Hepatocellular Organic Anion–Transporting Polypeptides (OATPs) and Multidrug Resistance–Associated Protein 2 (MRP2) Are Inhibited by Silibinin

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ABSTRACT

Silibinin has been reported to be a promising compound for hepatitis C treatment of nonresponders to standard treatment. Although administered silibinin is well tolerated, increased serum bilirubin levels have been observed during high-dose i.v. silibinin therapy. The mechanism of silibinin-induced hyperbilirubinemia in humans, however, has not been identified so far. The aim of this study was to investigate the effect of silibinin on hepatocellular uptake and efflux transport systems for organic anions to elucidate the cause of silibinin-induced hyperbilirubinemia. Therefore, the effect of silibinin on transport activity of the hepatocellular uptake transporters organic anion–transporting polypeptides (OATPs) OATP1B1, OATP1B3, and OATP2B1, as well as Na+-taurocholate cotransporting polypeptide (NTCP) and of the efflux transporters multidrug resistance–associated protein 2 (MRP2) and bile-salt export pump (BSEP) was studied. The effect of silibinin on OATPs and NTCP function was studied in stable transfected Chinese hamster ovary cells using the radiolabeled model substrates estrone-3-sulfate and dehydroepiandrosterone sulfate for OATPs and taurocholate for NTCP. Interaction of silibinin with MRP2 and BSEP was measured in vesicles isolated from ST21 or ST9 insect cells expressing these transporters using either estradiol-17β-glucuronide or taurocholate as substrates. OATP1B1, OATP1B3, and OATP2B1 were inhibited by silibinin, with OATP1B1 being inhibited by a complex mechanism(s). An inhibitory effect was also seen for MRP2. In contrast, the bile acid transporters NTCP and BSEP were not affected by silibinin. We concluded that silibinin-induced hyperbilirubinemia may be caused by an inhibition of the bilirubin–transporting OATPs and the efflux–transporter MRP2.

Introduction

Acute hepatitis C virus (HCV) infection evolves into chronic infection in about 75% of cases (Alter and Seeff, 2000; Seeff et al., 2001). Chronic HCV infection is frequently associated with cirrhosis, end-stage liver disease, and hepatocellular carcinoma (Alter and Seeff, 2000). Depending on the HCV genotype, the standard treatment, consisting of pegylated interferon-α and ribavirin, achieves sustained virological response in 54-63% of patients (Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004). However, for nonresponding or difficult-to-treat patients, alternative treatment options are rare. Because of its reported antiviral activity (Ferenci et al., 2008; Ahmed-Belkaecem et al., 2010), silibinin became a promising rescue compound. Consequently, its effectiveness in hepatitis C treatment is being investigated in several centers. Promising outcomes have been reported after silibinin treatment of chronic hepatitis C nonresponders (Ferenci et al., 2008; Biermer and Berg, 2009; Rutter et al., 2011). A positive effect of silibinin administration on hepatocellular C was also observed in patients undergoing liver transplantation, in whom silibinin prevents reinfection after transplantation (Neumann et al., 2010; Beinhardt et al., 2011; Eurich et al., 2011; Rutter et al., 2011; Marino et al., 2013). Although administration of silibinin was generally well tolerated, a significant increase in serum bilirubin levels was reported in several studies (Neumann et al., 2010; Beinhardt et al., 2011; Rutter et al., 2011; Marino et al., 2013). Currently, the underlying mechanism of serum bilirubin elevation during silibinin treatment of HCV patients is unknown.

Silibinin, a flavonolignan occurring in milk thistle seed extract, has been used for hepatobiliary diseases since the 16th century. Since the identification of silibinin as the major active component of milk thistle seed extracts in the 1960s and 1970s (Schuppan et al., 1999), antioxidative, anti-inflammatory, antibifictotic, and hepatoprotective properties have been reported for this compound (Abenavoli et al., 2010). The hepatoprotective activity of silibinin became of special interest for the treatment of intoxications, especially for death cap mushroom poisoning. Consequently, a pharmaceutical formulation of silibinin (Legalon SIL) was approved in 12 European countries for the treatment of Amanita phalloides intoxication (Mengs et al., 2012). Although the exact mechanism of silibinin’s hepatoprotective activity is still unclear, inhibition of hepatocellular transporters mediating death cap toxin uptake has been described (Letscher et al., 2006).

