGT drug substrates (Km approximately 50 μM to 37 mM) (Kiang et al., 2005; Ritter, 2007). Such high affinity, coupled with the fact that raloxifene appears to be glucuronidated primarily by two intestinal UGT isoforms (UGT1A8 and UGT1A10) (Kemp et al., 2002; Jeong et al., 2005b; Sun et al., 2013), suggests that a marked increase in raloxifene bioavailability could result from inhibition of enteric glucuronidation.

The objective of this work was to assess, systematically, potential pharmacokinetic consequences when milk thistle preparations are taken concomitantly with raloxifene (Fig. 1). The aims were to 1) identify individual milk thistle constituents with the potential to inhibit raloxifene glucuronidation at physiologically plausible concentrations; 2) recover tissue-, isoform-, and pathway-specific inhibitory kinetic parameters; and 3) predict clinical interaction risk using a mechanistic static model. The information gained from this work will help to provide critical, evidence-based recommendations to both clinicians and consumers about the risk or safety of taking milk thistle products with raloxifene and potentially other chemopreventive or therapeutic medications.

Materials and Methods

Materials and Chemicals. Human liver microsomes (HLMs; pooled from 50 donors, mixed sex) and human intestinal microsomes (HIMs; pooled from 13 donors, mixed sex) were purchased from Xenotech, LLC (Lenexa, KS). Human embryonic kidney 293 (HEK293) cells overexpressing individual UGT1A enzymes were harvested, and homogenates were prepared as described previously (Sun et al., 2013). Raloxifene was purchased from BIOTANG Inc. (Lexington, MA). Raloxifene 4'-glucuronic acid (R4G) and raloxifene 6-glucuronic acid (R6G) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Alnemethicin, bovine serum albumin, magnesium chloride, naringin, nicardipine, saccharalactone, silybin, and UDP-glucuronic acid were purchased from Sigma-Aldrich (St. Louis, MO). Silymarin was obtained from Euromed S.A. (Barcelona, Spain) and consisted of silybin A (16%), silybin B (24%), isosilybin A (6.4%), isosilybin B (4.4%), silydianin (17%), silychristin (12%), and isosilychristin (2.2%); the remainder consisted of the flavonoid taxifolin (1.6%) and uncharacterized polyphenols and aliphatic fatty acids (Davis-Scales et al., 2005). Individual flavonolignans were purified as described previously (Graf et al., 2007) and were >97% pure as determined by ultra-high-performance liquid chromatography (UHPLC) (Napolitano et al., 2013). Dimethylsulfoxide, methanol (liquid chromatography/mass spectrometry grade), ethanol, Tris-HCl, Tris base, and formic acid were purchased from Fisher Scientific (Waltham, MA).

**Determination of Raloxifene Glucuronidation Kinetics.** Incubation conditions were optimized for linearity with respect to protein concentration and time and ensuring less than 20% substrate depletion (data not shown). Km and Vmax were obtained by fitting the simple Michaelis–Menten equation to [raloxifene] versus metabolite formation velocity data using Phoenix WinNonlin (version 6.3; Certara, St. Louis, MO) as shown in eq. 1:

\[ v = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \]  

where v denotes the velocity of metabolite formation, S denotes nominal substrate concentration, and Km denotes the substrate concentration corresponding to 50% of maximum velocity, denoted Vmax. Kinetic parameters were recovered for R4G and R6G formation using HIMs, HLMs, and UGT1A1- and UGT1A10-overexpressing HEK293 cell lysates. Intrinsic clearance (CLint) for R4G and R6G formation was calculated as the ratio of Vmax to Km.

