DMD Fast Forward. Published on February 11, 2013 as doi:10.1124/dmd.112.048272

DMD #48272

Title Page

Interaction of silymarin flavonolignans with organic anion transporting polypeptides (OATPs)

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DMD #48272

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Running title: Silymarin flavonolignans and OATPs

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Text pages: 29

Number of tables: 3

Number of figures: 4

Number of references: 50

Words in abstract: 250

Words in introduction: 578

Words in discussion: 1533

Abbreviations: bromosulfophthalein (BSP), Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), dimethyl sulfoxide (DMSO), organic anion transporting polypeptide (OATP), sandwich-cultured hepatocytes (SCH), estradiol-17β-glucuronide ($E_2$17G), estrone-3-sulfate (E_1S)

Abstract

Organic anion-transporting polypeptides (OATPs) are multispecific transporters mediating the uptake of endogenous compounds and xenobiotics in tissues that are important for drug absorption and elimination, including the intestine and liver. Silymarin is a popular herbal supplement often used by patients with chronic liver disease; higher oral doses than those customarily used (140 mg three times/day) are being evaluated clinically. The present study examined the effect of silymarin flavonolignans on OATP1B1-, OATP1B3-, and OATP2B1-mediated transport in cell lines stably expressing these transporters, and in human hepatocytes. In overexpressing cell lines, OATP1B1- and OATP1B3-mediated estradiol-17β-glucuronide uptake and OATP2B1-mediated estrone-3-sulfate uptake were inhibited by most of the silymarin flavonolignans investigated. OATP1B1-, OATP1B3-, and OATP2B1-mediated substrate transport was inhibited efficiently by silymarin (IC_{50} values of 1.3, 2.2 and 0.3 μM, respectively), silybin A (IC_{50} values of 9.7, 2.7 and 4.5 μM, respectively), silybin B (IC_{50} values of 8.5, 5.0 and 0.8 μM, respectively), and silychristin (IC_{50} values of 9.0, 36.4 and 3.6 μM, respectively). Furthermore, silymarin, silybin A and silybin B (100 μM) significantly inhibited OATP-mediated estradiol-17β-glucuronide and rosuvastatin uptake into human hepatocytes. Calculation of the maximal unbound portal vein concentrations/IC_{50} values indicated a low risk for silymarin-drug interactions in hepatic uptake with a customary silymarin dose. The extent of silymarin-drug interactions depends on OATP isoform specificity and concentrations of flavonolignans at the site of drug transport. Clinical investigations that achieve higher concentrations with either increased doses of silymarin or formulations with improved bioavailability may enhance the potential risk of DDIs with OATP substrates.

Introduction

Silymarin, a purified extract from milk thistle (*Silybum marianum*), is a popular herbal supplement that is used by approximately one-third of patients with hepatitis C infection or chronic liver disease because of its reported hepatoprotective properties. (Seeff et al., 2008; Freedman et al., 2011) A standardized milk thistle extract contains at least 70% silymarin, a complex mixture composed mainly of the flavonolignans silybin A, silybin B, silydianin, silychristin, isosilybin A, and isosilybin B, as well a few flavonoids such as taxifolin. (Wen et al., 2008) (Figure 1) Legalon® SIL, a commercially available formulation of silybin A and silybin B (silibinin dihemisuccinate), has been used clinically for Amanita mushroom poisoning resulting in reduced mortality rates compared to control-treated patients. (Mengs et al., 2012) *In vitro* studies suggest that silibinin dihemisuccinate inhibits organic anion transporting polypeptide (OATP)-mediated hepatic uptake of the toxin amanitin. (Letschert et al., 2006)

OATPs are important membrane transport proteins expressed in key organs of drug disposition including the intestine, liver and kidneys, where they mediate the cellular uptake of a broad range of xenobiotics as well as endogenous compounds. Two members of this family of proteins, OATP1B1 and OATP1B3, are expressed predominantly in the basolateral membrane of the hepatocyte. (Hsiang et al., 1999; Konig et al., 2000a) Other members such as OATP2B1 and OATP1A2 show broader tissue specificity; OATP2B1, for example, is expressed in the intestine, placenta, brain, endothelial cells and platelets. (St-Pierre et al., 2002; Kobayashi et al., 2003; Grube et al., 2006b; Niessen et al., 2009) Substrates of OATP transport proteins include widely prescribed drugs such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), angiotensin converting enzyme (ACE) inhibitors as well as antibiotics and anticancer drugs. (Kobayashi et al., 2003; Hirano et al., 2004; Nozawa et al., 2005; Smith et al., 2005; Grube et al., 2006b; Ishiguro et al., 2006; Kitamura

et al., 2008) Furthermore, bile acids and steroid hormone conjugates are endogenous substrates for these transporters. (St-Pierre et al., 2002; Hagenbuch and Meier, 2004)

Concomitant administration of inhibitors and substrates of OATPs poses the risk of drug-drug interactions (DDIs), which can consequently result in changes in pharmacokinetics and an increased risk of adverse events and/or reduced efficacy. Numerous studies investigating the effect of genetic polymorphisms as well as DDIs have highlighted the impact of altered hepatic OATP function on the pharmacokinetics of commonly used drugs. For example, coadministration of gemfibrozil, rifampicin, or cyclosporine A with statins increased the area under the plasma concentration-time profile (AUC) of statins as well as the risk of developing rhabdomyolysis; these changes in statin disposition have been attributed primarily to decreased OATP-mediated hepatic uptake. [reviewed in (Koenen et al., 2011)]

In addition to drugs, several herbal ingredients such as flavonoids interact with drug uptake transport proteins of the OATP family. For example, the flavonoids apigenin, quercetin, and kaempferol inhibit the function of OATP1A2 and OATP2B1, which are localized in the apical membrane of the intestine. (Mandery et al., 2010) Furthermore, green tea catechins, herbal extracts, as well as citrus and grapefruit juice affect OATP-mediated transport. (Satoh et al., 2005; Fuchikami et al., 2006; Roth et al., 2011a; Roth et al., 2011b)

In light of recent trends in the clinic towards the use of higher doses and improved formulations of silymarin, the present study investigated the influence of silymarin flavonolignans on the transport function of the major hepatic OATP proteins, OATP1B1, OATP1B3 and OATP2B1, to gain insights into possible herb-drug interactions. The potential clinical implications of these findings are discussed.