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ABBREVIATIONS: ABC, ATP-binding cassette; BSEP, bile-salt export pump; CHO, Chinese hamster ovary; CHO WT, CHO wild-type cells; DHEAS, dehydroepiandrosteronesulfate; E17G, estradiol-17β-glucuronide; E3S, estrone-3-sulfate; HCV, hepatitis C virus; Kᵢ, inhibition constant; MRP, multidrug resistance associated protein; NTCP, Na+-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; TC, taurocholate; UGT, UDP-glucuronosyltransferase.
Silibinin, the degradation product of heme, is taken up into hepatocytes by family members of the organic anion–transporting polypeptides (OATPs) OATP1B1, OATP1B3, and OATP2B1 (Kullak-Ublick et al., 2001; Stieger et al., 2012). After UDP-glucuronosyltransferase (UGT) 1A1–mediated glucuronidation, silibinin monoglucuronides and diglucuronides are exported from hepatocytes into bile via the ATP-binding cassette (ABC) transporter multidrug resistance–associated protein (MRP) 2 (Kamisako et al., 1999). Patients with mild unconjugated hyperbilirubinemia resulting from Gilbert syndrome show deficient UGT activity but also a decreased bilirubin uptake into the liver (Martin et al., 1976; Persico et al., 2001). Therefore, a contribution of hepatocellular bilirubin uptake by transport processes to unconjugated hyperbilirubinemia has been suggested.

Although many natural products, including fruit juices, flavonoids, or green tea catechins (Roth et al., 2011), have been reported to interfere with OATP function, the interaction of silibinin with hepatocellular organic anion uptake and efflux transporters has not been studied in detail. Therefore, we tested in the present study the hypothesis that silibinin interacts with hepatocellular organic anion transporters expressed in the basolateral and canalicular hepatocyte membrane and thereby contributes to increased serum bilirubin levels in silibinin-treated patients.

Materials and Methods

Materials

We purchased [3H]estradiol-17β-glucuronide (E17BG, 41.8 Ci/mmole), [3H]estrone-3-sulfate (E3S, 45.6 Ci/mmole), [3H]taurocholate (TC, 5 Ci/mmole), and [3H]dehydroepiandrosteronesulfate (DHEAS, 60 Ci/mmole) from PerkinElmer (Schwerzenbach, Switzerland) and OATP substrates, including E3S, DHEAS, and E17BG, from Sigma-Aldrich (Buchs, Switzerland). The liquid scintillation cocktails Ultima Gold AB, used in cell uptake studies and Filter Count, used in vesicle uptake studies, were purchased from PerkinElmer. Supplements added to cell culture media were purchased from Invitrogen (Zug, Switzerland). Silibinin solutions were prepared by dissolving Legalon SIL (Rottapharm Madaus, Monza, Italy) in 0.9% sodium chloride.

Cell Cultures

Chinese Hamster Ovary Cells. OATP1B1-, OATP1B3-, and OATP2B1-transfected (Treiber et al., 2007; Gui et al., 2008) Chinese hamster ovary (CHO), wild-type (WT), and CHO FlpIn Na+-taurocholate cotransporting polypeptide (NTCP) (de Waart et al., 2010) cell lines were cultured in Petri dishes of 10-cm diameter (CytoOne, Starlab, Merenschwand, Germany) at 37°C in an atmosphere containing 5% CO2/95% relative humidity. The CHO WT cells were grown in Dulbecco’s modified Eagle’s medium containing penicillin/streptomycin (100 IU/ml), 10% fetal calf serum, and t-proline (0.05 mg/ml). The culture medium for the OATP-transfected cell lines additionally contained Gentamicin G-418 (500 μg/ml). CHO FlpIn cells stably expressing NTCP were grown in Ham’s F-12 medium containing 100 IU/ml penicillin/streptomycin, 10% fetal calf serum, 1 mM l-glutamine, and 500 μg/ml hygromycin B.

