OATP1B3-1B7 (LST-3TM12) Is a Drug Transporter That Affects Endoplasmic Reticulum Access and the Metabolism of Ezetimibe[§]

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ABSTRACT

Drug transporters play a crucial role in pharmacokinetics. One subfamily of transporters with proven clinical relevance are the OATP1B transporters. Recently we identified a new member of the OATP1B family named OATP1B3-1B7 (LST-3TM12). This functional transporter is encoded by *SLCO1B3* and *SLCO1B7*. OATP1B3-1B7 is expressed in hepatocytes and is located in the membrane of the smooth endoplasmic reticulum (SER). One aim of this study was to test whether OATP1B3-1B7 interacts with commercial drugs. First, we screened a selection of OATP1B substrates for inhibition of OATP1B3-1B7-mediated transport of dehydroepiandrosterone sulfate and identified several inhibitors. One such inhibitor was ezetimibe, which not only inhibited OATP1B3-1B7 but is also a substrate, as its cellular content was significantly increased in cells heterologously expressing the transporter. In humans, ezetimibe is extensively metabolized by hepatic and intestinal uridine-5'-diphospho-glucuronosyltransferases (UGTs), the catalytic site of which is located within the SER lumen. After verification of OATP1B3-1B7 expression in the small intestine, we determined in microsomes whether SER access can be modulated by inhibitors of OATP1B3-1B7. We were able to show that these compounds significantly reduced accumulation in small intestinal and hepatic microsomes, which

influenced the rate of ezetimibe β -D-glucuronide formation as determined in microsomes treated with bromsulphthalein. Notably, this molecule not only inhibits the herein reported transporter but also other transport systems. In conclusion, we report that multiple drugs interact with OATP1B3-1B7; for ezetimibe, we were able to show that SER access and metabolism is significantly reduced by bromsulphthalein, which is an inhibitor of OATP1B3-1B7.

SIGNIFICANCE STATEMENT

OATP1B3-1B3 (LST-3TM12) is a transporter that has yet to be fully characterized. We provide valuable insight into the interaction potential of this transporter with several marketed drugs. Ezetimibe, which interacted with OATP1B3-1B7, is highly metabolized by uridine-5'-diphospho-glucuronosyltransferases (UGTs), whose catalytic site is located within the smooth endoplasmic reticulum (SER) lumen. Through microsomal assays with ezetimibe and the transport inhibitor bromsulphthalein we investigated the interdependence of SER access and the glucuronidation rate of ezetimibe. These findings led us to the hypothesis that access or exit of drugs to the SER is orchestrated by SER transporters such as OATP1B3-1B7.

Introduction

Transporters play a major role in pharmacokinetics and pharmacodynamics, as their influx and efflux function influences absorption, distribution, and elimination of their substrates. Moreover, drug transporters are assumed to be key determinants in the compartmentalization of the organism, governing transbarrier transport and modulating the cellular amount of drugs at intracellular drug targets or metabolizing enzymes (Hillgren et al., 2013). In the last two decades, membrane proteins facilitating cellular entry have extended

our understanding of drug transport. Findings on their role in adverse drug events and drug-drug interactions finally resulted in the recommendation to also test new drug entities for interactions with a selection of influx transporters (Giacomini et al., 2010).

Part of this recommendation pertains to members of the 1B-subfamily of the organic anion transporting polypeptides (OATPs), namely OATP1B1 and OATP1B3. Both transporters are highly expressed in the liver (Abe et al., 1999, 2001) and transport a variety of compounds, including statins, sartans, angiotensinogen-converting enzyme inhibitors, and glinides (Maeda, 2015). Tirona et al. (2001) reported on function-impairing genetic polymorphisms of OATP1B1, setting the

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ABBREVIATIONS: BSP, bromsulphthalein; CI, confidence interval; CYP, cytochrome P450 isoform; DHEAS, dehydroepiandrosterone sulfate; G6PT, glucose-6-phosphatase; HBSS, Hanks' balanced salt solution; IS, internal standard; OATP, organic anion transporting polypeptide; SER, smooth endoplasmic reticulum; UGT, 5'-diphospho-glucuronosyltransferase.

stage for in vivo studies on the relevance of OATP1B1 in the hepatocellular entry of substrate drugs. In particular, the rs4149056 polymorphism (c.521T > C; p.Val174Ala) was shown to be linked to changes in pharmacokinetics (Kalliokoski and Niemi, 2009). For OATP1B3, there is less evidence from pharmacogenetic studies for its relevance in vivo. Even if there are polymorphisms that influence transport function in vitro (Picard et al., 2010; Schwarz et al., 2011), there are only limited reports on their impact on pharmacokinetics in humans. However, one variant—rs4149117 (c.334G > T; p.Ala112Ser)—has been reported to be predictive for altered pharmacokinetics of mycophenolic acid and mycophenol-glucuronide (Miura et al., 2008; Picard et al., 2010). However, considering that OATP1B transporters share most substrates and that OATP1B1 is more abundant in hepatocytes (Kunze et al., 2014), the impact of impaired OATP1B3 function is expected to only manifest for molecules that exhibit low affinity toward OATP1B1 (Yoshida et al., 2012).

