A Pharmacokinetic Natural Product-Disease-Drug Interaction: A Double Hit of Silymarin and Nonalcoholic Steatohepatitis on Hepatic Transporters in a Rat Model

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ABSTRACT

Patients with nonalcoholic steatohepatitis (NASH) exhibit altered hepatic protein expression of metabolizing enzymes and transporters and altered xenobiotic pharmacokinetics. The botanical natural product silymarin, which has been investigated as a treatment of NASH, contains flavonolignans that inhibit organic anion–transporting polypeptide (OATP) transporter function. The purpose of this study was to assess the individual and combined effects of NASH and silymarin on the disposition of the model OATP substrate pitavastatin. Male Sprague Dawley rats were fed a control or a methionine- and choline-deficient diet (NASH model) for 8 weeks. Silymarin (10 mg/kg) or vehicle followed by pitavastatin (0.5 mg/kg) were administered intravenously, and a control or a methionine- and choline-deficient diet (NASH model) for 8 weeks. Silymarin (10 mg/kg) or vehicle followed by pitavastatin (0.5 mg/kg) were administered intravenously, and the pharmacokinetics were determined. NASH increased mean total flavonolignan area under the plasma concentration-time curve (AUC0–120 min) 1.7-fold. Silymarin increased pitavastatin AUC0–120 min in both control and NASH animals approx. 2-fold. NASH increased pitavastatin plasma concentrations from 2 to 40 minutes, but AUC0–120 min was unchanged. The combination of silymarin and NASH had the greatest effect on pitavastatin AUC0–120 min, which increased 2.9-fold compared with control vehicle-treated animals. NASH increased the total amount of pitavastatin excreted into the bile 2.7-fold compared with control animals, whereas silymarin decreased pitavastatin biliary clearance approx. 3-fold in both control and NASH animals. This double hit of NASH and silymarin on hepatic uptake transporters is another example of a multifactorial pharmacokinetic interaction that may have a greater impact on drug disposition than each hit alone.

SIGNIFICANCE STATEMENT

Multifactorial effects on xenobiotic pharmacokinetics are within the next frontier for precision medicine research and clinical application. The combination of silymarin and NASH is a probable clinical scenario that can affect drug uptake, liver concentrations, biliary elimination, and ultimately, efficacy and toxicity.

Introduction

Nonalcoholic steatohepatitis (NASH) is a severe form of nonalcoholic fatty liver disease (NAFLD) (Chalasani et al., 2018). An emerging concern for NASH patients is altered expression and function of drug-metabolizing enzymes and transporters, potentially leading to altered pharmacokinetics of xenobiotics. For example, previous studies suggested that decreased sinusoidal expression of hepatic organic anion–transporting polypeptide (OATP) uptake transporters may be responsible for the net 1.4-fold increase in systemic concentrations of 99mTc-mebrofenin observed in NASH patients (Ali et al., 2017; Clarke et al., 2017). In addition, impaired canalicular localization of the efflux transporter multidrug resistance-associated protein (MRP)2 and increased sinusoidal expression of MRP3 may be responsible for higher plasma retention of morphine glucuronides in rodent models of NASH and in NASH patients (Hardwick et al., 2011, 2012, 2013; Dzierlenga et al., 2015; Ferslew et al., 2015). These changes in xenobiotic disposition may alter therapeutic and adverse drug responses in NASH patients.

The high clinical burden of NAFLD has spurred investigation of novel therapeutic agents, including the botanical natural product silymarin. Silymarin, an extract prepared from the seeds of milk thistle [Silybum marianum (L.) Gaertn.] as well as other milk thistle preparations have been used for centuries to treat liver and gallbladder disorders (Abenavoli et al., 2010). Milk thistle has remained in the top 20 best-selling herbal dietary supplements in...
the U.S. mainstream multoutlet channel for the past 30 years, with 16.8 million dollars in sales in 2017 (Smith et al., 2018). Evidence for the efficacy of silymarin in NAFLD patients is limited, although incorporation of silymarin into treatment plans, either alone or in combination with other agents, continues to be investigated (Abenavoli and Bellantani, 2013). Multiple studies have demonstrated that silymarin is well tolerated at doses greater than one gram, and in NAFLD patients specifically silymarin doses of 700 mg three times daily have been used safely (Haweke et al., 2010; Abenavoli and Bellantani, 2013; Colica et al., 2017; Fathallah et al., 2017; Wah Kheong et al., 2017). Thus, NAFLD patients are a target population for silymarin use and may be taking more than the 140-mg dose that is commonly recommended (Zhu et al., 2013). In vitro studies indicate that silymarin, which is a complex of at least seven flavonolignans, inhibits human OATP1B1, OATP1B3, and OATP2B1 (IC50: 0.3–3.2 μM) and the canalicular efflux transporter breast cancer–resistance protein (BCRP) (Kp, 97 μM) (Deng et al., 2008; Köck et al., 2013).