Insect Cells. Sf9 and Sf21 cells were grown in humidified atmosphere at 27°C on 10-cm Petri dishes in BD BaculoGold TNM-FH insect medium (BD Bioscience, Basel, Switzerland) supplemented with 100,000 IU/1000 ml penicillin/streptomycin.

MRP2 and Bile-Salt Export Pump (BSEP) Expression in Insect Cells

MRP2 (de Waart et al., 2006) was expressed in Sf21 cells and in BSEP (Noe et al., 2002) in Sf9 cells infected with respective baculoviruses at a multiplicity of infection of 5.0 and cultured for 3–5 days before harvesting. The isolation of the membrane vesicles from virus-infected cells was performed as previously described (Gerloff et al., 1998). The vesicles were resuspended in 50 mM sucrose, 100 mM KNO₃, 20 mM Hepes/Tris pH 7.4 and stored frozen in liquid nitrogen until use. Protein concentration was determined using the bicinchoninic acid assay with bovine serum albumin as standard (Smith et al., 1985). Expression levels of MRP2 and BSEP were controlled by Western blot analysis of the isolated membrane vesicles.

Cell Transport Studies

Determination of substrate uptake into OATP expressing CHO cells was performed as described previously (Schroeder et al., 1998). Briefly, cells were grown to confluency on 35-mm dishes. Twenty-four hours before the uptake experiment, cells were treated with 5 mM sodium butyrate.

Before the transport experiment, CHO cells were rinsed three times with 2 ml of prewarmed (37°C) uptake buffer (116.4 mM choline chloride or NaCl, 5.3 mM KCl, 1 mM KH₂PO₄ or NaH₂PO₄, 0.8 mM MgSO₄, 5.5 mM D-glucose, and 20 mM Hepes/Tris pH 7.4). Uptake was initiated by the addition of prewarmed 37°C uptake buffer containing [3H]labeled substrates supplemented with unlabeled compound to reach the concentrations indicated in figure legends. Transport was stopped after the desired time of incubation at 37°C by a quick aspiration of the radiolabeled solution and rinsing the cells 4 times with 2 ml of ice-cold uptake buffer. Thereafter, cells were solubilized for at least 30 minutes with 1 ml of 1% (wt/vol) Triton X-100; 500 μl of the cell suspension was used for radioactivity measurement by liquid scintillation counting. The protein concentrations of individual dishes were determined, and specific OATP-mediated uptake was determined by subtracting values from parallel experiments conducted with wild-type cells.

To study the effect of silibinin on OATP-mediated transport, silibinin solutions prepared as described in the Materials and Methods section were added in the desired concentrations (given in the figure legends) to the substrate containing uptake buffer.

Vesicle Transport Studies

ATP-dependent uptake of taurocholate for BSEP and E17BG for MRP2 was measured using the rapid filtration technique as previously described (Gerloff et al., 1998). Vesicles were diluted to a concentration of 3.5 μg/μl with 50 mM sucrose, 100 mM KNO₃, 20 mM Hepes/Tris pH 7.4; 20 μl of vesicles was prewarmed in a 37°C water bath, and uptake was initiated by the addition of the radiolabeled substrate containing uptake buffer (50 mM sucrose, 100 mM KNO₃, 12.5 mM Mg(NO₃)₂, 10 mM Hepes/Tris, pH 7.4). Substrate concentrations used are given in the figure legends. After the desired incubation time in the 37°C water bath, transport was stopped by adding 3 ml of ice-cold stop solution (50 mM sucrose, 100 mM KCl, 10 mM Tris/HCl, pH 7.4) and immediate filtration through 0.45-μm nitrocellulose acetate filter (Sartorius, Göttingen, Germany). In the case of taurocholate uptake studies, the filters were presoaked with 1 mM taurocholate solution. The tube was rinsed once with 3 ml of cold stop solution and also filtered. After filter washing with 3 ml of ice-cold stop solution twice, filter was dissolved in liquid scintillation fluid and radioactivity was measured. For inhibition studies with silibinin, 0, 10, or 100 μM silibinin was added to the uptake buffer. To measure ATP-dependent uptake, 5 mM ATP was added to the uptake buffer, and uptake rates were calculated as the difference in uptake in the presence and in the absence of ATP.