Most of the hitherto characterized drug transporters are located in the plasma membrane. However, the organization of a cell with intracellular compartments surrounded by lipid layers suggests that there are similar mechanisms directing a compound to the lumen of a compartment (Petzinger and Geyer, 2006). One intracellular compartment where mechanisms of entry and efflux are most likely is the smooth endoplasmic reticulum (SER). This organelle contains several drug-metabolizing enzymes such as cytochrome P450 enzymes (CYP) or the uridine-5'-diphosphoglucuronosyltransferases (UGT) (Matern et al., 1984; Coleman, 2010). Hepatocytes in particular exhibit a sophisticated SER that contains high amounts of metabolizing enzymes (Alberts et al., 2002). Among these, UGT enzymes are known to have their active site located within the lumen (Coleman, 2010). In regard to hepatic drug metabolism, cellular entry is often followed by functionalization and/or intramicrosomal glucuronidation (Coleman, 2010). This implies that a compound has to overcome not only the plasma membrane but also the SER membrane to be directed to its metabolic fate.

In terms of transporters that facilitate SER entry, we have recently reported on OATP1B3-1B7 (LST-3TM12). This novel member of the OATP1B family is a variant encoded by two gene loci. In detail, the first five exons of OATP1B3-1B7 (LST-3TM12, NCBI#AY257074 containing 2064 base pairs) originate from *SLCO1B3*, while *SLCO1B7* provides the remaining exons (exons 3–13 of *SLCO1B7*). The resulting OATP1B3-1B7 mRNA encodes for a functional transporter (687 AA), which recognizes dehydroepiandrosterone sulfate (DHEAS) and estradiol 17 β -D-glucuronide as substrates. Furthermore, we showed that the abundance of OATP1B3-1B7 is expressed in the liver, with localization in the SER of hepatocytes (Malagnino et al., 2018). Due to its localization, we hypothesized that OATP1B3-1B7 has a SER gateway function and influences metabolism by SER enzymes.

In the present study we tested whether OATP1B3-1B7 also interacts with molecules used in drug therapy. Thus, a selection of 19 compounds that have previously been reported to interact with OATP1B transporters was tested for their effect on OATP1B3-1B7 function. In addition, clozapine was included in the screening as adverse events

during clozapine treatment have been associated with polymorphism in the *SLCO1B7* gene region (Legge et al., 2017). One of the identified inhibitors was ezetimibe, which acts as a cholesterol uptake inhibitor that is extensively glucuronidated (Kosoglou et al., 2005). As we assumed that OATP1B3-1B7 could mediate access to the SER lumen and thus to the active site of UGTs, we investigated the putative SER gateway function of OATP1B3-1B7 on the example of ezetimibe.

Materials and Methods

Unless stated otherwise, all materials were purchased from Sigma-Aldrich (Buchs, Switzerland).

Cell Culture. HeLa cells (CCL-2 in passage 6–27; American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Amimend, Allschwil, Switzerland) and 1% glutamine (Bioconcept, Allschwil, Switzerland) in a humidified atmosphere at 37°C and 5% CO₂. Cell lines were assayed for mycoplasma with the PCR Mycoplasma Test Kit I/C (Promokine, Heidelberg, Germany) before experimentation.

Transport Inhibition Studies. HeLa cells were seeded at a density of 4×10^5 cells/well, which corresponds to a confluence of 80%, and were then transfected with OATP1B3-1B7-pEF6 or pEF6-control and infected with vTF-7 (VR-2153; American Type Culture Collection) as described elsewhere (Malagnino et al., 2018). Importantly, in this experimental system OATP1B3-1B7 also sorts to the plasma membrane (Malagnino et al., 2018). Cells were exposed to 0.1 nM DHEAS supplemented with [³H]-DHEAS as a tracer (specific activity 81.3 Ci/mmol, 100,000 DPM/well, NET860250UC; PerkinElmer, Schwerzenbach, Switzerland) in commercially obtained Hanks' balanced salt solution (HBSS, pH 7.4; Sigma-Aldrich).

In a preliminary screening, the test compound was added at a concentration of 25 or 100 μ M. After 10 minutes of incubation at 37°C, the cells were washed twice with ice-cold PBS and lysed in 0.2% SDS-5 mM EDTA (Carl Roth AG, Arlesheim, Switzerland). Five volume percent of the lysate was used for protein quantification by bicinchoninic acid assay (Pierce BCA Protein Assay Kit; Thermo Scientific, Reinach, Switzerland). The remaining lysate was transferred to a Rotiszinteco Plus scintillation buffer (Carl Roth AG). The β -decay was quantified with the liquid scintillation counter Tri-Carb 2900TR (TopLab, Rickenbach, Switzerland).

Compounds that exhibited statistically significant inhibition of OATP1B3-1B7-mediated DHEAS accumulation were further characterized by determining the respective half-maximal inhibitor concentration (IC₅₀). Here, cells were treated with ascending concentrations (ranges shown in Figs. 1 and 2) of the identified inhibitor. Data were analyzed by subtracting the transport rates of control transfected cells from the rates observed for the cells expressing OATP1B3-1B7. Data are shown as percent of solvent control, where the observed net transport rate was normalized to that of the solvent control. By using the statistical program GraphPad Prism version 6 (La Jolla, CA), the IC₅₀ was estimated with a "log (inhibitor) versus normalized response" curve fitting using a fixed slope of -1 .