**Initial Evaluation of Milk Thistle Flavonolignans and Extracts as Inhibitors of Raloxifene Glucuronidation.** Milk thistle flavonolignans and associated extracts (silibinin, silymarin) were evaluated as inhibitors of raloxifene glucuronidation using HIMs and UGT1A8- and UGT1A10-overexpressing HEK293 cell lysates. In addition, silybin A, silybin B, silibinin, and silymarin were evaluated as inhibitors of raloxifene glucuronidation using HLMs and UGT1A1-overexpressing cell lysates. Incubation mixtures (150 μl total volume) consisted of the following: HIMs (0.05 mg/ml), HLMs (0.05 mg/ml), or UGT1A1-overexpressing HEK293 cell lysates. Incubation mixtures (150 μl total volume) consisted of the following: HIMs (0.05 mg/ml), HLMs (0.05 mg/ml), or UGT1A1-overexpressing cell lysates (0.1, mg/ml for UGT1A1 and UGT1A8 and 0.05 mg/ml for UGT1A10); raloxifene at a concentration approximating the experimentally determined Km for each enzyme source (based on the above) [1 μM (HIMs), 4 μM (HLMs), 4 μM (UGT1A1), 15 μM (UGT1A8), or 0.9 μM (UGT1A10)]; flavonolignan/extract (1, 10, or 100 μM) or the prototypic UGT inhibitor, nicardipine (400 μM) (Latham et al., 2012); bovine serum albumin (0.05%); alamethicin (50 μg/ml protein); saccharalactone (100 μM); and Tris-HCl buffer supplemented with magnesium chloride (5 mM). HLMs, HIMs, and cell lysates were activated by incubating with alamethicin on ice for 15 minutes. Mixtures were equilibrated at 37°C for 5 minutes before initiating the reactions with UDP-glucuronic acid (2 mM final concentration). Reactions were terminated after 4 minutes (HIMs, HLMs, UGT1A10) or 6 minutes (UGT1A1, UGT1A8) by removing 100 μl from the incubation and diluting into 300 μl of ice-cold methanol containing internal standard (naringin, 100 nM). Samples were centrifuged (3000 × g, 10 minutes, 4°C), and 200 μl of supernatant was removed and transferred to clean 96-well plates with polypropylene inserts. Samples were dried under nitrogen purge and reconstituted in 100 μl of 40% methanol in water containing 0.1% formic acid for analysis by UHPLC coupled to tandem mass spectrometry (MS/MS).

**Quantification of R4G and R6G by UHPLC-MS/MS.** Chromatographic separation was achieved using an HSS T3 column (1.8 μM, 2.1 × 50 mm) with a Vanguard Pre-Column (2.1 × 5 mm) Waters Corporation, Waltham, MA) heated to 45°C and a binary gradient at a flow rate of 0.35 ml/min. R4G and R4G retention times were 2.3 and 4.0 minutes, respectively, which are identical to both commercially acquired and experimentally generated glucuronide metabolites. The gradient elution began with 60:40 water/methanol (each with 0.1% formic acid) and increased linearly to 50:50 over 10 minutes before returning to initial conditions over 0.5 minutes and holding for 0.5 minutes; the total run time was 11 minutes. Samples were analyzed (3 μl injection volume) using the QTRAP 6500 UHPLC-MS/MS system (AB Sciex, Framingham, MA) with the turbo electrospray source operated in negative ion mode. The declustering potential and collision energy were set at −38 V and −25 mV, respectively. R4G (648.1→472.1 m/z),
IC$_{50}$s were estimated from the initial two- or three-point inhibitor screening concentrations by UHPLC-MS/MS. Initial estimates of apparent $K_m$ and $V_{max}$ were derived from Michaelis–Menten fits of the velocity versus [substrate] data in the absence of inhibitor. Initial estimates of apparent $K_m$ and/or $K_i$ were derived from Lineweaver–Burk plots of velocity$^{-1}$ versus [substrate]$^{-1}$. Kinetic parameters ($K_m$, $V_{max}$, $K_i$, $K_a$) were obtained by fitting eqs. 2, 3, or 4 to untransformed data via nonlinear least-squares regression using Phoenix WinNonlin software:

**Competitive**

$$v = \frac{V_{max} \times [S]}{K_m(1 + \frac{I}{K_i})}$$

(2)

**Uncompetitive**

$$v = \frac{V_{max} \times [S]}{K_m + [S](1 + \frac{I}{K_i})}$$

(3)

**Mixed**

$$v = \frac{V_{max} \times [S]}{K_m(1 + \frac{I}{K_i}) + [S](1 + \frac{I}{K_i})}$$

(4)

where $[I]$ denotes inhibitor concentration, $K_m$ denotes the affinity of the inhibitor toward the “free” enzyme, and $K_i$ denotes the affinity of inhibitor toward the enzyme-substrate complex. The best-fit equation was determined by visual inspection of Lineweaver–Burk plots and corresponding slope and intercept replots and the randomness of the residuals, Akaike information criteria, and S.E.s of the parameter estimates generated from the nonlinear regression procedure.