Data Analysis

The inhibition constant (Kᵢ) of silibinin in OATP-mediated E3S uptake was evaluated using Dixon plot analysis. E3S uptake was measured using two different concentrations of substrate in the presence of different concentrations (indicated in the figure legends) of silibinin. Data were plotted as 1/transported activity (y-axis) as a function of inhibitor concentration (x-axis) to perform Dixon plot analysis. Linear regression was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA).

For IC₅₀ determination of silibinin inhibition, nonlinear regression analysis was performed using GraphPad Prism 5 software and eq. 1:

\[ y = y_m + \frac{(y_m - y_0)}{1 + 10^{(\log_{10} IC_{50} - X)/\alpha}} \]

where \( \alpha \) is the Hill Slope.
Uptake values are expressed as means ± S.D. Uptake rates in presence of silibinin were compared with uptake rates in the absence of silibinin using one-way analysis of variance test (Dunnett’s multiple comparison test). P values less than 0.05 were indicated as a statistically significant difference.

Results

Interaction of Silibinin with Basolateral Uptake Transporters

OATP1B1-, OATP1B3-, and OATP2B1-Mediated Uptake. To study a possible interaction of silibinin with liver OATPs, E3S and DHEAS were used as substrates.

Fig. 1. Effect of silibinin on OATP1B3 (A) and OATP2B1 (B). Dixon plots were performed in CHO OATP1B3 (A) and CHO OATP2B1 (B) cells using E3S as substrate. (A) Uptake of 30 µM (●) or 120 µM (■) E3S in OATP1B3-transfected and CHO WT cells in the presence and absence of 2, 4, or 6 µM silibinin. E3S uptake was measured after an incubation time of 40 seconds at 37°C. (B) Uptake of 6 µM (●) or 30 µM (■) E3S in OATP2B1-transfected and CHO WT cells in the presence and absence of 15, 30, or 45 µM silibinin as outlined in Materials and Methods. E3S uptake was measured after an incubation time of 40 seconds at 37°C. Specific OATP-mediated uptake was calculated by subtracting uptake values from CHO WT cells from values measured in OATP-transfected CHO cells. Data are shown for a representative sample of two individual experiments as 1/substrate transported per silibinin concentration. Data are given as picomoles per milligram of protein from triplicate measurements.

Fig. 2. Effect of silibinin on OATP1B1. Uptake of either 0.1 µM (A) or 10 µM (B) E3S in OATP1B1-transfected and CHO WT cells in the presence and absence of different silibinin concentrations ranging from 0.001 to 100 µM. E3S uptake was measured after an incubation time of 1 minute at 37°C as described in Materials and Methods. Specific OATP1B1-mediated uptake was calculated by subtracting uptake values from CHO WT cells from values measured in OATP1B1 CHO cells. Uptake rates are shown for a representative sample of three individual experiments. Data are given as mean picomoles per milligram of protein ± S.D. calculated from triplicate measurements.
Fig. 3. Effect of silibinin on NTCP. NTCP-FlpIn CHO cells were incubated with 1 ml of incubation buffer containing a final substrate concentration of 2.5 μM TC in the absence and presence of sodium. Uptake was measured after an incubation time of 1 or 5 minutes at 37°C in the absence and presence of either 10 or 100 μM silibinin as described in Materials and Methods. Specific NTCP-mediated uptake was calculated by subtracting uptake values in the absence of sodium from values measured in the presence of sodium. Uptake rates are shown for the representative sample of two individual experiments and given as mean picomoles per milligram of protein ± S.D. calculated from triplicate measurements.

Unlike OATP1B1 and OATP1B3, silibinin did not affect OATP2B1-mediated E3S uptake after 1 minute of incubation. However, inhibition was seen by 100 μM silibinin after 5 minutes of incubation. As previously reported (Kullak-Ublick et al., 2001), DHEAS was not transported by OATP2B1 (unpublished data; Kullak-Ublick et al., 2001).