Materials and Methods

Materials

[^{3}H]-Estrone-3-sulfate (E_1S; 53.4 Ci/mmol) and [^{3}H]-estradiol-17β-glucuronide ($E_2$17G; 50.3 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA); [^{3}H]-rosuvastatin (10 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled E_1S, $E_2$17G, silymarin, and bromosulfophtalein were purchased from Sigma-Aldrich (St. Louis, MO). Silychristin and silydianin were purchased from ChromaDex (Santo Anna, CA) and U.S. Pharmacopoeia (Rockville, MD), respectively. Isosilybin A and isosilybin B were a generous gift from Ulrich Mengs (Madaus GmbH, Germany). Silybin A and silybin B were isolated and purified as previously described. (Graf et al., 2007) Cell culture supplies were purchased from Gibco® (Life Technologies, Grand Island, NY). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fairlawn, NJ). Bio-Safe II liquid scintillation mixture was obtained from Research Products International (Mt. Prospect, IL). All other materials were purchased from Sigma-Aldrich (St. Louis, MO) or Invitrogen (Carlsbad, CA). Silymarin flavonolignans were dissolved in DMSO and stock solutions were stored at -20°C.

Cell culture

The HEK293-Mock, HEK293-OATP1B3, and HEK293-OATP1B1 cell lines were kindly provided by Dr. Dietrich Keppler (German Cancer Research Center, Germany). The MDCKII-OATP2B1 cell line was kindly provided by Dr. Markus Grube (University of Greifswald, Germany). HEK293-OATP1B1, HEK293-OATP1B3 and HEK293-Mock, MDCKII-OATP2B1 and the parental MDCKII cell lines were grown in 75 cm² cell culture flasks in Dulbecco's modified Eagle's medium containing 10 mM L-glutamine and 10 % fetal calf serum. The transfected cell lines were maintained in medium containing 350 µg/ml Hygromycin B (MDCKII cell lines) or 600 µg/ml G418 (HEK293 cell lines). Cells were

incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Freshly isolated human hepatocytes provided by Life Technologies (Durham, NC) and Triangle Research Labs, LLC (Research Triangle Park, NC) were seeded in 24-well plates at a seeding density of 350,000 cells per well in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) fetal bovine serum, 10 µM insulin, 1 µM dexamethasone, 1% (v/v) minimum essential medium (MEM) non-essential amino acids, 2 mM L-glutamine, 100 units penicillin G and 100 µg streptomycin sulphate (seeding medium). After 1 h incubation at 37°C, 5% CO_2 in a humidified incubator, medium was aspirated to remove dead and unattached cells and replaced with fresh seeding medium. At day 1, either an uptake experiment was performed or hepatocytes were overlaid with Matrigel^{TM} (0.25 mg/ml) and cultured in Dulbecco's modified Eagle's medium containing 1% insulin/transferrin/selenium (ITS^{TM} + Premix), 0.1 µM dexamethasone, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units of penicillin G and 100 µg of streptomycin; medium was changed every day. Human hepatocytes were cultured up to 8 days to allow polarization and canalicular network formation. The demographics of the human liver donors are shown in Table 1.

Transport assays

For transport studies using cell lines, cells were seeded in 24-well plates and cultured to confluence; after removing the medium, cells were washed twice with PBS and incubated at 37°C with Hank's balanced salt solution (HBSS) containing the substrate (pH 7.4). At specified time points, the transport buffer was aspirated rapidly, and cells were washed four times with ice-cold HBSS. Cells were solubilized in 0.5% Triton X-100 in phosphate-buffered saline, an aliquot was dissolved in 2 ml scintillation cocktail (Rotiszint, Roth, Karlsruhe, Germany), and samples were measured in a scintillation β-counter. In all cases, the amount of substrate transported was normalized for protein content determined by the

DMD Fast Forward. Published on February 11, 2013 as DOI: 10.1124/dmd.112.048272
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bicinchoninic acid (BCA) protein assay. To determine the concentration-dependent effects of compounds on OATP-mediated uptake, experiments were performed as described above except that specified concentrations of silymarin and silymarin flavonolignans (0-100 µM) were used.

For hepatocyte experiments, hepatocytes were rinsed twice with Hank's balanced salt solution (HBSS) at 37°C. Subsequently, hepatocytes were incubated with [^{3}H]-rosuvastatin (0.5 µM, 1.5 min) or [^{3}H]-$E_2$17G (1 µM, 1 min) in the presence of test compound or vehicle in HBSS at 37°C. After incubation, the dosing solution was aspirated and cells were washed 3 times with ice-cold HBSS. Cells were lysed in 0.2 ml of 0.5% (v/v) Triton-X100 in phosphate buffered saline and an aliquot was measured in a scintillation β-counter.

Since substrate uptake experiments were performed under sink conditions, apparent hepatic uptake clearance ($CL_{uptake,app}$) of substrates, which includes total cellular accumulation due to passive diffusion, active transport, binding and/or membrane partitioning, was calculated according to equation 1:

$$CL_{uptake,app} = \frac{Accumulation_{Hepatocytes}}{C_{incubation\ buffer} * incubation\ time} \qquad (1).$$

Inhibition of OATP-mediated uptake of $E_2$17G and rosuvastatin by silymarin flavonolignans in human hepatocytes was determined after subtraction of the non-OATP-mediated component of accumulation, which was determined as the accumulation of the respective substrate in the presence of 100 µM BSP (an inhibitor of OATPs). For each experiment, this OATP-mediated accumulation value was set at 100%, data were expressed as a percentage of vehicle control, and the mean and SEM of three experiments was reported. Uptake was corrected for non-specific binding by incubating collagen-coated wells without hepatocytes. Furthermore, accumulation and clearance values were normalized for protein content measured by BCA.