**Mechanistic Static Model Prediction of the Milk Thistle–Raloxifene Interaction.** Identification of marker constituents within a natural product represents an attractive approach to characterize and predict dietary substance–drug interactions or natural product–drug interactions (Ainslie et al., 2014). Identification and isolation of such constituents facilitates adaptation of established systems for the prediction of drug–drug interactions to predict the magnitude and likelihood of natural product–drug interactions (Brantley et al., 2014a). The impact of reversible inhibition of raloxifene intestinal metabolism by milk thistle flavonolignans and extracts on raloxifene systemic exposure was predicted using a mechanistic static model (eqs. 5 and 6) (Fahmi et al., 2009):

$$\frac{AUC_i}{AUC} = \frac{1}{A_k(1 - F_i) + F_i}$$

(5)

$$A_k = \frac{1}{1 + \frac{I}{K_i}}$$

(6)

where AUC$_i$/AUC is the ratio of the area under the victim drug (raloxifene) plasma concentration–time curve in the presence to the absence of inhibitor; $A_k$ is the term denoting reversible inhibition of gut metabolism; $F_i$ is the fraction of drug escaping intestinal extraction, estimated to be 5.4% for raloxifene (Mizuma, 2009); $I$ is the inhibitor concentration in the gut, estimated at 140 μM for silibinin (Hoh et al., 2006) and silymarin and assumed to be approximately 70 μM for silybin A or silybin B alone based upon their relative contribution to silibinin content (Davis-Searles et al., 2005; Kroll et al., 2007); and $K_i$ is the experimentally determined parameter recovered with pooled HIMs.

**Statistical Analysis.** Data are presented as means ± S.D.s of triplicate incubations unless noted otherwise. $K_i$s are presented as estimates ± S.E.s.

**Results**

**Raloxifene Glucuronidation Is Tissue and Isoform Dependent.** Consistent with previous reports (Kemp et al., 2002; Jeong et al., 2005b; Dalvie et al., 2008; Chang et al., 2009; Trdan Lusin et al., 2011; Sun et al., 2013), R4G was the dominant metabolite formed in all enzyme systems except UG1T1A1-overexpressing HEK293 cell lysates (Fig. 2; Table 1). UGT1A10-overexpressing cell lysates generated both R4G and R6G, in contrast with a previous report of selective R4G formation in this system (Sun et al., 2013). This discrepancy is likely attributable to enhanced analytical sensitivity, permitting detection of the lesser formed R6G (approximately one-tenth versus R4G formation) (Table 1). Considering the relative expression of UGT1A8 and UGT1A10 in HIMs (Harbort et al., 2012; Fallon et al., 2013; Sun et al., 2013; Wu et al., 2013), calculated CL$_{int}$s from the HEK293 cell lysates represented a reasonable depiction of the contribution of each isoform to raloxifene glucuronidation in HIMs. Raloxifene glucuronidation demonstrated substrate inhibition kinetics at supraphysiologic substrate concentrations.
 (>20 μM) in both HIMs and HLMs (data not shown). As observed previously (Kemp et al., 2002; Trdan Lusin et al., 2011; Sun et al., 2013), raloxifene was metabolized more efficiently by HIMs compared with HLMs, due largely to the much lower K_{in} recovered from HIMs than from HLMs (Table 1).