These data indicate that silibinin affects OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake. To confirm the inhibitory effect of silibinin and to obtain information on the mode of interaction of silibinin with the OATP transporters, Dixon plot analyses were performed to estimate the $K_i$ of silibinin. Dixon plots for OATP1B3 and OATP2B1 showed clear inhibition of E3S uptake by silibinin with $K_i$ values of 5 and 3.64 μM, respectively (Fig. 1). Interestingly, Dixon plots could not be analyzed for OATP1B1, suggesting a complex mode of inhibition not following classic Michaelis-Menten kinetics. OATP1B1 has previously been shown to have a low and high binding affinity component for E3S with a $K_i$ value of 45 μM for the low and 0.23 μM for the high affinity component (Nöe et al., 2007). Consequently, we tested the effect of various silibinin concentrations (0.001–100 μM) on the uptake of 0.1 and 10 μM E3S (Fig. 2). At low E3S concentration (0.1 μM, Fig. 2A), silibinin showed an IC$_{50}$ value of 3.28 ± 2.7 μM (mean of IC$_{50}$ measurement in three individual experiments). In contrast, the transport of 10 μM E3S was slightly but consistently ($n = 3$) stimulated when silibinin was present at concentrations ≤1 μM. Only higher silibinin concentrations showed an inhibitory effect on OATP1B1-mediated E3S transport (Fig. 2B).

**NTCP-Mediated TC Uptake.** NTCP does not exclusively transport bile salts; rather, it also transports other organic anions (de Graaf et al., 2011; Steiger, 2011); therefore, a possible interference of silibinin with NTCP was assessed in NTCP-expressing CHO FlpIn cells. No significant difference in TC uptake rates was seen in the presence of either 10 or 100 μM silibinin. Neither after 1 minute nor after 5 minutes of incubation time was a change in TC transport observed (Fig. 3). The transport capacity of NTCP is therefore not affected by silibinin.

As a positive control for the inhibition of NTCP, indocyanine green was used (unpublished data).

**Interaction of Silibinin with Canalicular Efflux Transporters**

**MRP2-Mediated E17βG Transport.** A possible interaction of silibinin with the canalicular efflux transporter MRP2 was measured by using E17βG as substrate. The effect of 10 or 100 μM silibinin on MRP2-mediated E17βG uptake was studied after incubation times of 1 and 10 minutes. Silibinin caused a significant decrease in E17βG transport at both concentrations after 10 minutes of incubation (unpublished data).

MRP2 has been shown to be an allosteric transporter, which is most likely due to two substrate binding sites (Zelcer et al., 2003). This may be the likely explanation that Dixon plots could not be analyzed for silibinin inhibition of MRP2 (unpublished data). Therefore, an IC$_{50}$ value of 6.79 ± 2.60 μM was determined for 5 μM E17βG at an incubation time of 5 minutes (Fig. 4).

**BSEP-Mediated TC Transport.** BSEP-mediated TC uptake was measured in the absence or presence of two different silibinin concentrations (10 and 100 μM). No changes in TC transport rates were observed in presence or absence of silibinin. Therefore, silibinin does not interfere with this hepatocellular efflux transporter (Fig. 5).

Cyclosporine was used as positive control for BSEP inhibition (unpublished data).

**Discussion**

In the present study, we investigated the effect of silibinin on hepatocellular uptake and efflux transporters for organic anions. This study was motivated by the clinical observation that patients treated with high i.v. doses of silibinin often have elevated bilirubin levels (Neumann et al., 2010; Beinhardt et al., 2011; Rutter et al., 2011; Marino et al., 2013). Table 1 shows total serum bilirubin levels of 14 patients treated with high-dose i.v. silibinin (P.F.). In all patients, the total serum bilirubin levels were above normal values (0.0–1.2 mg/dl) before silibinin treatment, which is caused by their underlying liver disease. However, in 78% of the patients, total serum bilirubin levels
further increased between 1.2- and 6-fold during silibinin treatment, which has also been reported in other cases (Neumann et al., 2010; Beinhardt et al., 2011; Rutter et al., 2011; Marino et al., 2013). High interindividual variability is observed in serum bilirubin levels during silibinin treatment over the monitored period. Whereas three patients (no. 1, 9, and 11) showed no increase in bilirubin levels, bilirubin increase during silibinin treatment was transient in four patients (no. 4–7). In five patients (no. 2, 10, 12, 13, and 14), serum bilirubin increased consistently, with the highest values at the last time point of monitoring. This interindividual variability may, among other factors, be due to the known interindividual transporter expression in human hepatocytes (Meier et al., 2006).