Statistics

The OATP-mediated net uptake was calculated by subtracting the substrate uptake in the parental cell lines (OATP-negative) from the uptake in OATP-expressing cells. Uptake inhibition, presented as percentage of control, was calculated from control experiments in the presence of vehicle, which was set as 100% uptake. Results are presented as mean ± SD, mean ± range, or mean ± SEM, as indicated. The half-maximal inhibitory concentration (IC_{50}) values were calculated by fitting dose-response curves to the data by non-linear regression using Graph-Pad Prism software 5.0 (GraphPad Software Inc., La Jolla, CA) employing three models: (a) four parameter fit, (b) three parameter fit with a fixed bottom, and (c) three parameter fit with a fixed top. IC_{50} values are reported from the model that best described each data set based on goodness of fit parameters.

Statistical analysis of the data was conducted using a repeated-measures ANOVA followed by Dunnet's post hoc test. Differences were considered statistically significant when $p \leq 0.05$.

Estimation of portal vein concentrations

Maximal unbound portal vein concentrations ($C_{u,max,in}$) were estimated using the method described by Ito et al. to assess the relevance of the inhibitory effect of silymarin flavonolignans on the transport function of the investigated OATPs according to equation 2. (Ito et al., 1998)

$$C_{u,max,in} = (C_{max} + \frac{(k_a * D)}{Q} * F_a) * f_u \qquad (2)$$

The maximal plasma concentration (C_{max}), the dose (D), the fraction absorbed from the gastrointestinal tract (F_a), and hepatic blood flow (Q) were obtained from the literature (Table 3). We previously determined an absorption rate constant (k_a) of 0.669 hr^{-1} for silybin A in patients with liver disease using a population pharmacokinetic approach (unpublished data).

However, because this study was conducted in a diseased population, and the absorption rate constant (k_a) for other silymarin flavonolignans has not been described in the literature, k_a was set to the theoretical maximal value (0.1 min^{-1}, which is the maximal gastric emptying rate constant in humans). (Table 3) The fraction unbound (f_u) of silibinin (silybin A and silybin B 1:1) was 0.05-0.1 (personal communication to RH from Dr. Ulrich Mengs, Madaus GmbH).

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Results

Characterization of OATP-overexpressing cell lines

HEK293-OATP1B1, HEK293-OATP1B3 as well as MDCKII-OATP2B1 cells have been described previously. (Konig et al., 2000a; Konig et al., 2000b; Grube et al., 2006a) Uptake of E_1S (1 µM; OATP2B1) and $E_2$17G (1 µM; OATP1B1 and OATP1B3) was linear up to 5 min (data not shown). Therefore, subsequent experiments using these cell lines were performed at 3 min.

Influence of silymarin flavonolignans on OATP-mediated uptake in overexpressing cell lines

To determine the effect of silymarin on OATP function, OATP-mediated uptake of $E_2$17G (OATP1B1, OATP1B3) and E_1S (OATP2B1) was measured in the presence of silymarin or individual silymarin flavonolignans (10 µM each). As illustrated in Figure 2, all tested silymarin flavonolignans significantly inhibited OATP2B1-mediated E_1S uptake. For OATP1B1, all flavonolignans except silychristin inhibited $E_2$17G uptake, while OATP1B3-mediated $E_2$17G transport was significantly inhibited only by silybin A, isosilybin B, silydianin, and silymarin.

The concentration-dependent inhibition of OATP-mediated substrate uptake was investigated further for silymarin as well as the individual flavonolignans silybin A, silybin B, and silychristin, which are the main constituents of silymarin each comprising up to 35% of the mixture. As shown in Figure 3 and Table 2, the interaction of individual silymarin flavonolignans with OATP2B1, compared to inhibition of OATP1B1- and 1B3-mediated substrate uptake, appeared to be very potent, in general. Silymarin was the strongest inhibitor of all OATPs (IC_{50} values of 1.3 µM, 2.2 µM, and 0.3 µM for OATP1B1, OATP1B3, and OATP2B1, respectively). Silybin B inhibited OATP2B1 uptake more than silybin A or silychristin (IC_{50} values of 0.8 µM vs. 4.5 µM and 3.6 µM, respectively). Silybin A, silybin B

and silychristin, were almost equipotent in inhibiting OATP1B1-mediated substrate uptake, whereas OATP1B3-mediated substrate uptake was inhibited more strongly by silybin A and silybin B (IC_{50} values of 2.7 µM and 5.0 µM, respectively) compared to silychristin (IC_{50} value of 36.4 µM).

Estimation of portal vein concentrations

To evaluate the potential impact of silymarin flavonolignans on inhibition of hepatic OATPs, maximal unbound portal vein concentrations ($C_{u,max,in}$) were estimated based on a method described by Ito et al. (Ito et al., 1998) Since the absorbed fraction of silymarin flavonolignans in humans has not been reported, absolute bioavailability data from rats were used under the assumption that the fraction absorbed is at least equal to absolute bioavailability, and similar in rat and human. To determine the inhibition potential for hepatic OATPs, the unbound fraction must be taken into account as well. Although no protein binding data have been reported in humans, the protein binding of silibinin (silybin A and silybin B) is 70% in rat. (Wu et al., 2007) Using the ADMET predictor™, the unbound fraction of silybin A and silybin B was estimated to be approximately 1.5% in humans. An unbound fraction between 5-10% in human plasma was determined experimentally for silibinin (silybin A and B 1:1) (personal communication to RH from Dr. Ulrich Mengs, Madaus GmbH). To calculate the unbound portal vein concentration an unbound fraction of 5% was assumed. (Table 3)

Influence of silymarin flavonolignans on $E_2$17G and rosuvastatin uptake in human hepatocytes

$E_2$17G and rosuvastatin were utilized to investigate the effect of silymarin, silybin A, and silybin B on OATP-mediated uptake in human hepatocytes. BSP, a potent inhibitor of OATPs, was used to assess passive permeation and other active uptake processes in the

absence of OATP-mediated substrate transport. BSP (100 µM) decreased substrate uptake into OATP1B1, OATP1B3 and OATP2B1-overexpressing cell lines to values observed in the parental cell lines (data not shown).