**Milk Thistle Flavonolignans and Extracts Differentially Inhibit Raloxifene Glucuronidation.** Relative to vehicle control, milk thistle flavonolignans and extracts inhibited raloxifene glucuronidation in a concentration-dependent manner (Fig. 3). Based on results from the initial screen with HIMs, silybin A and silybin B (the most abundant individual flavonolignans present in silibinin and silymarin; Davis-Searles et al., 2005; Kroll et al., 2007) were characterized further for inhibitory potential using UGT1A8-, UGT1A10-, and UGT1A1-overexpressing cell lysates and HLMs (Fig. 3). The inhibition kinetics of these flavonolignans and the two extracts toward raloxifene glucuronidation were described best by a competitive inhibition model. Silybin A and silybin B inhibited both glucuronidation pathways with comparable potency; likewise, silibinin and silymarin inhibited these pathways with comparable potency (Table 2). The K_{in} of the flavonolignans and extracts were up to 50 times lower than reported average silibinin concentrations measured in colorectal tissue specimens (approximately 140 μM) (Hoh et al., 2006). The flavonolignans/extracts were most potent toward UGT1A1-mediated raloxifene glucuronidation (K_{in} of 3.2–8.3 μM) (Fig. 4; Table 2), consistent with previous reports involving other UGT substrates (Sridar et al., 2004; Gufford et al., 2014a). The K_{in} recovered with HIMs (27–66 μM) approximated the mean of the K_{in} recovered with UGT1A8- and UGT1A10-overexpressing cell lysates (19–73 μM and 65–120 μM, respectively), providing further evidence of the dominant contribution of these two isoforms to raloxifene intestinal glucuronidation (Sun et al., 2013). Potent inhibition of UGT1A1 and UGT1A8, isoforms highly expressed in the jejunum that are critical to raloxifene clearance (Sun et al., 2013), prompted prediction of interaction risk of concomitant raloxifene and milk thistle usage.

A Mechanistic Static Interaction Model Predicts Milk Thistle Constituents to Have High Interaction Risk with Raloxifene. The mechanistic static interaction model (eq. 5) predicted a 4.7- or 4.3-fold increase in raloxifene area under the curve in the presence of silymarin or silibinin, respectively, indicating high interaction risk (Food and Drug Administration Center for Drug Evaluation and Research, 2012). The model predicted a 3-fold increase in the raloxifene area under the curve in the presence of silybin A or silybin B alone, supporting these two constituents as potential marker constituents predictive of the interaction risk of the more complex mixtures.

**Discussion**

Potential interactions between conventional medications and natural products, including herbal products, are increasingly recognized in clinical practice. Despite concerns of clinicians, consumers, and regulators regarding unwanted natural product–drug interactions, systematic approaches to identify and characterize the risk of these interactions remain elusive. Approaches focusing on the adaptation of established paradigms used to assess drug–drug interaction risk have been proposed to elucidate natural product–drug interaction risk (Won et al., 2010, 2012; National Center for Complementary and Alternative Medicine, 2012; Brantley et al., 2013, 2014a,b; Ainslie et al., 2014; Gufford et al., 2014a; Barr et al., 2015). Assessment of these interactions involves challenges that extend beyond drug–drug interactions (Gufford et al., 2014b). Unlike drugs, natural products frequently are mixtures of potentially bioactive constituents with immense compositional variation both within and between lots. A proposed framework (Fig. 1) entails evaluation of isolated constituents and complex mixtures to ascertain the relative contribution of each constituent in a mixture. Identification of marker constituents predictive of the likelihood and magnitude of complex interactions enables simplified and reproducible assessment of natural product–drug interaction risk. This framework was used in the current work to evaluate silybin A and silybin B as potential marker constituents representative of the interaction potential of semipurified (silibinin) and crude (silymarin) milk thistle extracts. These constituents were selected based on relative inhibition potency, abundance in milk thistle extracts, and available pharmacokinetic data to facilitate model predictions of interaction risk.

Studies in breast cancer cell lines have demonstrated antioxidant, antiproliferative, and cytotoxic effects of silibinin and silymarin (Scambia et al., 1996; Zi et al., 1998; Agarwal et al., 2006; Provinciali et al., 2007; Ramasamy and Agarwal, 2008). Although these potentially beneficial effects have not been characterized rigorously in the clinic, patients often purchase over-the-counter, perceived-as-safe supplements to augment prescribed therapeutic regimens without consulting their healthcare providers (Gardiner et al., 2006; Kennedy et al., 2008). Women aged 30–69 years who are current or former smokers and have chronic health conditions are some of the most common consumers of herbal products, and these individuals are also at increased risk for developing breast cancer (Barnes et al., 2008). These observations raise concern for interactions between pharmaceutical interventions to reduce breast cancer risk and loosely regulated, poorly understood herbal products.