Transport Experiments with Ezetimibe. HeLa cells, which transiently express OATP1B3-1B7, were prepared to validate the transport of ezetimibe. Ten minutes before exposure to ezetimibe, the cells were preincubated with HBSS. The cells were then exposed to 100 nM unlabeled ezetimibe supplemented with [³H]-labeled tracer (specific activity 20–40 Ci/mmol, 100,000 DPM/well, ART 1463; Hartmann ANALYTIC, Braunschweig, Germany). The cells were incubated for 10 minutes at 37°C and were subsequently washed twice with ice-cold PBS. The amount of radiolabel was quantified by liquid scintillation counting as described earlier.

Western Blot Analysis. For Western blot analysis we used 5–10 μ g of protein of liver homogenate from healthy human subjects

Fig. 1. Concentration-dependent effects of selected therapeutic agents on OATP1B3-1B7-mediated DHEAS transport. The concentration-dependent impact of atorvastatin (A), fluvastatin (B), simvastatin (C), irbesartan (D), telmisartan (E), valsartan (F), glibenclamide (G), troglitazone (H), mifepristone (I), clozapine (J), cyclosporine (K), and rifampicin (L) on the OATP1B3-1B7-mediated accumulation of DHEAS was determined. Normalized data were used to estimate the IC_{50} with a “log (inhibitor) versus normalized response” curve fitting that uses a fixed slope of -1 . Data are shown as mean % of solvent control $\pm$ S.D. of at least three independent experiments, each performed in triplicate.

(BioChain, Newark, NJ), pooled microsomes from human liver (Ultra Pool; Corning/Thermo Fisher Scientific, Corning, NY), duodenum (BioChain), or pooled human small intestinal microsomes (Corning). The Western blot analysis was performed as described elsewhere (Malagnino et al., 2018). Equal loading of the samples was ascertained by Ponceau S staining (Carl Roth AG). The commercial supplier and the dilutions of the antibodies are listed in Supplemental Table 1.

Immunohistological and Immunofluorescent Staining of Human Tissue Samples. Localization of OATP1B3-1B7 was determined by immunohistochemistry and immunofluorescent staining of liver (male; PRIMACYT Cell Culture Technology, Schwerin, Germany) or duodenum (male; AMSBIO, Abingdon, United Kingdom) tissue slides, performed as described elsewhere (Malagnino et al., 2018) and using the antibodies summarized in Supplemental Table 1. In controls, serum of rabbits not exposed to the epitope was used.

The images were acquired with a Leica DMi8 Microscope (Leica Microsystems, Heerbrugg, Switzerland).

The cellular localization of OATP1B3-1B7 in duodenum slices was tested by analyzing its colocalization with the SER marker glucose-6-phosphatase (G6PT, *SLC37A4*). A Zeiss LSM800 (Carl Zeiss Microscopy, Munich, Germany) was used for image acquisition. Z-stacks were recorded at a resolution of 123 nm/pixel with a section distance of 50 nm (Z-sectioning). Images were processed with the Zeiss Airyscan method, which directly deconvolves the images. Pearson's correlation coefficient and Mander's overlap coefficient were calculated with the open source program Fiji and the JACoP plugin (Bolte and Cordelières, 2006; Schindelin et al., 2012).

Microsomal Transport Assays. Human liver microsomes (Corning) and pooled human small intestinal microsomes (Corning) were used for microsomal transport assays. The mean diameter of the

Fig. 2. Interaction of ezetimibe and ezetimibe β -D-glucuronide with OATP1B3-1B7. The concentration-dependent inhibition of DHEAS transport in OATP1B3-1B7 overexpressing HeLa cells was determined for ezetimibe (A) and ezetimibe β -D-glucuronide (B). The IC_{50} of ezetimibe and ezetimibe β -D-glucuronide were estimated by “log (inhibitor) versus normalized response” curve fitting with a fixed slope of -1 . Data are shown as mean % of solvent control $\pm$ S.D. of four independent experiments, each conducted in triplicate.

microsomes was determined by dynamic light scattering with a Zeta Sizer nano ZS (Malvern Instruments, Malvern, United Kingdom). For the microsomal transport assay, 40 μ g (hepatic) or 20 μ g (intestinal) of microsomes were suspended in a Tris-Sucrose buffer containing 0.1 μ M ezetimibe (3 H]-tracer, 100,000 DPM/reaction). Uptake was inhibited by the addition of 100 μ M of the OATP inhibitors/substrates atorvastatin, bromsulphthalein (BSP), or DHEAS to the reaction mix. After incubating the microsomes for 10 minutes at 37°C and 620 rpm, we stopped the reaction by filtering the solution through a Millipore Durapore filter (pore size of 0.22 μ m; Millipore, Zug, Switzerland) followed by a washing step using 3 ml of ice-cold PBS. The filters were transferred to a 3-ml Rotiszinteco Plus scintillation fluid (Carl Roth AG) and incubated for at least 30 minutes at room temperature under constant shaking. Accumulation was determined by scintillation counting with the Tri-Carb 2900TR.