The objective of this study was to determine the effects of NASH and silymarin, both alone and in combination, on the pharmacokinetics of the clinical OATP probe substrate pitavastatin (Prueksaritanont et al., 2014). Using an established rat model of NASH (Canet et al., 2014), the study was designed to mimic systemic concentrations of silymarin flavonolignans in NAFLD patients taking 700 mg of silymarin. An intravenous administration route was selected to isolate hepatic uptake and efflux processes by bypassing potential variability in oral absorption of pitavastatin and silymarin. Results suggest that the presence of NASH may increase the risk of disease-drug interaction for this OATP-mediated natural product.

**Materials and Methods**

**Animals.** Handling, care, and maintenance of the animals took place in the Program of the Laboratory Animal Resources facility of Washington State University, Spokane, which is accredited by the Association for the Assessment of Laboratory Animal Care International. All animals were maintained in 12-hour light and dark cycles for the duration of the study. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Washington State University. Eight-week-old male Sprague-Dawley rats (n = 16) were purchased from Envigo (Huntingdon, Cambridgeshire, UK). Animals were randomly placed in cages (two per cage) with diamond particle bedding and fed either a control diet (cat. no. D518754; Dyets Inc., Bethlehem, PA) or a methionine- and choline-deficient diet (cat. no. D518810; Dyets Inc.). The latter diet is the best rat model for NASH and silymarin, both alone and in combination, on the pharmacokinetics of the clinical OATP probe substrate pitavastatin (Prueksaritanont et al., 2014). Using an established rat model of NASH (Canet et al., 2014), the study was designed to mimic systemic concentrations of silymarin flavonolignans in NAFLD patients taking 700 mg of silymarin. An intravenous administration route was selected to isolate hepatic uptake and efflux processes by bypassing potential variability in oral absorption of pitavastatin and silymarin. Results suggest that the presence of NASH may increase the risk of disease-drug interaction for this OATP-mediated natural product.

**Western Blot Analysis.** Livers were homogenized in NP-40 lysis buffer with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche, Basel, Switzerland) (100 mg tissue/1 ml buffer) using a TissueLyzer II (Qiagen, Hilden, Germany) with two metal beads (2.4 mm) and the following protocol: 30 Hz for 3 minutes, transfer to ice for 5 minutes, 30 Hz for 3 minutes. Cellular debris was removed by centrifugation at 15,000 × g for 10 minutes at 4°C. Ten micrograms of total protein were loaded into 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes with a Bio-Rad (Hercules, CA) Trans-Blot Turbo system at 25 V/1.0A for 30 minutes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/ Tween 20 and incubated with the following antibody conditions: OATP1B1 (cat. no. D376904, 1:1000; mouse secondary 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) and extracellular signaling-regulated kinase (Erk) 1 and 2 (cat. nos. 271269 and 271202; 1:1000; Santa Cruz Biotechnology). Signal was detected with SuperSignal West Femto (ThermoFisher Scientific, Waltham, MA) and images captured using a Bio-Rad ChemiDoc imager. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