This work is the first to rigorously evaluate the tissue-specific (HIMs and HLMs), isoform-specific (UGT1A1, UGT1A8, and UGT1A10), and pathway-specific (R4G versus R6G formation) UGT inhibition of a clinically relevant probe substrate by individual natural product constituents and related extracts. Potent inhibition of raloxifene glucuronidation by milk thistle extracts/constituents with pooled HIMs was further supported by inhibition observed with UGT1A1-overexpressing HEK293 cell lysates. Although UGT1A10 most efficiently converts raloxifene to the predominant circulating metabolite, R4G, the approximately 10–20 times lower jejunal expression compared with UGT1A1 and UGT1A8 suggests that UGT1A1 and UGT1A8 have

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>R4G</th>
<th>R6G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_{in}</td>
<td>V_{max}</td>
</tr>
<tr>
<td>HIMs</td>
<td>0.81 ± 0.11 μM</td>
<td>750 ± 22 µmol/min per mg</td>
</tr>
<tr>
<td>HLMs</td>
<td>3.0 ± 0.73 μM</td>
<td>850 ± 53 µmol/min per mg</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>4.3 ± 0.51 μM</td>
<td>88 ± 2.7 µmol/min per mg</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>5.5 ± 0.48 μM</td>
<td>550 ± 14 µmol/min per mg</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>0.87 ± 0.08 μM</td>
<td>1100 ± 28 µmol/min per mg</td>
</tr>
</tbody>
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important roles in the intestinal clearance of raloxifene (Sun et al., 2013). The $K_i$ of silybin A and silybin B toward UGT1A1- and UGT1A8-mediated raloxifene glucuronidation were well below mean silibinin concentrations measured in colorectal tissue specimens obtained from cancer patients administered oral silibinin (1400 mg) (<50 μM versus approximately 140 μM) (Hoh et al., 2006).

Model predictions indicated that concomitant administration of raloxifene with silibinin or silymarin may increase raloxifene systemic exposure by 4-fold to 5-fold, indicating high interaction risk (Food and Drug Administration Center for Drug Evaluation and Research, 2012). In addition, silybin A or silybin B alone was predicted to increase raloxifene systemic exposure by approximately

![Image](image_url)

**Fig. 3.** Screening of milk thistle flavonolignans and extracts as inhibitors of raloxifene glucuronidation using HIMs (upper panel) and an abbreviated screening using UGT1A8-, UGT1A10-, and UGT1A1-overexpressing cell lysates or HLMs (lower panel). Values are expressed as a percent of the activity compared with vehicle control (blue bar, 0.1% dimethylsulfoxide). Black, gray, and white bars denote 100 μM, 10 μM, and 1 μM, respectively. Red bars denote the positive control UGT inhibitor nicardipine (400 μM). Bars and error bars denote means and S.D.s, respectively, of triplicate incubations. CTRL, control.

**TABLE 2**

$K_i$ determinations for selected milk thistle flavonolignans and extracts toward raloxifene glucuronidation by HIMs or UGT1A-overexpressing HEK293 cell lysates

Apparent $K_i$ were determined by fitting eq 2 with observed metabolite (R4G or R6G) formation velocities under varying substrate and inhibitor conditions. Values represent the $K_i$ estimate ± S.E. from nonlinear least-squares regression using Phoenix WinNonlin software (version 6.3).

<table>
<thead>
<tr>
<th>Flavonolignan/Extract</th>
<th>HIMs</th>
<th>UGT1A1</th>
<th>UGT1A8</th>
<th>UGT1A10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silybin A</td>
<td>59 ± 6.1</td>
<td>56 ± 5.1</td>
<td>5.0 ± 0.65</td>
<td>4.2 ± 0.48</td>
</tr>
<tr>
<td>Silybin B</td>
<td>66 ± 6.0</td>
<td>31 ± 3.2</td>
<td>3.3 ± 0.39</td>
<td>2.8 ± 0.34</td>
</tr>
<tr>
<td>Silibinin</td>
<td>33 ± 3.2</td>
<td>27 ± 2.9</td>
<td>3.2 ± 0.33</td>
<td>2.8 ± 0.25</td>
</tr>
<tr>
<td>Silymarin</td>
<td>28 ± 3.1</td>
<td>27 ± 2.7</td>
<td>8.3 ± 1.1</td>
<td>6.7 ± 0.85</td>
</tr>
</tbody>
</table>

µM

79 ± 7.9 72 ± 7.7
74 ± 8.6 65 ± 7.6
120 ± 19 86 ± 12
91 ± 14 81 ± 12
3-fold, supporting these constituents as marker constituents predictive of the complex mixtures.