Estimation of Enzymatic Activity of UGTs by Microsomal Assays. Hepatic and small intestinal microsomes, UGT reaction mixture solution A, and the UGT reaction mix solution B with alamethicin were obtained from Corning. In addition, UGT reaction mix solution B without alamethicin was prepared according to the manufacturer's protocol (250 mM Tris-HCl, 40 mM $MgCl_2$, pH 7.5). The UGT reaction mix solution A, UGT reaction mix solution B (with or without alamethicin), and ezetimibe were mixed according to the manufacturer's protocol and preheated to 37°C (ThermoMixer Comfort; Eppendorf, Hamburg, Germany). The reaction was started by adding microsomes to the mixture, which resulted in a protein content of 0.5 and 0.25 mg/ml for hepatic and small intestinal microsomes, respectively. At indicated time points an aliquot of 50 μ l was drawn and combined with 250 μ l methanol supplemented with the internal standard (IS) efavirenz-d5 (concentration 100 ng/ml; Toronto Research Chemicals, Ontario, Canada).

The samples were vigorously mixed and then centrifuged for 30 minutes at 3200g and 10°C. An aliquot of 2.5 μ l supernatant was injected into the high-pressure liquid chromatography with tandem mass spectrometry system, which consisted of a Shimadzu (Kyoto, Japan) high-pressure liquid chromatography connected to an API 4000 Qtrap mass spectrometer (AB Sciex, Concord, ON, Canada). The system was operated with the Analyst software 1.6.2 (AB Sciex).

Ezetimibe, ezetimibe β -D-glucuronide, and efavirenz-d5 (IS) were separated on a Luna PFP(2) analytical column (50 $\times$ 2 mm, 3 μ m; Phenomenex, Torrance). Mobile phase A and B were water and methanol, respectively. Both mobile phases were supplemented with 0.1% formic acid. The samples were loaded at 5% mobile B onto the analytical column. Mobile phase B was increased to 95% between 0.5 and 2 minutes. The column was washed for 1 minute at 95% B and reconditioned at 5% B for another 0.5 minutes. Ezetimibe, ezetimibe β -D-glucuronide, and efavirenz-d5 eluted after 2.17, 2.28, and 2.31 minutes, respectively.

The analytes were ionized in the negative mode by electrospray ionization and detected by multiple reaction monitoring. A transition of 584 $\rightarrow$ 271 m/z , 408 $\rightarrow$ 271 m/z , 319 $\rightarrow$ 248 m/z was used for ezetimibe, ezetimibe β -D-glucuronide, and efavirenz-d5, respectively. The calibration curves of ezetimibe, ezetimibe β -D-glucuronide were prepared in PBS plus 0.05% Triton X. The calibration covered a range of 5–10,000 nM for ezetimibe and ezetimibe β -D-glucuronide. The calibration samples were processed as the samples of the microsomal assay.

Ezetimibe and ezetimibe β -D-glucuronide were quantified based on a linear regression of the analyte to IS peak area ratio (y) and the nominal concentration (x). The regressions were weighted by $1/x^2$ and a coefficient of variation (R) above 0.99 was accepted. The glucuronidation rates of the different experimental conditions were calculated with a nonlinear one-phase-association curve fit (eq. 1) with GraphPad Prism version 6:

$$y = y_0 + \text{Plateau} (1 - \exp(-kx)) \quad (1)$$

where y_0 is the y value when the time is zero, Plateau is the y value at infinite times, and k is the rate constant.

Statistical Analysis. $P < 0.05$ was considered to be statistically significant. GraphPad prism version 6 was used for all statistical analyses.

Results

Inhibitor Screening and IC_{50} Determination. We screened 19 compounds shown to interact with OATP1B1 or OATP1B3 and clozapine (20 compounds in total) for their impact on OATP1B3-1B7-mediated cellular accumulation of DHEAS. Clozapine was included in our analysis, as occurrence of adverse events during clozapine treatment is associated with polymorphisms in the gene region of *SLCO1B7* (Fig. 1J) (Legge et al., 2017).

As summarized in Table 1, atorvastatin, troglitazone, and rifampicin inhibited OATP1B3-1B7 significantly at 25 μ M, while 13 of the 20 test compounds—namely, atorvastatin, fluvastatin, simvastatin, irbesartan, telmisartan, valsartan, glibenclamide, troglitazone, mifepristone, cyclosporine, rifampicin, ezetimibe, and, ezetimibe β -D-glucuronide—significantly changed the amount of DHEAS in the cells at a concentration of 100 μ M (Table 1), suggesting rather low inhibitory potency.

For all compounds exhibiting a statistical significant influence at 100 μ M concentration and clozapine, we assessed the concentration-dependence of their impact on OATP1B3-1B7-mediated DHEAS transport (Figs. 1 and 2). In our

TABLE 1

Overview of the 20 compounds tested for interaction with OATP1B3-1B7 mediated uptake of dehydroepiandrosterone sulfate (DHEAS)

The data are reported as mean percent of solvent control $\pm$ S.D. Each compound was tested at two concentrations (25 and 50 μ M) in three to four independent experiments each performed in triplicate. For statistical analysis of the data, the one-sample *t* test for normalized data was used.