**Liquid Chromatography/Mass Spectrometry for Pitavastatin.** Quantification of pitavastatin was adapted from published methods (Qi et al., 2013). Chromatographic separation and quantification was performed on a QTRAP 6500 UHPLC-MS/MS system (AB Sciex, Framingham, MA). A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was passed through an HSS T3 column (1.8 μm, 2.1 × 50 mm) (Waters Corporation, Waltham, MA) at 50°C under the following conditions: 0–1 minute, ramp from 50% to 78% B; 1–1.5 minutes 90% B; 1.5–2 minutes, 50% B; flow rate 0.5 ml/min. The autosampler was maintained at 4°C, and the sample injection volume was 2 μl. The turbo electrospray source was operated in positive ionization mode. The following parameters in multiple reaction monitoring were used for compound detection: pitavastatin, 422.1 → 290.1 m/z, declustering potential (DP) 111 V, collision energy (CE) 37 V; pitavastatin lactone (Cayman Chemicals, Ann Arbor, MI), 404.1 → 290.1 m/z, DP 111 V, CE 35 V; pitavastatin-d5 (Toronto Research Chemicals, Ontario, Canada), 426.9 → 293.8 m/z, DP 136 V, CE 41 V; pitavastatin lactone-d5 (Toronto Research Chemicals), 409.1 → 295.2, DP 81 V, CE 37 V. Plasma and bile samples were processed by mixing 10 μl of plasma or 10 μl of prediluted bile (20-fold) in water with 50 μl of 0.1% formic acid in water containing 0.24 μM pitavastatin-d4 and 1.24 μM pitavastatin lactone-d4 deuterated internal standards, followed by addition of 40 μl of 0.1% formic acid in acetonitrile. Samples were homogenized with a vortex mixer and centrifuged at 13,000g for 10 minutes at 4°C. The supernatant was transferred to an autosampler vial for analysis. Liver samples were pulverized with a mortar and pestle under liquid nitrogen. Powdered tissue was mixed with 0.1% formic acid in water containing 1.2 μM pitavastatin-d4 and 6.2 μM pitavastatin lactone-d4 deuterated internal standards at a ratio of 50 mg tissue to 100 μl of 0.1% formic acid in water and homogenized with a vortex mixer. The mixture was snap frozen in liquid nitrogen, thawed, homogenized with a vortex mixer,
and centrifuged at 13,000g for 10 minutes at 4°C. Ten microliters of the supernatant were mixed with 80 µl of 0.1% formic acid in acetonitrile, homogenized with a vortex mixer, and centrifuged at 13,000g for 10 minutes at 4°C. The clean supernatant (70 µl) was transferred to an autosampler vial for analysis. Quantification of unknowns was accomplished using a calibration curve that was linear from 0.018 to 4.75 µM and quality control samples were run periodically to ensure consistent instrument performance.

**Liquid Chromatography/Mass Spectrometry for Silymarin Flavonolignans.** Quantification of silymarin flavonolignans was adapted from previous methods (Gufford et al., 2014). All purified standards were provided by Dr. Nicholas Oberlie (University of North Carolina at Greensboro, Greensboro, NC). The same UPLC-MS/MS system and column used for pitavastatin was used for silymarin flavonolignans. A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at 50°C was used under the following conditions: 0 to 0.1 minute, ramp from 5% to 30% B; 0.1–5.5 minutes ramp from 30% to 55% B; 5.5–6.5 minutes, 5% B; flow rate 0.5 ml/min. The autosampler was maintained at 4°C, and the sample injection volume was 10 µl. The turbo electrospray source was operated in the negative ionization mode. The following parameters in multiple reaction monitoring were used for source was operated in the negative ionization mode.

**Pharmacokinetic Analysis.** The pharmacokinetics of the silymarin flavonolignans and pitavastatin were determined via non-compartmental methods using Phoenix WinNonlin (version 7.0; Certara, Princeton, NJ). Area under the plasma concentration-time curve (AUC) from 0 to 120 minutes (AUC0–120 min) was determined using the trapezoidal method. Terminal slope (λz) was calculated via linear regression of at least the last three data points. AUC from 0 to infinite time (AUC0–inf) was calculated as the sum of AUC0–120 min and the ratio of the concentration at 120 minutes to λz. Systemic clearance (Cl) was calculated as the ratio of dose to AUC0–inf. Volume of distribution at steady state (Vss) was calculated as the product of Cl and mean residence time (MRT), where MRT is the ratio of area under the moment curve from zero to infinite time to AUC0–inf. Terminal half-life (t1/2) was calculated as the ratio of 0.693 to λz. One of the silymarin-treated NASH animals received the incorrect dose of pitavastatin and was excluded from pitavastatin analyses because the actual dose could not be determined with confidence.