Transport proteins, including organic anion-transporting polypeptides (OATPs), multidrug resistance proteins, and P-glycoprotein (P-gp) have been reported to contribute to the disposition of raloxifene, R4G, R6G (Jeong et al., 2004, 2005a; Chang et al., 2006; Trdan Lušin et al., 2012a, b), and milk thistle flavonolignans (Miranda et al., 2008; Köck et al., 2013; Wlcek et al., 2013). The risk of interactions via milk thistle flavonolignan inhibition of OATP1B1- and OATP1B3-mediated hepatic uptake is believed to be low, based on limited systemic exposure (Köck et al., 2013). Similarly, silibinin may inhibit multidrug resistance protein 2-mediated canalicular efflux of raloxifene and its conjugates, but hepatocellular concentrations are likely too low to elicit clinically meaningful effects (Wlcek et al., 2013). Inhibition of P-gp-mediated raloxifene intestinal efflux by milk thistle flavonolignans may contribute to the overall effect of the milk thistle–raloxifene interaction (Trdan Lušin et al., 2012a). However, oral administration of silymarin did not significantly alter the pharmacokinetics of the P-gp probe substrate digoxin in healthy volunteers (Gurley et al., 2006). Collectively, because available data suggest a limited contribution of transport proteins to a potential milk thistle–raloxifene interaction, modulation of raloxifene transport was not included in the assessment of interaction risk. Nevertheless, modulation of transporters by flavonolignan glucuronides, which are the major circulating metabolites (Schrieber et al., 2008; Wen et al., 2008; Zhu et al., 2013), cannot be dismissed. Quantitative evaluation of flavonolignan glucuronides as modulators of transport activity would enhance the understanding of complex mechanisms underlying glucuronide disposition and potential interactions. However, lack of commercially available flavonolignan glucuronides precluded this effort.

Increased exposure to intact raloxifene may place patients at increased risk for adverse events, including hot flashes and venous thromboembolism. However, previous studies have demonstrated that intact raloxifene has an approximately 100-fold higher affinity for the ER than R4G (Sun et al., 2013). This observation suggests that the presumed silibinin–raloxifene interaction could be beneficial by enhancing the therapeutic effects of raloxifene at the ER by boosting systemic exposure to intact raloxifene. Application of this concept would require thorough characterization of the pharmacodynamic and therapeutic consequences of this pharmacokinetic natural product–drug interaction (Won et al., 2010, 2012; Brantley et al., 2014a).

In summary, milk thistle constituents and extracts inhibited the primary intestinal UGT1A isoforms responsible for the detoxification of raloxifene at concentrations encompassed by those measured in human intestinal tissue specimens. Using a mechanistic static model, the milk thistle products silymarin and silibinin were predicted to increase raloxifene systemic exposure by 4- to 5-fold. This systematic investigation of the potential interaction between a widely used herbal product and chemopreventive agent underscores the importance of understanding natural product–drug interactions in the context of cancer prevention. Dynamic modeling and simulation approaches (e.g., physiologically based pharmacokinetic modeling), and potentially clinical assessment, are needed to elucidate the clinical consequences of these complex interactions and provide recommendations to clinicians and consumers that will help guide therapeutic decisions.

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Authorship Contributions
Participated in research design: Gufford, Paine.
Conducted experiments: Gufford, Vergara.
Contributed new reagents or analytic tools: Chen, Lazarus, Oberlies, Vergara.
Performed data analysis: Gufford, Paine.
Wrote or contributed to the writing of the manuscript: Gufford, Chen, Lazarus, Oberlies, Paine.

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