Compound	Mean DHEAS Accumulation, Percentage of Solvent Control $\pm$ S.D.	
	Tested at 25 μ M	Tested at 100 μ M
Statins		
Atorvastatin ^a	38.8 $\pm$ 8.9 ^b	8.3 $\pm$ 10.5 ^b
Fluvastatin ^a	90.9 $\pm$ 22.6	20.5 $\pm$ 14.1 ^b
Rosuvastatin	100.9 $\pm$ 31.6	62.9 $\pm$ 25.9
Simvastatin ^a	91.1 $\pm$ 15.0	4.9 $\pm$ 9.5 ^b
Angiotensin II receptor antagonists		
Irbesartan ^a	63.9 $\pm$ 38.9	41.9 $\pm$ 24.5 ^b
Telmisartan ^a	146.5 $\pm$ 27.9	63.5 $\pm$ 14.7 ^b
Valsartan ^a	114.1 $\pm$ 36.6	56.0 $\pm$ 6.3 ^b
Angiotensin-converting enzyme inhibitors		
Enalapril	100.9 $\pm$ 18.5	100.5 $\pm$ 25.7
Sulfonylureas		
Glibenclamide ^a	69.7 $\pm$ 14.4	45.1 $\pm$ 24.4 ^b
Thiazolidinediones		
Troglitazone ^a	1.6 $\pm$ 3.0 ^b	3.0 $\pm$ 6.3 ^b
Steroidal antiprogesterones		
Mifepristone ^a	68.1 $\pm$ 30.0	43.9 $\pm$ 11.1 ^b
Anticonvulsives		
Carbamazepine	72.2 $\pm$ 21.5	75.0 $\pm$ 23.2
Atypical antipsychotics		
Clozapine ^a	131.4 $\pm$ 30.2	103.8 $\pm$ 54.1
Immunosuppressives		
Cyclosporine ^a	137.5 $\pm$ 24.1	135.6 $\pm$ 16.1 ^b
Chemotherapeutics		
Paclitaxel	106.3 $\pm$ 29.6	81.1 $\pm$ 12.5
Erythromycin	65.7 $\pm$ 54.4	109.3 $\pm$ 12.9
Antibiotics		
Doxorubicin	150.9 $\pm$ 109.3	82.6 $\pm$ 18.1
Rifampicin ^a	37.8 $\pm$ 17.8 ^b	35.2 $\pm$ 16.1 ^b
Azetidinones		
Ezetimibe ^a	96.7 $\pm$ 29.4	43.8 $\pm$ 32.0 ^b
Ezetimibe β -D-glucuronide ^a	72.1 $\pm$ 24.7	29.9 $\pm$ 5.7 ^b

^aCompound selected for determination of concentration dependence of the effect.

^bIndicates $P < 0.05$.

experimental set up, only atorvastatin, fluvastatin, glibenclamide, troglitazone, and rifampicin reached 90% inhibition of DHEAS accumulation, and the experimental data were used to calculate the respective IC₅₀ value. For simvastatin, irbesartan, valsartan, mifepristone, clozapine ezetimibe, and ezetimibe β -D-glucuronide the IC₅₀ values were computed based on the obtained experimental data. The IC₅₀ values were estimated applying a “log (inhibitor) versus normalized response” curve fitting using a fixed slope of -1 . The fixed slope was used as fewer than 12 experimental data points were used to fit the data.

Table 2 summarizes the obtained IC₅₀ values, the 95% confidence intervals (CI), and the respective R^2 values for each compound included in the analysis. Although telmisartan and cyclosporine enhanced DHEAS accumulation, this effect was not dose dependent.

Transport Experiments with Ezetimibe. Another identified inhibitor was ezetimibe, for which the reported IC₅₀ was of the same magnitude as observed for OATP1B1 and OATP1B3 (Oswald et al., 2008). Considering that glucuronidation mediated by UGTs is a major metabolic pathway of ezetimibe (Ghosal et al., 2004) and that formation of ezetimibe β -D-glucuronide occurs in the SER, we selected this compound to further investigate the influence of

OATP1B3-1B7 on SER access. At first, we determined the concentration-dependent influence of ezetimibe and of ezetimibe β -D-glucuronide on OATP1B3-1B7-mediated cellular accumulation of DHEAS. The IC₅₀ was 37.5 μ M (95% CI, 18.7–75.1 μ M) for ezetimibe and 99.7 μ M (95% CI, 66.7–149 μ M) for ezetimibe β -D-glucuronide (Fig. 2; Table 2).

In the next step, we tested whether OATP1B3-1B7 transports ezetimibe. First, the ezetimibe uptake experiments had to be extensively optimized as ezetimibe exhibited extensive unspecific binding (see Supplemental Fig. 1; Supplemental Methods). Eventually we were able to show a statistically significant increase in cellular accumulation at an ezetimibe concentration of 0.1 μ M of about 16% compared with control (mean % of pEF6-control $\pm$ S.D.; 116.4% $\pm$ 14.8% $n = 6$ independent experiments each performed in triplicates, $P < 0.05$ one sample *t* test).

Abundance of OATP1B3-1B7 in the SER. Expression of OATP1B3-1B7 in the microsomal preparations of human liver and small intestine was verified by Western blot analysis, showing an enriched band at around 90 kDa (Fig. 3A). Similar results had been previously observed for the transporter, of which the molecular size was predicted to be 76 kDa (Clone Manager Professional 8; Sci-Ed Software, Denver). Moreover, Western blot analysis of the V5-tagged transporter revealed

TABLE 2

Summary of estimated half-maximal inhibitory concentration (IC₅₀)The IC₅₀ was estimated with the log (inhibitor) versus normalized response curve applying a fixed slope of -1 . Provided are the obtained values for each compound, the respective 95% confidence interval (CI), and the goodness of fit (R^2 value).