**IC50 Determination.** All cells were maintained at 37°C in a humidified 5% CO2 atmosphere. Human embryonic kidney (HEK) 293T/17 cells were seeded at 1.5 × 105 cells per well onto poly-D-lysine-coated 12-well plates and were transiently transfected with 0.75 µg of rat OATP1B2-expressing plasmid (pdDNA5/FRT vector) for 4 hours using jetPRIME (Polyplus Transfection, Illkirch, France). After incubation, the medium was changed to fresh medium with no transfection reagent. Cells were grown for an additional 24 hours before the transport assays were performed. Chinese hamster ovary (CHO) cells stably expressing human OATP1B1 or OATP1B3 were provided by Dr. Bruno Stieger (University of Zurich, Zurich, Switzerland). CHO cells were grown in Dulbecco’s modified Eagle’s medium (low-glucose) containing 25 mM HEPES, supplemented with 10% fetal bovine serum, and 50 µg/ml L-proline. Cells were seeded onto 12-well plates at a density of 1 × 105 cells per well. Transporter expression was induced in CHO cells by addition of 5 mM sodium butyrate to the culture media for 24 hours before transport experiments.
were performed. Cells were washed with prewarmed uptake buffer (135 mM sodium chloride, 1.3 mM HEPES, 2.8 mM d-glucose, 0.5 mM potassium chloride, 0.25 mM calcium chloride, 0.12 mM magnesium chloride, 80 μM magnesium sulfate, pH 7.4) three times and allowed to equilibrate in the final wash for 5 minutes. Uptake was initiated by addition of prewarmed transport buffer containing 1 μM pitavastatin, and transport was terminated at 3 minutes with ice-cold uptake buffer. Cells were washed quickly three times with ice-cold uptake buffer before being lysed with 110 μl of HPLC water. Plates were subjected to a freeze-thaw cycle at −80°C before the wells were scraped and the sample transferred to a microcentrifuge tube. Eighty microliters of the supernatant were mixed with 320 μl of acetonitrile containing 0.04 μM pitavastatin-d5 and 0.2 μM pitavastatin lactone-d6. Samples were homogenized with a vortex mixer and centrifuged at 13,000g for 5 minutes at room temperature. The supernatant (380 μl) was dried under vacuum at 37°C, then reconstituted in 50 μl of 20% acetonitrile with 0.1% formic acid and analyzed for pitavastatin as described above.

The percent extrapolation from AUC0–120 min to AUC120 min–inf for the silymarin flavonolignans ranged from 0.23% to 18%. Data represent mean and S.E.M. of four rats. Statistical Analysis. All data represent mean ± S.E.M. Two-way analysis of variance (ANOVA) P values are shown in tables accompanying the respective graph. Tukey’s post-hoc test was used to compare groups: a = P value ≤0.05 versus respective vehicle group within each diet group; b = P value ≤0.05 versus vehicle control; c = P value ≤0.05 versus silymarin control. Comparisons of silymarin flavonolignans between control and NASH groups were made using the unpaired Student’s t test.

Results

Pharmacokinetics of Silymarin Flavonolignans. The percent extrapolation from AUC0–120 min to AUC120 min–inf for the silymarin flavonolignans ranged from 0.23% to 18%. Average AUC0–120 min of silybin A, silybin B, isosilybin A, and isosilybin B was 1.5- to 2.0-fold higher in the NASH group compared with the control group (Fig. 1; Table 1). Cl of each flavonolignan decreased by 40%–69% in the NASH group compared with the control group (Table 1). The terminal t1/2 of silybin B and isosilybin A increased 1.7- and 1.8-fold, respectively, in the NASH group compared with the control group. Vss of each flavonolignan decreased by 40%–69% in the NASH group compared with the control group.

Average AUC0–120 min of silybin A-4′′-O-β-glucuronide, silybin B-4′′-O-β-glucuronide, and silybinin-7-O-β-glucuronide was approximately 2-fold higher in the NASH group compared with the control group (Fig. 2). Average total flavonolignan AUC0–120 min (flavonolignans plus glucuronides) increased 1.7-fold in the NASH group compared with the control group (594 ± 71 vs. 353 ± 32 nmol × min/ml, mean ± S.E.M.) (P < 0.001).