In sandwich-cultured human hepatocytes, the apparent uptake clearance of $E_2$17G was 9.7 µl/mg protein/min and 7.5 µl/mg protein/min, for day 1 and day 8 of culture, respectively. For rosuvastatin, apparent uptake clearance values of 52.6 µl/mg protein/min and 21.7 µl/mg protein/min on day 1 and day 8, respectively, were observed. BSP inhibited the apparent uptake clearance of $E_2$17G in sandwich-cultured hepatocytes to ~50% and ~60% of control values on day 1 and day 8 of culture, respectively; BSP inhibited the apparent uptake clearance of rosuvastatin to ~27% and ~48% of uptake in control-treated cells on day 1 and day 8 of culture, respectively. These data suggested that some passive diffusion, non-OATP-mediated transport, and/or binding/partitioning processes were involved in accumulation of these probe substrates. (Figure 4A and 4B) On day 1, OATP-mediated uptake of $E_2$17G and rosuvastatin was inhibited significantly by silymarin, silybin B, and silybin A (100 µM) as well as 10 µM silymarin. (Figure 4C and 4D) Similar results were obtained for sandwich-cultured hepatocytes on day 8; however, in contrast to day 1, the $E_2$17G uptake was not significantly inhibited by silybin A at a concentration of 100 µM (inhibition to 58% ± 35% of control) (data not shown).

Discussion

Silymarin, the extract of milk thistle, is widely used as a dietary supplement by patients with liver and biliary tract disease due to its reported hepatoprotective properties. Because of the common perception that herbal supplements are generally safe, silymarin is often co-medicated with conventional drugs, raising the potential for herb-drug interactions. Members of the OATP family of transport proteins are responsible for the hepatic uptake of many clinically important drugs including statins, angiotensin-converting enzyme inhibitors and anticancer therapeutics, as well as some endogenous compounds such as bilirubin. Several clinical studies have shown that the activity of these transporters can determine the efficacy as well as adverse events of drugs. (Koenen et al., 2011) Therefore, the goal of the present study was to investigate the interaction potential of individual constituents of silymarin extract with hepatic OATPs to gain insights into possible silymarin-drug interactions.

The present study indicated that silymarin flavonolignans significantly inhibited OATP transport in over-expressing cell lines as well as human hepatocytes (Figures 2-4). Interestingly, despite the structural similarity and identical molecular weight (Figure 1), the individual silymarin flavonolignans differentially inhibited OATP-mediated transport. These findings suggest that stereo- and regiochemistry modify the interaction potential with OATP transport proteins; IC_{50} values varied ~10-fold among the individual flavonolignans (Table 2). This is consistent with results reported from recent clinical and *in vitro* metabolism studies in which the diastereomers of silybin (A and B) and the isomers isosilybin A and isosilybin B exhibited different pharmacokinetic properties and inhibition potential for CYP-mediated metabolism. (Brantley et al., 2010; Hawke et al., 2010)

To assess the clinical interaction potential of drugs/compounds with uptake transport proteins, the International Transporter Consortium recently recommended a cut-off value of $[I]/IC_{50} > 0.1$, where [I] represents the inhibitor concentration, for performing *in vivo* drug

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interaction studies. (Giacomini et al., 2010) It should be noted that the total (bound and unbound) systemic concentrations of silymarin flavonolignans are generally low. The maximal steady-state concentrations (C_{max}) of total unconjugated silybin A and silybin B, after a dose of 140 mg of silymarin three times per day in patients with chronic hepatitis C was 40 ng/ml (0.08 µM) and 8 ng/ml (0.016 µM), respectively, which is at least 10-fold lower than the IC_{50} values reported for OATP inhibition in the current study. (Hawke et al., 2010) However, in a recently completed large, placebo-controlled clinical trial in patients with HCV receiving silymarin doses of 420 and 700 mg three times per day, plasma concentrations up to 2048 ng/ml (4.2 µM) were observed for silybin A (Fried et al., 2012). Assuming an unbound fraction of 5% (personal communication, Dr. Ulrich Mengs, Madaus GmbH, Germany), the unbound concentration of silybin A achieved in this study is approximately 10- to 50-fold lower than the concentrations associated with the IC_{50} values observed for inhibition of OATP-mediated uptake in our experiments. Since the low customary doses of silymarin, which are used by patients with liver disease, do not achieve high systemic concentrations of silymarin flavonolignans, the potential for DDIs appears to be low. However, systemic concentrations may not be the best measure to assess the interaction potential with hepatic uptake transport proteins; for compounds that undergo extensive presystemic elimination, unbound portal vein concentrations are more applicable. Based on estimated unbound portal vein concentrations (Ito et al., 1998), the silybin B concentrations following high-dose silymarin supplementation are within the same micromolar range as the IC_{50} value determined for inhibition of OATP2B1. (Table 2 and 3) Furthermore, the overall lower IC_{50} values for silymarin compared to the individual flavonolignans suggest that there might be synergy between the silymarin constituents with respect to OATP inhibition. However, due to differential pharmacokinetics of these compounds, the actual unbound portal vein concentrations of each component are difficult to

assess in humans. Based on estimations, the maximal total unbound portal vein concentrations of the major flavonolignans in the systemic circulation (silybin A and silybin B) add up to ~0.17 and 0.92 µM for a dose of 140 mg and 700 mg silymarin, respectively; the ratio $[I]/IC_{50}$ observed for inhibition of OATP-mediated transport by silymarin (IC_{50} values of 1.3 µM, 2.2 µM, and 0.3 µM for OATP1B1, OATP1B3, and OATP2B1, respectively) yielded values that were above the cut-off of 0.1 recommended by the International Transporter Consortium to initiate further studies to assess the interaction potential with uptake transport proteins. Other flavonolignans present in the silymarin extract were excluded from this calculation because their systemic exposure is negligible after oral silymarin administration. However, this does not exclude the possibility that portal concentrations of these flavonolignans could be significant and contribute to inhibition of OATPs.