Compound	IC ₅₀ (μM)	CI (μM)	Number of Independent Experiments	R ² Value
Atorvastatin	7.2	4.2–11.6	3	0.93
Fluvastatin	30.7	24.4–38.8	3	0.92
Simvastatin	39.6 ^a	27.3–57.8	3	0.78
Irbesartan	59.0 ^a	35.5–97.4	3	0.80
Valsartan	293.0 ^a	136.6–628.4	4	0.56
Glibenclamide	12.7	7.7–20.9	3	0.79
Troglitazone	0.6	0.5–1.1	3	0.83
Mifepristone	706.7 ^a	381.6–1309.0	3	0.33
Clozapine	137.5 ^a	91.0–207.7	4	0.7
Rifampicin	13.6	9.6–19.6	5	0.89
Ezetimibe	37.5 ^a	18.7–75.1	5	0.63
Ezetimibe β-D-glucuronide	99.7 ^a	66.7–149.4	5	0.69

^aIndicates where IC₅₀ values were computed without reaching 90%.

an intense band at around 90 kDa and a faint band at around 58 kDa, when lysates of heterologously expressing cells were probed for V5 (Malagnino et al., 2018). Importantly, for the liver we observed an enrichment of the known SER proteins CYP3A4 and UGT1A1, comparing liver lysate with hepatic microsomes.

In accordance with OATP1B3 being localized in the plasma membrane, we observed a depletion of OATP1B3 in the microsomal fraction. In small intestinal microsomes we detected the microsomal enzymes and OATP1B3-1B7. However, we were not able to show enrichment of any of the tested markers compared with a commercially obtained total protein sample from human duodenum. Because the microsomes are prepared from mature jejunal and duodenal enterocytes, the total duodenum protein may not be a representative sample to control for ER enrichment.

We performed immunohistological and immunofluorescent stainings of human duodenal sections to investigate the cellular localization of OATP1B3-1B7 in the small intestine. Figure 3B shows that OATP1B3-1B7 is expressed in the liver and duodenum; in both tissues OATP1B3-1B7 is mostly located intracellularly. This finding was corroborated when we made an immunofluorescent staining of OATP1B3-1B7 in the duodenum (Fig. 3C). To verify that the intracellular structure is part of the intestinal SER we detected OATP1B3-1B7 in a costaining with the SER marker G6PT.

Figure 3D depicts a Z-section of the colocalization study, which was analyzed calculating the Pearson's and Mander's coefficient of colocalization of the fluorophores. The Pearson's coefficient (0.59 ± 0.14 S.D.) indicated that 59% of the detected proteins share the same localization, while the Mander's overlap coefficient for OATP1B3-1B7 was 0.80 ± 0.18 ($\pm$ S.D.), suggesting that 80% of all areas positive for OATP1B3-1B7 were also positive for G6PT. In contrast, the Mander's overlap coefficient for G6PT was 0.48 ± 0.13 , which showed that 48% of the G6PT-positive areas also contained OATP1B3-1B7. This seems reasonable, considering that G6PT marks the entire SER, while OATP1B3-1B7 would be expected to be present in specialized regions of the SER.

Microsomal Accumulation of Ezetimibe. Before assessing the accumulation of ezetimibe, we determined the diameters of hepatic and small intestinal microsomes to be

234.9 and 243.7 nm, respectively, suggesting that vesicle transport studies could be conducted. To test whether accumulation of ezetimibe into microsomes may be influenced by OATP1B3-1B7 inhibition, we measured the accumulation of ezetimibe in hepatic or small intestinal microsomes in the presence and absence of the OATP inhibitors atorvastatin and BSP, or the reported OATP1B3-1B7 substrate (DHEAS) (Malagnino et al., 2018). For both the hepatic (Fig. 4A) and intestinal (Fig. 4B) microsomes, we observed a statistically significant reduction of the intramicrosomal accumulated amount of ezetimibe in the presence of the tested compounds.

Inhibition of OATP1B3-1B7 Transport and Its Consequences for UGT-Mediated Metabolism. We have conducted metabolism studies in microsomes in which we determined the conversion rate of ezetimibe to ezetimibe β-D-glucuronide by UGT isoforms. Drugs have to overcome the SER membrane to be glucuronidated because the enzymatically active sites of UGT isoforms lie inside the SER. Therefore, it is standard procedure to permeabilize microsomes for glucuronidation assays with the intention to increase the metabolic rates. To test whether the transmembrane transport is the rate-limiting step in the glucuronidation process of ezetimibe, we compared the formation of ezetimibe β-D-glucuronide in the presence and absence of the pore-forming agent alamethicin, which is commonly used in microsomal studies.

When we subjected ezetimibe to nonpermeabilized microsomes, we saw that the initial conversion rate dropped by more than half in hepatic and small intestinal microsomes. In liver microsomes, the glucuronidation rate was $0.19 \text{ nmol mg}^{-1} \text{ min}^{-1}$ (95% CI, $0.18\text{--}0.22 \text{ nmol mg}^{-1} \text{ min}^{-1}$) and $0.03 \text{ nmol mg}^{-1} \text{ min}^{-1}$ (95% CI, $0.02\text{--}0.06 \text{ nmol mg}^{-1} \text{ min}^{-1}$) in the case of permeabilized and nonpermeabilized membranes, respectively (Fig. 5A). In permeabilized intestinal microsomes, the rate was $0.023 \text{ nmol mg}^{-1} \text{ min}^{-1}$ (95% CI, $0.01\text{--}0.04 \text{ nmol mg}^{-1} \text{ min}^{-1}$) and in intact membranes $0.012 \text{ nmol mg}^{-1} \text{ min}^{-1}$ (95% CI, $0.00\text{--}0.03 \text{ nmol mg}^{-1} \text{ min}^{-1}$) (Fig. 5C).