The main findings of the present study are that silibinin showed an inhibitory effect on the hepatocellular OATP uptake transporters (OATP1B1, OATP1B3, OATP2B1) and the efflux transporter MRP2, whereas the bile acid transporters NTCP and BSEP were not affected. Under physiologic conditions, bilirubin, the oxidative end-product of heme metabolism, is taken up (at least in part) into liver cells via OATPs. After its uptake into hepatocytes, UDP-glucuronosyltransferase 1A1 (Bosma et al., 1994) conjugates bilirubin to monoglucuronides and diglucuronides (Gollan, 1985). Bilirubin glucuronides are then excreted into bile via the canalicular export pump MRP2 (ABCC2) (Kamisako et al., 1996). In contrast, unconjugated hyperbilirubinemia is caused by the complete absence of UGT1A1 (Crigler-Najjar syndrome type 1) or decreased activity (10% activity in Crigler-Najjar type 2 and 30% activity in Gilbert syndrome) of this enzyme (Seppen et al., 1994; Bosma et al., 1995). In addition to deficient UGT-mediated glucuronidation, decreased uptake from the blood into the liver was also suggested to play a role in unconjugated hyperbilirubinemia (Martin et al., 1976; Persico et al., 2001), which has been shown for OATP1B1, where polymorphisms in this transporter’s gene are associated with elevated serum bilirubin levels (Roth et al., 2012). Furthermore, drug-induced unconjugated hyperbilirubinemia by rifampicin SV, indinavir, and cyclosporin A was reported to be due to an inhibition of this OATP family member (Campbell et al., 2004). Indeed, these drugs have been described to cause unconjugated hyperbilirubinemia in patients (Campbell et al., 2004), and rifampicin and rifamycin have been shown to inhibit rat and human OATPs (Fattinger et al., 2000; Vavricka et al., 2002). Besides their role in unconjugated hyperbilirubinemia, OATP1B1 and OATP1B3 deficiency has been identified as a cause of Rotor syndrome, a benign hereditary hyperbilirubinemia (van de Steeg et al., 2012) that shows altered clearance of the prototypic OATP substrate bromosulfophthalein (Wolpert et al., 1977).

As we show here, silibinin acts as an inhibitor of OATP1B1, OATP1B3, and OATP2B1. In the present study, OATP1B3 and OATP2B1 are competitively inhibited when E3S is used as substrate (K_i values of 5 and 3.64 μM, respectively). Interestingly, Dixon plots were not suitable to characterize an inhibition effect of silibinin on OATP1B1-mediated E3S transport, which is best explained by two different binding sites of OATP1B1 for E3S (Noe et al., 2007). With respect to the high affinity binding site, silibinin acts as inhibitor. Interestingly, the interaction with the low affinity binding site turns out to be complex. Low silibinin concentrations (<1 μM) show a slight stimulation of E3S transport, whereas at higher silibinin concentrations, OATP1B1-mediated transport of 10 μM E3S was impaired. Our data do not, however, rule out the possibility that silibinin does not directly bind to the low-affinity E3S binding site but that it modulates this binding site from a distant interaction site. Furthermore, the fact that Dixon plot analysis of OATP1B1 inhibition
by silibinin was not feasible may indicate that the high- and the low-affinity E3S binding sites are allosterically interacting. To account for possible different substrate binding sites, we included DHEAS as a second OCTP substrate into our inhibition studies. With exception of OCTP2B1, which showed no transport activity for this compound (Kullak-Ublick et al., 2001), OCTP1B1- and OCTP1B3-mediated DHEAS transport was also significantly inhibited by silibinin. These findings support a contribution of these transporters to hyperbilirubinemia observed during silibinin treatment (Neumann et al., 2010; Beinhardt et al., 2011; Rutter et al., 2011; Marino et al., 2013) and are in accordance with the observation that flavonoids, a class of natural products to which silibinin belongs, are able to inhibit OCTP function (Roth et al., 2012). Under high-dose silibinin treatment regimens, plasma Cmax values of up to 30 μM (P.F., unpublished data) are observed in the range of the observed inhibitory concentrations of silibinin against OCTPs and MRPs ranging from 3 to 7 μM (OATP1B1; IC50 = 3.28 μM; OATP1B3: Ki = 5 μM; OCTP2B1: Ki = 3.64 μM; MRP2: IC50 = 6.79 μM).