Several *in vivo* studies have investigated the interaction between silymarin co-administration with drugs such as digoxin, nifedipine, indinavir, ranitidine, and rosuvastatin. [see (Wu et al., 2009)]. Of those drugs, only digoxin and rosuvastatin have been described as OATP substrates, although the role of OATPs in digoxin transport has been questioned. (Taub et al., 2011) Silymarin administration (140 mg three times per day) did not affect the pharmacokinetics of the OATP substrate rosuvastatin in healthy subjects despite inhibition of OATP1B1 function by silymarin in OATP1B1-overexpressing oocytes. (Deng et al., 2008) The authors concluded that pre-treatment with silymarin does not result in a risk for drug interactions between silymarin and rosuvastatin *in vivo*. However, in the study by Deng et al., a customary dose of 140 mg silymarin was administered three times daily. We previously demonstrated that silymarin flavonolignans do not reach peak plasma concentrations above 0.2 µM using this dose regimen (Hawke et al., 2010); these concentrations are significantly lower than those associated with drug interaction risk identified in the present study. However, higher oral doses, as well as improved formulations of silymarin (e.g.

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nanoemulsions) are being evaluated to increase systemic/tissue concentrations to achieve desired clinically outcomes. (Flaig et al., 2010; Li et al., 2010; Wang et al., 2012) Based on the present study, these higher doses and/or improved formulations may increase the risk of OATP-mediated interactions.

In hepatocytes at day 1 of culture, 100 µM of silymarin, silybin A or silybin B significantly inhibited $E_2$17G and rosuvastatin transport. These concentrations were above the observed IC_{50} value for all investigated OATPs; thus almost complete ablation of OATP-mediated transport would be expected. Silymarin and individual flavonolignan concentrations of 10 µM are close to the observed IC_{50} values for OATP inhibition, resulting in less pronounced inhibition of transport (Figure 4 and Table 2). Indeed, for silybin B, no significant reduction in substrate uptake was observed. In order to assess the overall effect on hepatic substrate uptake, it is important to appreciate the contribution of individual transport proteins to substrate uptake, the relative protein expression levels, and the potency of the inhibitor toward the respective transport protein. For example, OATP1B1 is primarily involved in rosuvastatin uptake, with only 16 to 34% attributable to OATP1B3. Due to the lower expression levels of OATP2B1 in hepatocytes, the contribution of this transport protein to hepatic uptake of rosuvastatin was deemed negligible. (Kitamura et al., 2008). However, for other substrates, the contribution of the individual transport proteins might differ. Compounds will affect the overall substrate uptake differently, based on their inhibition potential for the individual proteins, which was highlighted by predictions in the study by Karlgren et al. (Karlgren et al., 2012)

Due to the low estimated portal vein concentrations (Table 3), interaction with hepatic OATP-mediated uptake processes is relatively unlikely, especially at the low dose of silymarin recommended for supplementary use. However, our results demonstrate that silymarin flavonolignans not only inhibit OATP1B1 but also OATP2B1. While OATP1B1

and OATP1B3 are expressed almost exclusively in hepatocytes, OATP2B1 exhibits ubiquitous expression and localization in the apical plasma membrane of enterocytes where this transport protein is believed to play an important role in uptake of substrates from the intestinal lumen into enterocytes. (Nozawa et al., 2004) A dose containing 140 mg silymarin extract as a dietary supplement would result in a theoretical maximal gastrointestinal concentration of ≈1 mM if taken with 250 ml water. Given silymarin's low water solubility of 0.4 mg/ml (Woo et al., 2007), concentrations in the range of 0.8 mM may be more realistic. Regardless, the estimated maximal gastrointestinal concentration of silymarin is significantly greater than the IC_{50} for OATP2B1 inhibition, which could result in lower bioavailability of OATP2B1 substrates when administered orally with silymarin. Although drugs that are predominately absorbed by an OATP2B1-mediated process have yet to be identified, current drugs that may be partly dependent on OATP2B1-mediated transport include aliskiren, montelukast, and glibenclamide. (Vaidyanathan et al., 2008; Mougey et al., 2009; Mougey et al., 2011; Tapaninen et al., 2011) Recently, scutallarin, an active flavonoid in *Erigheron brevisacapus* extract was demonstrated to be a specific substrate for OATP2B1. (Gao et al., 2012)

In conclusion, the present data suggest that silymarin flavonolignans inhibit the transport of OATP substrates in over-expressing cell lines as well as in human hepatocytes. Estimations of the maximal portal vein concentrations indicate a low risk for silymarin-drug interactions at the hepatic transport protein level, especially at the recommended silymarin dose of 140 mg. However, the use of higher silymarin doses or silymarin formulations with improved bioavailability might increase portal vein concentrations and thus, may increase the risk of OATP-mediated drug interactions.

Acknowledgements

The HEK293-Mock, HEK293-OATP1B1 and HEK293-OATP1B3 cell lines were kindly provided by Dr. Dietrich Keppler (German Cancer Research Center, Germany). The MDCKII-OATP2B1 cells were kindly provided by Dr. Markus Grube (University of Greifswald, Germany). Freshly isolated human hepatocytes were generously provided by LifeTechnologies (Durham, NC) and Triangle Research Labs, LLC (Research Triangle Park, NC).

Authorship Contributions

Participated in research design: Köck, Brouwer, Ying, and Hawke

Conducted experiments: Köck

Contributed new reagents or analytical tools: Oberlies

Performed data analysis: Köck

Wrote or contributed to the writing of the manuscript: Köck, Brouwer, Ying, Oberlies, and Hawke

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Footnotes

The research reported in this publication was supported by the National Institutes of Health through a grant awarded by the National Center for Research Resources and the National Center for Advancing Translational Sciences [Grant UL1TR000083]; and a grant awarded by the National Institute of General Medical Sciences [Grant: R01GM41935]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This research also was supported by Deutsche Forschungsgemeinschaft (DFG) [Grant Ko4186/1-1].

DMD Fast Forward. Published on February 11, 2013 as DOI: 10.1124/dmd.112.048272
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Legends for Figures

Fig 1. Chemical structure of silymarin flavonolignans. The composition of individual flavonolignans in silymarin is indicated in parentheses.