To estimate the contribution of a SER transporter, we added BSP to the nonpermeabilized microsomes. Here, we observed a significant decrease in the conversion rates in hepatic as well as small intestinal microsomes (Fig. 5, B and D), suggesting that a BSP inhibitable protein grants access to the metabolic site of UGT isoforms.

Fig. 3. Detection of OATP1B3-1B7 in liver and small intestinal tissue. (A) Western blot analysis of total liver, hepatic microsomes (L. Micro) (6–3 blots), small intestine duodenum (Sm. Intes.), and small intestinal microsomes (Intes. Micro) (three blots) was performed to assess the amount of OATP1B3-1B7, CYP3A4, and UGT1A1. Moreover, in samples derived from the liver, OATP1B3 was quantified. The proteins were detected on single films (single exposure), and the protein load was 10 μ g for all investigated proteins except CYP3A4, for which the load was 5 μ g. (B) Detection of OATP1B3-1B7 localization in the human liver or duodenum by immunohistochemistry. (C) Immunofluorescent staining of OATP1B3-1B7 (red) in the duodenum. Nuclei are labeled with 4',6-diamidino-2-phenylindole (blue). In control images, rabbit serum was used instead of the primary antiserum, and these are shown in the right corner of each image. (D) Representative Z section of a costaining showing the transporter OATP1B3-1B7 in red and the smooth endoplasmic reticulum marker G6PT in green. In the control image the primary antibodies were omitted.

Discussion

In this study, we report that various drugs interact with OATP1B3-1B7 (LST-3TM12), a novel member of the OATP1B-family that is located in the SER membrane of liver tissue. For a preliminary screening, we selected drugs described to act as either substrates or inhibitors of OATP1B1 and OATP1B3, and identified several inhibitors of OATP1B3-1B7. Moreover, we have also assessed the interaction between clozapine and OATP1B3-1B7 because polymorphisms in the gene region of *SLCO1B7* have been associated with an increased risk for adverse events (Legge et al., 2017).

For all identified inhibitors and clozapine we determined the IC_{50} . Notably, the obtained experimental data allowed the calculation of the IC_{50} for five compounds while for the other compounds the respective data were computed as they did not reach 90% inhibition in our experimental setup. On average the observed IC_{50} values were two magnitudes above the expected maximal plasma concentrations (Heikinheimo, 1997; Siekmeier et al., 2001; Goodman et al., 2005; Masubuchi, 2006; Guo et al., 2015).

However, it would be too early to consider the interaction with OATP1B7-1B3 as of no relevance for the in vivo situation because we do not know the intracellular concentration of the compounds. According to Matsuda et al. (2012), the intestinal

and portal concentrations of orally administered compounds may be higher than the maximal systemic concentrations. Moreover, in the employed expression system OATP1B3-1B3 is not only expressed at the cell membrane but also intracellularly (Malagnino et al., 2018), which may have resulted in an underestimation of the inhibitory potency.

In accordance with recent findings by Dickens et al. (2018) for OATP1B1 and OATP1B3, we did not observe inhibition of OATP1B3-1B7-mediated DHEAS transport by clozapine. However, considering the function of OATP1B3-1B7 as a SER access mechanism, one may speculate that the contribution of transporters to an adverse event is not driven by the interaction with the parent compound but with its metabolite *N*-desmethylclozapine, which is known to undergo hepatic glucuronidation (Schaber et al., 2001).

For the tested HMG-CoA reductase inhibitors, we observed that those statins known to be highly metabolized by CYPs and/or by UGTs (Dain et al., 1993; Prueksaritanont et al., 2002; Lennernäs, 2003) interact with OATP1B3-1B7, while rosuvastatin, for which metabolism plays a minor role in elimination (Martin et al., 2003), did not impact OATP1B3-1B7 mediated transport. However, for the angiotensin II type 1 receptor inhibitors tested in this study, the association of metabolism with OATP1B3-1B7 interaction no longer holds.

Fig. 4. Impact of known inhibitors of OATP1B3-1B7 on microsomal accumulation of ezetimibe. Hepatic (A) and small intestinal microsomes (B) were exposed to 0.1 μM ezetimibe in the absence or presence of 100 μM bromsulphthalein, atorvastatin, or dehydroepiandrosterone sulfate, and the microsomal accumulation was quantified. Data of $n = 3$ independent experiments are shown as mean % of solvent control $\pm$ S.D. For statistical analysis, the one-sample t test was applied (* $P < 0.05$).

Here we found inhibition of OATP1B3-1B7 by irbesartan and valsartan. Irbesartan undergoes extensive hydroxylation and glucuronidation (Chando et al., 1998), whereas valsartan is only minimally metabolized (Nakashima et al., 2005). Taken together, this suggests that microsomal metabolism is not a valid criterion to predict interaction with OATP1B3-1B7.

One feature that may be predictive is the presence of a steroid scaffold. Indeed, mifepristone, a progesterone receptor antagonist (Wildschut et al., 2011), was identified as an OATP1B3-1B7 inhibitor. However, it was the weakest inhibitor, with a maximal inhibition of about 50% at the maximum concentration (1000 μM) tested. Nevertheless, the steroid scaffold in mifepristone appears in the previously reported OATP1B3-1B7 substrates DHEAS and estradiol 17 β -D-glucuronide (Malagnino et al., 2018). Further studies would certainly be warranted to strengthen this assumption.