Pharmacokinetics of Pitavastatin. Pitavastatin lactone was below the detection limit in all samples. The NASH vehicle group showed higher pitavastatin concentrations from 2 to 40 minutes but did not significantly change AUC0–120 min compared with the control vehicle group (Fig. 3, A and B). Average pitavastatin AUC0–120 min increased 1.8-fold in the control silymarin group and 2.9-fold in the NASH silymarin group compared with the control vehicle group (Fig. 3B). The amount of pitavastatin in bile was increased by NASH and decreased by silymarin at the early time points (Fig. 4A). Bile flow was increased by NASH and decreased by silymarin at the early time points (Fig. 4B). The total amount of pitavastatin excreted into bile was increased owing to NASH and decreased by silymarin in both control and NASH groups (Fig. 4C). Pitavastatin biliary clearance was decreased approx. 70% by silymarin in both control and NASH groups (Fig. 4D). The amount of pitavastatin in bile was associated with bile flow (Fig. 4, E and F). At the terminal time point, the total amount of pitavastatin in the liver and the pitavastatin liver-to-plasma ratio were lower in the NASH groups (Fig. 5, A and B). Total pitavastatin in the gastrocne-mius (leg muscle) and pitavastatin muscle-to-plasma ratio were not different between the groups (Fig. 5, C and D).

OATP1B2 Protein Expression in NASH and Inhibitory Effects of Silymarin on OATPs. OATP1B2 protein expression decreased in the NASH group compared with the control group (Fig. 3B). The total amount of pitavastatin in the liver and the pitavastatin liver-to-plasma ratio were lower in the NASH groups (Fig. 5, A and B). Total pitavastatin in the gastrocne-mius (leg muscle) and pitavastatin muscle-to-plasma ratio were not different between the groups (Fig. 5, C and D).

TABLE 1
Silymarin flavonolignan pharmacokinetics

The percent extrapolation from AUC0–120 min to AUC120 min–inf for the silymarin flavonolignans ranged from 0.23% to 18%. Data represent mean and S.E.M. of four rats.

<table>
<thead>
<tr>
<th>Silybin A</th>
<th>Silybin B</th>
<th>Silychristin</th>
<th>Silydianin</th>
<th>Isosilybin A</th>
<th>Isosilybin B</th>
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<tbody>
<tr>
<td>Control</td>
<td>Control</td>
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<tr>
<td>AUC0–inf</td>
<td>t1/2</td>
<td>Cl</td>
<td>Vss</td>
<td>AUC0–inf</td>
<td>t1/2</td>
</tr>
<tr>
<td>18.1 ± 1.9</td>
<td>14.9 ± 2.4</td>
<td>41.8 ± 5.3</td>
<td>0.51 ± 0.04</td>
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<tr>
<td>27.7 ± 2.8*</td>
<td>29.1 ± 5.8</td>
<td>17.4 ± 2.1</td>
<td>0.27 ± 0.03*</td>
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<tr>
<td>33.6 ± 2.3</td>
<td>20.8 ± 2.2</td>
<td>39.4 ± 3.2</td>
<td>0.61 ± 0.06</td>
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<tr>
<td>50.2 ± 5.7*</td>
<td>34.6 ± 4.4*</td>
<td>17.2 ± 1.9</td>
<td>0.28 ± 0.04*</td>
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<tr>
<td>105.6 ± 3.7</td>
<td>48.1 ± 11.8</td>
<td>13.2 ± 0.7</td>
<td>0.32 ± 0.01</td>
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<tr>
<td>112.4 ± 4.2</td>
<td>61.2 ± 6.6</td>
<td>7.9 ± 0.4*</td>
<td>0.19 ± 0.01*</td>
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<td>11.2 ± 0.6</td>
<td>40.2 ± 4.2</td>
<td>30.5 ± 1.6</td>
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<td>14.8 ± 1.8</td>
<td>54.2 ± 8.5</td>
<td>15.2 ± 1.9*</td>
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<tr>
<td>9.6 ± 0.8</td>
<td>17.4 ± 2.9</td>
<td>46.5 ± 4.5</td>
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<tr>
<td>19.4 ± 1.9*</td>
<td>30.8 ± 4.4*</td>
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<td>0.28 ± 0.04*</td>
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<tr>
<td>9.9 ± 0.9</td>
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<tr>
<td>20.1 ± 1.8*</td>
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<td>6.8 ± 0.5*</td>
<td>0.13 ± 0.02*</td>
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AUC0–inf, area under the plasma concentration-time curve from time zero to infinite time; t1/2, terminal half-life.