Fig 2. Effect of silymarin flavonolignans on OATP-mediated substrate uptake. Cells were incubated with [^{3}H]-$E_2$17G (1 μM; OATP1B1, OATP1B3) or [^{3}H]-E_1S (1 μM, OATP2B1) and 10 μM of the flavonolignans indicated, or the vehicle control for 3 min at 37ºC. OATP-mediated uptake was calculated after correcting for protein by subtracting uptake into empty vector (OATP1B1, OATP1B3), or non-transfected control cells (OATP2B1). Values are expressed as a percentage of control; each value is presented as the mean ± S.D. of at least three independent experiments performed in triplicate. * $p \leq 0.05$ compared to control

Fig 3. Concentration-dependent modulation of E_1S and $E_2$17G uptake into OATP over-expressing cell lines by silymarin and silymarin flavonolignans. Cells were co-incubated with silymarin flavonolignans (0-100 μM) and [^{3}H]-E_1S (0.25 μCi/ml; 1 μM; OATP2B1) or [^{3}H]-$E_2$17G (0.25 μCi/ml; 1 μM; OATP1B1, OATP1B3) for 3 min at 37°C. Values are expressed as percentage of vehicle control; each value represents the mean ± range of two independent experiments performed in duplicate.

Fig 4. Inhibition of OATP-mediated $E_2$17G and rosuvastatin accumulation in sandwich-cultured human hepatocytes by silymarin and silymarin flavonolignans. A and B. Apparent uptake clearance ($CL_{uptake,app}$) of $E_2$17G (0.25 μCi/ml; 1 μM, 1 min) and rosuvastatin (0.05 μCi/ml; 0.5 μM, 1.5 min) in sandwich-cultured human hepatocytes on day 1 and day 8 of culture determined in the absence (control, solid bars) or presence (hatched bars) of 100 μM BSP. Values represent the mean ± SEM of three (rosuvastatin) or four ($E_2$17G) independent experiments performed in triplicate. **C and D.** Human hepatocytes (day

1) were co-incubated with silymarin flavonolignans and [^{3}H]-$E_2$17G (0.25 µCi/ml; 1 µM) for 1 min or [^{3}H]-rosuvastatin (0.05 µCi/ml; 0.5 µM) for 1.5 min at 37°C. To calculate OATP-mediated uptake, substrate accumulation was determined in the presence of 100 µM BSP; after subtraction of this value, accumulation in the presence of silymarin or silymarin flavonolignans was expressed as a percentage of vehicle control. Values represent the mean ± SEM of three independent experiments performed in triplicate. * $p \leq 0.05$ compared to control

Tables

Table 1. Demographics of human liver donors.

Donor	Gender	Race	Age	BMI	Alcohol/Smoking
Hu1369	Female	Caucasian	47	19.4	1 Beer/day
Hu1416	Male	Caucasian	58	28.1	-
Hu1434	Male	Caucasian	55	25.6	Yes
RTL Hu 4014	Male	Caucasian	20	19	Not reported

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Table 2. IC_{50} values and 95% confidence intervals (95% CI) for inhibition of OATP-mediated transport ($E_2$17G for OATP1B1 and OATP1B3, and E_1S for OATP2B1).

Cell line	Compound	IC_{50} (μM)	95% CI
OATP1B1	Silymarin	1.3[a]	1.0 – 1.6
	Silybin A	9.7[b]	5.3 – 17.7
	Silybin B	8.5[b]	5.6 – 12.9
	Silychristin	9.0[b]	6.0 – 13.4
OATP1B3	Silymarin	2.2[c]	1.1 – 4.7
	Silybin A	2.7[c]	0.7 – 11.0
	Silybin B	5.0[c]	0.5 – 52.4
	Silychristin	36.4[c]	1.6 – 855
OATP2B1	Silymarin	0.3[a]	0.2 – 0.7
	Silybin A	4.5[b]	2.7 – 7.8
	Silybin B	0.8[b]	0.6 – 1.1
	Silychristin	3.6[a]	2.5 – 4.0

[a] four parameter fit; [b] three parameter fit with fixed bottom;

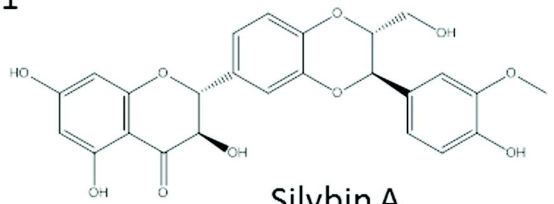
[c] three parameter fit with fixed top

Table 3. Estimation of portal vein concentrations for individual parent flavonolignans.

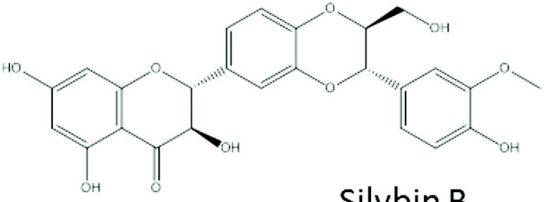
		Silybin A		Silybin B		Reference
Dose of silymarin (mg)		700	140	700	140	(Hawke et al., 2010)
Dose of individual flavonolignans (mg)		*116*	*23.2*	*160*	*32*	
C_{max} (µg/ml)		0.58	0.04	0.22	0.008	(Hawke et al., 2010)
k_a (Absorption rate constant; min^{-1})		0.1				(Ito et al., 1998)
Q (Hepatic blood flow; ml/min)		1500				(Wynne et al., 1989)
F_u (Fraction unbound)		0.05				Dr. Mengs, Madaus GmbH, Germany
F_a (Fraction absorbed)		0.2		0.62		From rat (Pade, 2007)
$C_{u,max,in}$ (unbound portal vein conc.; µM)		0.22	0.04	0.70	0.13	Calculated
[I]/IC50	OATP1B1	0.02	0.004	0.08	0.02	
	OATP1B3	0.08	0.01	**0.14**	0.03	
	OATP2B1	0.05	0.01	**0.88**	**0.16**	

A bold $[I]/IC_{50}$ number indicates that the value is above the cut-off of 0.

