Prediction of OATP1B1 and OATP1B3 mediated hepatic uptake of statins based on transporter protein expression and activity data

Annett Kunze, Jörg Huwyler, Gian Camenisch, Birk Poller

Division of Drug Metabolism and Pharmacokinetics, Drug-Drug Interactions
Section, Novartis Institutes for BioMedical Research, CH-4056 Basel,
Switzerland (A.K, G.C, BP);

Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology,
University of Basel, CH-4056 Basel, Switzerland (A.K., J.H.)

Running Title: Prediction of OATP mediated hepatic uptake of statins

Corresponding author:

Birk Poller

Novartis Pharma AG

Fabrikstrasse 14

CH-4056 Basel

e-mail: birk.poller@novartis.com

Mobile: +41796822472

Fax: +41613248001

Number of text pages: 45

Number of tables: 3

Number of figures: 4

Number of references: 30

Number of words in abstract: 247

Number of words in introduction: 748

Number of words in discussion: 1458

List of abbreviations:

CCK8	cholecystokinin octapeptide
DDI	drug-drug interaction
DMEM	Dulbecco's Modified Eagle Medium
E3S	estrone-3-sulfate
FBS	fetal bovine serum
exp	transporter protein expression
HEK	human embryonic kidney
HMG-CoA	hydroxymethylglutaryl-coenzyme A
IVIVE	<i>in vitro-in vivo</i> extrapolation
KHB	Krebs-Henseleit Buffer
K_m	Michaelis-Menten constant
LC-MS/MS	liquid chromatography- tandem mass spectrometry
LSC	liquid scintillation counting
MRM	multiple reactions monitoring
NSB	nonspecific binding
NTCP	sodium taurocholate co-transporting polypeptide
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT	organic cation transporter
PBS	phosphate-buffered saline
PS_{act}	active uptake clearance
$PS_{act,max}$	maximal transporter activity
PS_{app}	apparent uptake clearance

PS_{pas}	passive uptake clearance
PS_{tot}	total uptake clearance
RAF	relative activity factor
REF	relative expression factor
QTAP	quantitative targeted absolute proteomics
V_{app}	uptake rate

Abstract

Organic anion transporting polypeptides (OATP) 1B1 and OATP1B3 are drug transporters mediating the active hepatic uptake of their substrates. Since they exhibit overlapping substrate specificities the contribution of each isoform to the net hepatic uptake needs to be considered when predicting drug-drug interactions. The relative contribution of OATP1B1 and OATP1B3-mediated uptake of statins into hepatocytes was estimated based on either relative transporter protein expression data or relative activity data. Therefore, kinetics of eight statins and OATP1B1 and OATP1B3-specific reference substrates were determined in OATP1B1 and OATP1B3 expressing HEK293 cells and in human cryopreserved hepatocytes. Absolute OATP1B1 and OATP1B3 protein abundance was determined by liquid chromatography- tandem mass spectrometry in all expression systems. Transporter activity data generated in recombinant cell lines were extrapolated to hepatocyte values using relative transporter expression factors (REF) or relative activity factors (RAF). Our results showed a pronounced OATP1B1 and comparatively low OATP1B3 protein expression in the investigated hepatocyte lot. Based on REF-scaling, we demonstrated that the active hepatic uptake clearances of reference substrates, atorvastatin, pravastatin, rosuvastatin, and simvastatin were well predicted within two-fold error demonstrating that OATP1B1 and OATP1B3 were major contributors. For other statins, the net hepatic uptake clearance was under-predicted, suggesting the involvement of other hepatic uptake transporters. Summarized, we showed that REF and RAF-based predictions were highly

similar indicating a direct transporter expression-activity relationship. Moreover, we demonstrated that the REF-scaling method provided a powerful tool to quantitatively assess the transporter-specific contributions to the net uptake clearance of statins in hepatocytes.

Introduction

Human drug uptake transporters are membrane-bound proteins that facilitate the active cellular uptake of compounds which cannot cross cellular membranes by passive diffusion due to their physiochemical properties. Expressed at the basolateral membrane of hepatocytes, organic anion transporting polypeptides (OATPs) mediate the uptake of mainly anionic drugs from the blood into the liver. OATP inhibition due to drug-drug interactions (DDI) can lead to increased plasma concentrations levels of drugs thus posing a potential risk for toxicity in peripheral organs. Following co-medication involving hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), muscle toxicity and severe myopathy are reported risks that have been partially attributed to inhibition of OATPs (Staffa et al., 2002; Neuvonen et al., 2006; Shitara and Sugiyama, 2006). Thus, predicting the transporter-mediated DDI risk is a necessity for the development of new molecular entities.