*p value ≤0.05 vs. control (unpaired t test).
Silymarin inhibited rat OATP1B2- and human OATP1B1- and OATP1B3-mediated uptake of pitavastatin, with IC50 values of 13.8, 6.5, and 12.7 μM, respectively (Fig. 7).

Discussion

A previous clinical study reported no pharmacokinetic interaction between silymarin and an OATP substrate (rosuvastatin) in healthy subjects (Deng et al., 2008). We hypothesized that the presence of NASH may increase the risk for an OATP-mediated silymarin-drug interaction compared with a healthy condition owing to decreased hepatic OATP protein expression and increased systemic silymarin flavonolignan concentrations (Schrieber et al., 2008; Clarke et al., 2017). The present study was designed to address this hypothesis by mimicking key aspects of a clinical NAFLD scenario in an established rodent model of human NASH (Clarke et al., 2014).

Pitavastatin has been proposed as an effective probe drug for OATP1B function because it is minimally metabolized and is primarily dependent on OATPs for hepatic sinusoidal uptake (Hirano et al., 2004, 2005; Prueksaritanont et al., 2014, 2017). Although we confirmed that silymarin inhibits rat OATP1B2, a limitation of using a rodent model to test our hypothesis is that other rodent orthologs beyond OATP1B2 (e.g., OATP1A isoforms) may have been contributing to the interaction.
disposition of pitavastatin (Chang et al., 2019). Silymarin increased pitavastatin exposure in both the control and NASH groups, suggesting that silymarin doses at or above 700 mg may precipitate OATP-mediated drug interactions. These data are noteworthy because higher doses of silymarin, as well as milk thistle formulations designed to improve flavonolignan relative oral bioavailability, are increasingly available on the market. In addition, the combination of NASH and silymarin had the greatest effect on pitavastatin exposure, suggesting that the combined effects of NASH-mediated decrease in the protein expression of and silymarin-mediated inhibition of hepatic OATPs may place NASH patients at the highest risk for this OATP-mediated silymarin-drug interaction. These data are consistent with a previous study indicating that a double hit on hepatic...
OATP-mediated uptake has a greater effect on drug exposure than each hit alone and provide additional impetus for careful evaluation of multifactorial effects on pharmacokinetics (Clarke et al., 2014).

In addition to sinusoidal uptake and efflux, the current data suggest that pitavastatin biliary excretion is affected predominately by bile flow. In the NASH groups, increased bile flow probably contributed to increased pitavastatin biliary excretion. NASH alone did not increase pitavastatin biliary clearance, potentially the result of variability in these data caused by capturing fewer than five half-lives. In the silymarin-treated groups, decreased bile flow probably contributed to decreased pitavastatin biliary excretion. These opposing effects of NASH and silymarin on bile flow contrast with their combined effects on pitavastatin systemic exposure. Interestingly, the effect of bile flow on pitavastatin in bile is not a linear relationship, such that at lower pitavastatin concentrations, the relationship has a steeper slope compared with the relationship at higher concentrations. The silymarin-mediated leftward shift in the NASH pitavastatin data (Fig. 4E vs. Fig. 4F) suggest that the previously reported increase in BCRP protein expression in NASH potentially plays a role in producing high pitavastatin biliary concentrations (Hardwick et al., 2011; Toth et al., 2018). The silymarin-mediated decrease in bile flow observed in the present study is inconsistent with a previous report showing that intraperitoneal administration of silymarin increased bile flow (Croceeni et al., 2000). The reason for this discrepancy is unclear but may be related to the strain of rats used (Wistar vs. Sprague Dawley) or route of silymarin administration (intraperitoneal vs. intravenous). Silymarin-mediated inhibition of BCRP is not expected to contribute to altered biliary disposition of pitavastatin because of the high \( K_i \) reported against BCRP (97 \( \mu \)M) (Deng et al., 2008). Collectively, these data indicate that opposing effects of NASH and silymarin on bile flow influence pitavastatin biliary disposition in a rodent model.