Troglitazone exhibited the highest inhibitory potency in our primary screening with an IC_{50} value of about 0.6 μM . This peroxisome proliferator-activated receptor- γ agonist developed for the treatment of diabetes was withdrawn from the market in 2000 due to severe idiosyncratic hepatotoxicity (Ogimura et al., 2017). The mechanisms underlying the drug-induced liver injury are still a focus of ongoing research programs, but they are assumed to involve interactions with multiple cellular pathways (Masubuchi, 2006). Considering that troglitazone itself is highly metabolized (He et al., 2004), it could be possible that OATP1B3-1B7 plays a role in its metabolism. However, this remains to be determined.

In this study, we further investigated the role of OATP1B3-1B7 in SER access and metabolism using ezetimibe, for which

the reported IC_{50} value was similar to that observed for OATP1B1 (14.8 μM) or OATP1B3 (8.9 μM) (Oswald et al., 2008). Moreover, ezetimibe is extensively glucuronidated (Ghosal et al., 2004), which allows indirect testing of the relation between SER access and the glucuronidation rate of a compound. Ezetimibe not only impaired OATP1B3-1B7-mediated DHEAS uptake, but our data also suggested that it is a substrate of OATP1B3-1B7. Once absorbed, ezetimibe is extensively metabolized by glucuronidation (Patel et al., 2003). The major metabolite created is ezetimibe β -D-glucuronide, which not only exhibits pharmacologic activity comparable to the parent compound but also is known to be part of the extensive enterohepatic recycling of ezetimibe (Patel et al., 2003; Kosoglou et al., 2005).

Importantly, glucuronidation of ezetimibe is not limited to the liver; it has also been shown for the small intestine. Considering that SER access would also be of relevance in the intestine, we confirmed the presence of OATP1B3-1B7 in enterocytes and small intestinal microsomes. In accordance with previously published findings where OATP1B3-1B7 was found to be located in the SER of hepatocytes (Malagnino et al., 2018), we observed the majority of the transporter to be present in the SER. Also important is that SER access is a rate-limiting step for glucuronidation. In metabolic studies with microsomes the pore-forming substance alamethicin is commonly used, which results in an increase of the observed glucuronidation rates (Fisher et al., 2000). By testing the influence of alamethicin on the glucuronidation of ezetimibe, we observed this effect of alamethicin in both small intestinal and hepatic microsomes.

To link OATP1B3-1B7 to SER access of ezetimibe, we tested the influence of OATP inhibitors or substrates of the transporter on the accumulation of ezetimibe in liver and small intestine microsomes. We observed a significant reduction of ezetimibe accumulation in their presence. This finding suggested that an OATP transporter might be involved in the SER entry of ezetimibe in the intestine and liver. Furthermore, we were able to show that the diminished accumulation of ezetimibe into microsomes also affected glucuronidation rates of ezetimibe. Indeed, in the presence of BSP, a nonglucuronidated OATP-inhibitor (Grotsky et al., 1959; Guofeng and Morris, 2014), the glucuronidation rates of ezetimibe were significantly decreased.

Taken together, our data suggest that ezetimibe is a substrate of OATP1B3-1B7, for which OATP1B3-1B7 could mediate SER access of ezetimibe and thereby facilitate admission to the active site of UGT isoforms. Considering that ezetimibe β -D-glucuronide is the major metabolite, we tested whether this molecule also interacts with the transport function of OATP1B3-1B7. Here, we observed moderate inhibition. Although we were able to link glucuronidation of ezetimibe with OATP inhibition in the SER, we are not able to define whether OATP1B3-1B7 mediates SER entry or exit; both processes would have an influence on the enzymatic rates of glucuronidation, considering the possibility of product inhibition of the involved UGT enzymes.

In conclusion, we showed that OATP1B3-1B7 interacts with several drugs. One of the identified inhibitors was ezetimibe, which is also a substrate of OATP1B3-1B7. Moreover, ezetimibe glucuronidation rates were decreased by BSP, which is a OATP1B3-1B7 inhibitor. Thus, we hypothesize, that OATP1B3-1B7 may function as a SER gateway for ezetimibe.

hepatic microsomes

intestinal microsomes

Fig. 5. Ezetimibe glucuronidation in microsomes. The ezetimibe glucuronidation rates in hepatic (A and B) and small intestinal microsomes (C and D) were assessed in the presence or absence of the pore-forming molecule alamethicin (A and C) and in the presence or absence of the OATP inhibitor BSP (B and D). The rate of ezetimibe glucuronidation was calculated applying the nonlinear one-phase association curve fit. Data are reported as mean $\pm$ S.D. of $n = 3$ independent experiments. n.a., not applicable.

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Authorship Contributions

Participated in research design: Malagnino, Duthaler, Meyer zu Schwabedissen.

Conducted experiments: Malagnino, Duthaler, Seibert.

Contributed new reagents or analytic tools: Krähenbühl, Meyer zu Schwabedissen.

Performed data analysis: Malagnino, Duthaler, Meyer zu Schwabedissen.

Wrote or contributed to the writing of the manuscript: Malagnino, Duthaler, Meyer zu Schwabedissen.

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