Cryopreserved human hepatocytes are a common tool to assess the hepatic uptake of compounds. Hepatocytes co-express various uptake transporters including OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*), OATP2B1 (*SLCO2B1*), the organic anion transporter 2 (OAT2; *SLC22A7*), the organic cation transporter 1 (OCT1; *SLC22A1*), and the sodium taurocholate co-transporting polypeptide (NTCP; *SLC10A1*). Since a variety of compounds including statins exhibit an overlap of transporter specificity, compound uptake in hepatocytes reflects the sum of all transporter-specific contributions (Neuvonen et al., 2006; Shitara and

Sugiyama, 2006; Noe et al., 2007; Kalliokoski and Niemi, 2009; Knauer et al., 2010; Bi et al., 2013; Shitara et al., 2013).

To assess the relative contribution of specific transporters to the net hepatic uptake in vitro, methods based on relative transporter expression and transporter activity have been introduced. Hirano et al. established a method that allows the estimation of the contribution of OATP1B1 and OATP1B3 mediated uptake in hepatocytes based on relative activity factors (RAF) (Hirano et al., 2004). Determined as ratios of the uptake transporter activity of transporter specific substrates in hepatocytes relative to the activity in recombinant cell lines, the RAF method has been widely used to estimate the contribution of OATP1B1 and OATP1B3 to the hepatic uptake of various compounds (Hirano et al., 2004; Shimizu et al., 2005; Shitara and Sugiyama, 2006; Kitamura et al., 2008; Williamson et al., 2013). In addition, Hirano et al. used protein expression data from Western Blot analysis to estimate relative expression factors (REFs) to determine the contribution of OATP1B1, OATP1B3, and OATP2B1 to the net hepatic uptake of pitavastatin and estradiol 17 β -D-glucuronide (Hirano et al., 2004; Hirano et al., 2006). While the predicted transporter contributions based on RAFs and REFs were within a comparable range, net hepatic uptake clearances estimated from REFs were significantly over-predicted compared to observed values. Recently, the contribution of OATP1B1-mediated hepatic uptake of five substrates was investigated using a gene knockdown approach (Williamson et al., 2013). Compared with a RAF-based method highly similar results were obtained in predicting the transporter-specific contributions to the net hepatic uptake. Yet, extending the described approaches to any transport protein of

interest is challenging due to practical limitations, such as the need for specific antibodies for Western Blots, transporter-specific substrates for RAFs, and gene-specific knockdown. Moreover, the RAF-based and siRNA-based approaches are restricted to investigated cell systems (i.e. hepatocytes) and do not allow the extrapolation of transporters activities to any tissue based on in vitro experiments.

Recently, novel quantitative targeted absolute proteomics (QTAP) methods, based on liquid chromatography- tandem mass spectrometry (LC-MS/MS), have been used to determine the absolute transporter protein abundance in plasma membrane samples of various human tissues including liver and brain (Kamiie et al., 2008; Sakamoto et al., 2011; Uchida et al., 2011; Ohtsuki et al., 2012; Schaefer et al., 2012). Moreover, REFs determined by LC-MS/MS based approaches are used in first studies to determine the specific contribution of hepatic uptake transporters in cryopreserved hepatocytes and human liver (Karlgren et al., 2012; Kimoto et al., 2012; Bi et al., 2013; Vildhede et al., 2014).

It was the aim of the present study to determine the contribution of OATP1B1 and OATP1B3 to the net hepatic uptake clearance of atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. For this purpose protein expression levels of OATP1B1 and OATP1B3 were measured in cryopreserved human hepatocytes and in recombinant HEK293 cell lines. Subsequently, we determined the uptake clearances of statins and used REFs derived from QTAP analysis to extrapolate OATP1B1 and OATP1B3 activities obtained in recombinant cells to hepatocyte values. Finally, in order to further validate the REF-based scaling method, we assessed the correlation

between uptake transporter activity and their relative protein abundance by comparing RAF and REF-based predictions.

Materials and Methods

Compounds

[³H]atorvastatin calcium (0.37 MBq/nmol), [³H]cholecystokinin octapeptide (CCK8; 3.65 MBq/nmol), [³H]cerivastatin sodium (0.185 MBq/nmol), [³H]fluvastatin sodium (0.74 MBq/nmol) [³H]lovastatin acid (0.37 MBq/nmol), [³H]pitavastatin calcium (0.185 MBq/nmol), [³H]pravastatin sodium (0.185 MBq/nmol), [³H]rosuvastatin calcium (0.37 MBq/nmol), and [³H]simvastatin acid (0.37 MBq/nmol) were obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO). [³H]estrone-3-sulfate ammonium (E3S; 1.67 MBq/nmol) was purchased from PerkinElmer (Boston, MA). All other compounds and reagents were of analytical grade and purchased from commercial sources.