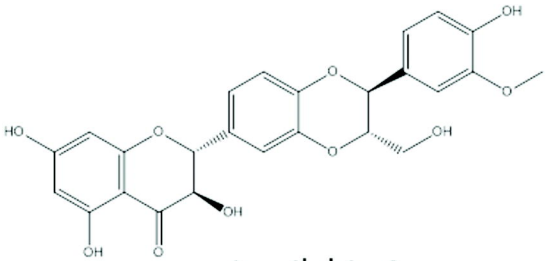
Figure 1



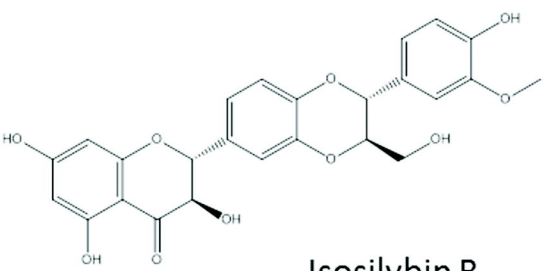
Silybin A
(18-22%)



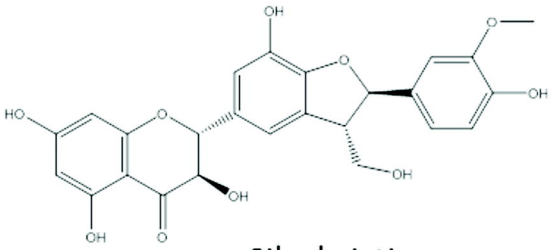
Silybin B
(30-35%)



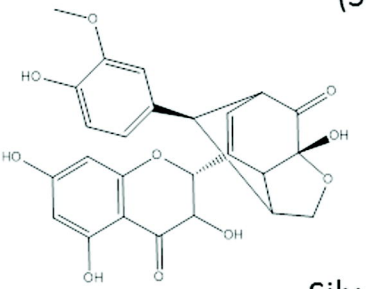
Isosilybin A
(8-9%)



Isosilybin B
(3-4%)

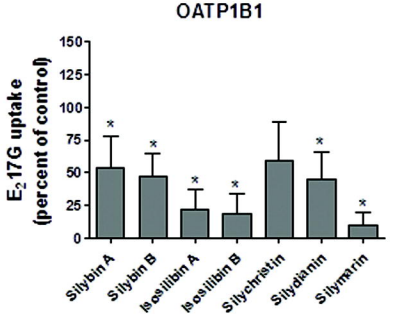


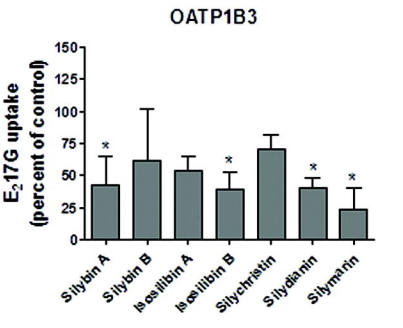
Silychristin
(22-24%)



Silydianin
(9-15%)

Figure 2


OATP1B1
E2 17G uptake (percent of control)
150
125
100
75
50
25
0
Silybin A
Silybin B
Isosilibin A
Isosilibin B
Silychristin
Silydianin
Silymarin


OATP1B3
E2 17G uptake (percent of control)
150
125
100
75
50
25
0
Silybin A
Silybin B
Isosilibin A
Isosilibin B
Silychristin
Silydianin
Silymarin

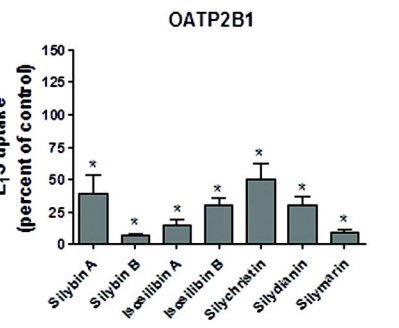

OATP2B1
E1S uptake (percent of control)
150
125
100
75
50
25
0
Silybin A
Silybin B
Isosilibin A
Isosilibin B
Silychristin
Silydianin
Silymarin