As the basolateral OCTPs show overlapping substrate specificity with the canalicular bilirubin conjugate efflux transporter MRPs (Suzuki and Sugiyama, 1999), it is not surprising that we also find inhibition of MRP2-mediated transport by silibinin. Although MRP2 has been reported to play an important role in the excretion of silibinin conjugates, but not of silibinin itself, in rats (Miranda et al., 2008), data about the interaction of silibinin with efflux transporters, belonging to the ABC family, focused on P-glycoprotein (MDR1) (Zhang and Morris, 2003a,b; Wu et al., 2008), Mrp1 (Nguyen et al., 2003; Lania-Pietrzak et al., 2005; Wu et al., 2005), Mrp4 (Wu et al., 2005), and Mrp5 (Wu et al., 2005). In the cited studies, the flavonolignan complex of milk thistle silymarin, which constitutes 60% of silibinin (Loguerio and Festi, 2011), also showed an inhibitory effect on the mentioned ABC transporters. These findings, together with our findings, demonstrate that in particular members of the ABCB family may be subject to inhibition by silibinin.

Both OCTPs and MRPs contribute to the bile acid–independent bile flow (Kepler, 2011), whereas the determinant transporters for bile acid dependent bile flow are NTCP and BSEP (Stieger, 2011). Consequently, basolateral uptake and canalicular efflux transporters are important determinants for the vectorial transport of cholephile compounds and, therefore, the bile flow. As the major organic components of human bile are bile salts (Esteller, 2008), we were also interested in whether silibinin affects bile acid uptake (NTCP) and efflux (BSEP) transporters. Neither NTCP nor BSEP transport was influenced by silibinin, indicating that the bile acid transport across hepatocytes is not affected. These findings are in accordance of the knowledge that MRP2 and BSEP show different substrate specificity. Whereas OCTP2B1 predominantly mediates the transport of bile salts (Stieger, 2011), MRP2 is known to transport particularly glutathione, glucuronide, and sulfate conjugates of lipophilic compounds (Nies and Keppler, 2007). The lack of interaction of silibinin with BSEP also supports a contribution of these transporters to hyperbilirubinemia observed during silibinin treatment (Neumann et al., 2010; Beinhardt et al., 2011; Rutter et al., 2011; Marino et al., 2013) and are in accordance with the observation that flavonoids, a class of natural products to which silibinin belongs, are able to inhibit OCTP function (Roth et al., 2012). Under high-dose silibinin treatment regimens, plasma Cmax values of up to 30 μM (P.F., unpublished data) are observed in the range of the observed inhibitory concentrations of silibinin against OCTPs and MRPs ranging from 3 to 7 μM (OATP1B1; IC50 = 3.28 μM; OATP1B3: Ki = 5 μM; OCTP2B1: Ki = 3.64 μM; MRP2: IC50 = 6.79 μM).

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While this manuscript was in revision, Köck et al. (2013) reported inhibition of OATP1B1, OATP1B3, and OCTP2B1 transport function by silibinin A and B, which is in accordance with our findings.

In summary, we showed a clear inhibitory effect of silibinin on hepatocellular OCTP and MRP2 transport function, although the bile acid transporters NTCP and BSEP were not affected. Our data suggest that increased bilirubin levels observed during silibinin treatment are due to simultaneous or individual inhibition of OATPs and MRPs in hepatocytes.