The previous clinical study that found no pharmacokinetic interaction between silymarin (140 mg three times daily) and an OATP/BCRP substrate, rosuvastatin (10 mg single dose), has a number of limitations, some of which arose after the study was completed over a decade ago (Deng et al., 2008).
In addition to the limitations discussed by the authors of that study, many studies performed after 2008 have used silymarin doses greater than 140 mg three times daily (e.g., 700 mg three times daily) and/or formulations that increase flavonolignan plasma concentrations and relative oral bioavailability (Kumar et al., 2014; Poruba et al., 2015; Liang et al., 2018). These doses and formulations that can produce higher plasma concentrations, coupled with the higher plasma concentrations observed in NAFLD patients, suggest that silymarin-mediated OATP substrate interactions may occur in this multifactorial scenario. Although we were able to match maximum silybin A plasma concentrations reported for NAFLD and hepatitis C virus-infected patients taking 560–700 mg of silymarin (present study: 1.7–2.7 μM; clinical studies: 0.9–4.2 μM), an important limitation of our study is that intravenous administration of silymarin produces higher plasma concentrations of the other silymarin flavonolignans than are typically observed after oral administration (Hawke et al., 2010; Schrieber et al., 2011; Fried et al., 2012; Köck et al., 2013). Without IC₅₀ values for each of the flavonolignans it is difficult to determine how each flavonolignan may be contributing to the inhibition of OATPs and increased pitavastatin systemic exposure. It remains to be determined whether these current preclinical data and the limitations of the previous clinical pharmacokinetic interaction study necessitate a re-evaluation of the risk for this natural product-drug interaction.

One of the challenges facing pharmacokinetic natural product-drug interaction research is the large variability in the composition of commercially available products. The Center of Excellence for Natural Product-Drug Interaction Research (NaPDI Center) was created to address this challenge and others by developing Recommended Approaches for studying pharmacokinetic natural product-drug interactions (Paine et al., 2018). In the present study, the composition of the silymarin product was characterized and compared with previously published data on the composition of other silymarin products. The product used in this study contained less than 50% silybin A and silybin B (Fig. 8), which contrasts with a silymarin capsule and another lot of silymarin from the same source, both of which contained more than 55% silybin A and silybin B (Wen et al., 2008). In addition, the silymarin product used in the current study contained more than 30% silychristin, whereas all other products contained approx. 20% silychristin (Wen et al., 2008; Hawke et al., 2010). The IC₅₀ values for silymarin-mediated inhibition of pitavastatin uptake by human OATP1B1 (6.5 μM) and OATP1B3 (12.7 μM) were higher than those previously reported using estrone-3-sulfate as the probe substrate (1.3 and 2.2 μM, respectively) (Köck et al., 2013). Another group, who used a mix of disuccinated silybin A and silybin B, reported an IC₅₀ of 3.3 μM for OATP1B1 and a Ki of 5 μM for OATP1B3 using estradiol 17β-glucuronide as the probe substrate (Wlcek et al., 2013). These differences may reflect different compositions of the silymarin products and/or the substrate used. In addition, the higher percentage of silychristin in the current study may have contributed to the higher IC₅₀ values, as previous data suggest that silychristin may be a less potent OATP inhibitor (Köck et al., 2013). Unfortunately, a direct comparison between the previously published and current IC₅₀ values cannot be made because the composition of the product used in the previous study was not reported and the probe substrates were different (Köck et al., 2013). These data highlight the importance of characterizing the constituent composition of a given natural product and its interaction with the probe drug of interest in pharmacokinetic natural product-drug interaction studies.

In conclusion, these data provide further evidence that double hit pharmacokinetic interactions can have a greater effect on drug exposure than each hit alone. This may be important for patients with chronic diseases such as NASH, because these patients are often subject to polypharmacy to manage and/or treat comorbidities. In addition, patients afflicted with multiple chronic diseases would be more inclined to take botanical dietary supplements, potentially placing them at greatest risk for adverse drug effects (Dickinson and MacKay, 2014; Patel et al., 2017; Chalasani et al., 2018).
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Authorship Contributions

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References


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