Figure 3

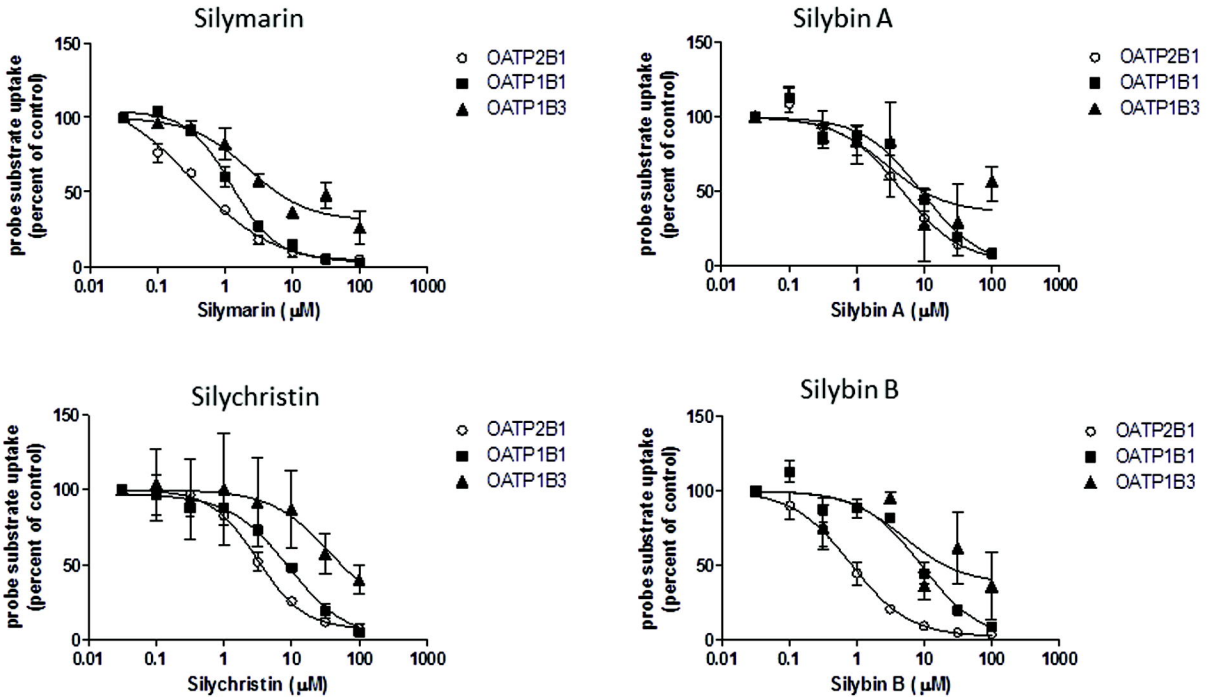
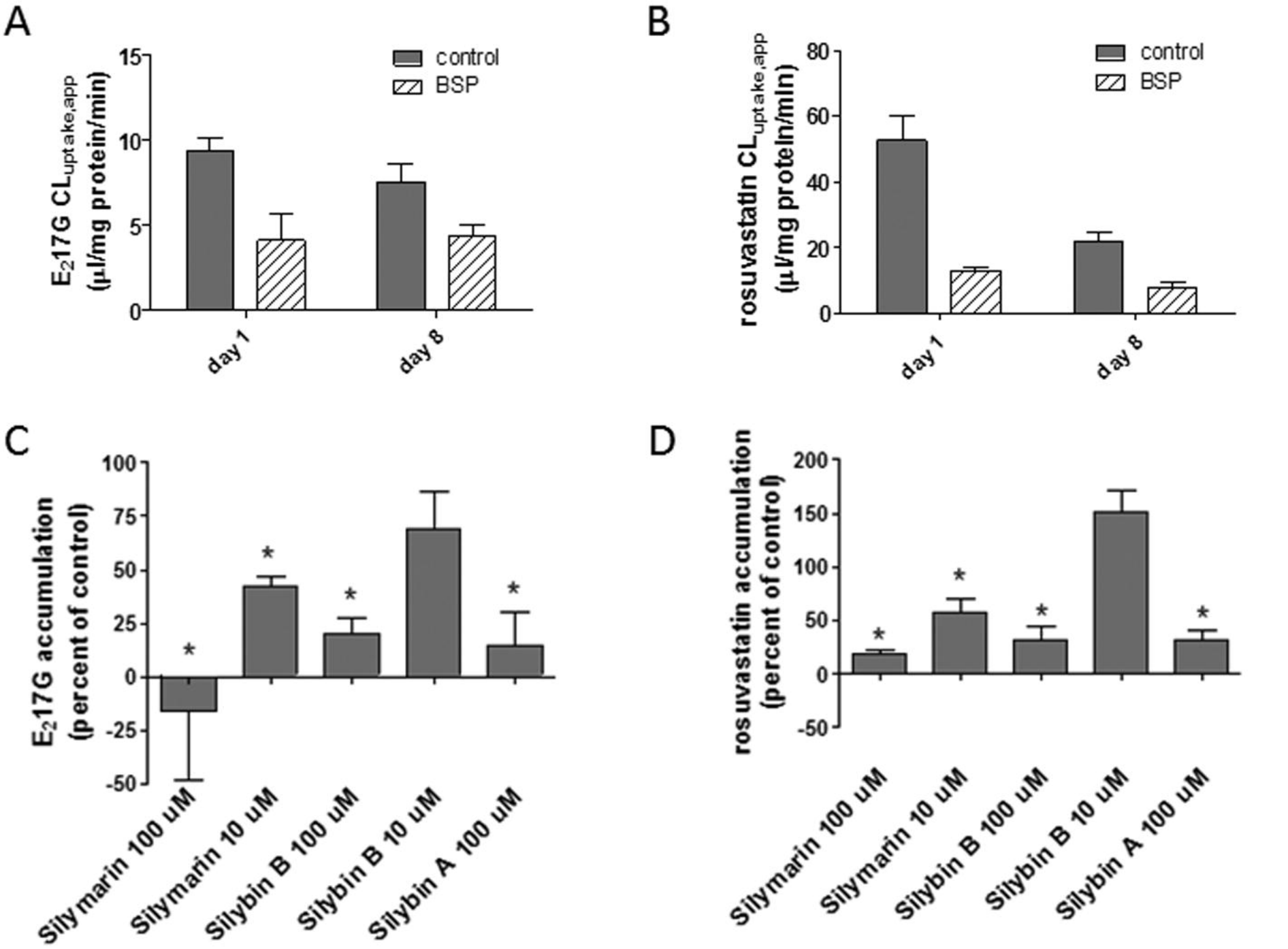

Silymarin
probe substrate uptake (percent of control)
150
100
50
0
0.01
0.1
1
10
100
1000
Silymarin (μM)
OATP2B1
OATP1B1
OATP1B3
Silybin A
probe substrate uptake (percent of control)
Silybin A (μM)
OATP2B1
OATP1B1
OATP1B3
Silychristin
probe substrate uptake (percent of control)
Silychristin (μM)
OATP2B1
OATP1B1
OATP1B3
Silybin B
probe substrate uptake (percent of control)
Silybin B (μM)
OATP2B1
OATP1B1
OATP1B3

Figure 4


A
control
BSP
$E_2$17G $CL_{uptake,app}$ (µl/mg protein/min)
15
10
5
0
day 1
day 8
B
control
BSP
rosuvastatin $CL_{uptake,app}$ (µl/mg protein/min)
80
60
40
20
0
day 1
day 8
C
$E_2$17G accumulation (percent of control)
100
75
50
25
0
-25
-50
*
Silymarin 100 uM
Silymarin 10 uM
Silybin B 100 uM
Silybin B 10 uM
Silybin A 100 uM
D
rosuvastatin accumulation (percent of control)
200
150
100
50
0
-50
*
Silymarin 100 uM
Silymarin 10 uM
Silybin B 100 uM
Silybin B 10 uM
Silybin A 100 uM