Cell systems

LiverPoolTM cryopreserved human hepatocytes (lot PQP) were obtained from Celsis, In Vitro Technologies (Brussels, Belgium). The hepatocyte pool was derived from non-transplantable fresh liver tissues of twenty donors (gender: 10 male and 10 female; age: 17-75, average age: 52; ethnic background: 16 Caucasians, 2 Blacks, and 2 Hispanics). A HEK293 cell line stably expressing human OATP1B3 (polybrene transfection method) was purchased from Deutsches Krebsforschungszentrum (Heidelberg, Germany) (König et al., 2000). A recombinant HEK293 cell line with stable expression of human OATP1B1 was

generated in-house using the Flp-In™ system (Invitrogen by Life Technologies, Paisley, United Kingdom) as previously described (Kunze et al., 2012).

All HEK293 cell lines were cultivated in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. For HEK293 cells expressing OATP1B1 or OATP1B3, 100 ng/μL hygromycin B or 800 ng/μL geneticin, respectively, was added to the cultivation medium.

The protein content of solubilized cells (solved in 0.2 N NaOH) was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories AG, Hercules, CA) according to the manufacturer's recommendations.

Determination of absolute transporter protein abundance

Absolute protein expression levels of human OATP1B1 and OATP1B3 in the membrane fractions of recombinant HEK293 cells and human cryopreserved hepatocytes were determined by peptide-based LC-MS/MS. The preparation of membrane fractions as well as the QTAP analysis was performed by BertinPharma (Montigny le Bretonneux, France) according to established methods by the group of Terasaki, Sakamoto, and Ohtsuki et al. (Sakamoto et al., 2011; Ohtsuki et al., 2012).

Three samples containing each approximately 80 million HEK293-OATP1B1 or HEK293-OATP1B3 cells were quickly harvested in ice-cold lysis buffer [Tris-HCl 10 mM, 250 mM sucrose, Complete Protease Inhibitor (Roche, Basel, Switzerland)]. Subsequently, the samples were centrifuged (537g, 4°C, 5 min) and the supernatant was aspirated. Two samples of human cryopreserved hepatocytes (approximately 30 million cells per sample) were thawed and

immediately suspended in *InVitroGRO*TM HT Medium (BioreclamationIVT; Baltimore, MD). Samples were then centrifuged (50g; 4°C; 5 min) and the supernatant was aspirated. All cells were stored as dry pellet at -80°C and were shipped to BertinPharma on dry ice.

All cell samples were processed by BertinPharma following the published protocols from the Terasaki group (Sakamoto et al., 2011; Ohtsuki et al., 2012). The cell pellets were suspended in lysis buffer followed by homogenization. Thereafter, sub-mitochondrial fractions were isolated by centrifugation (25 min; 10.800 g; 4°C). The supernatant was collected and the microsomal fractions were obtained by centrifugation (60 min; 100.000 g; 4°C). The microsomal pellet was suspended in 10 mM Tris-HCl buffer (pH 7.4; 250 mM sucrose). The plasma membrane fractions were obtained by ultracentrifugation of the microsomal fractions through a 38% (w/v) sucrose solution (Sakamoto et al., 2011; Ohtsuki et al., 2012).

Total protein contents were determined by Lowry's method before and after each fractionation step. The absolute transporter protein abundance in the respective plasma membrane fractions were determined using simultaneous QTAP based on LC-MS/MS with multiple reactions monitoring (MRM) (Sakamoto et al., 2011; Ohtsuki et al., 2012). The same reference peptides were selected as previously published by Uchida et al.: LNTVGIK for OATP1B1; IYNSVFFGR for OATP1B3; VLLQTLR for OATP2B1; NVALLALPR for OAT2; LSPSFADLFR for OCT1; and GIYDGDLEK for NTCP (Uchida et al., 2011).

The respective transporter protein expression (exp) was obtained as the amount of transporter protein (fmol) per amount of plasma membrane protein (μg

prot_{mem}). To determine the transporter expression per amount of total protein [fmol/(μg prot)], exp was multiplied with the amount of plasma membrane protein obtained per amount of total sample protein.

Uptake studies in suspended human hepatocytes

Hepatocyte uptake of reference compounds (E3S; CCK8) and the statins was assessed by the oil spin method as previously described (Umehara and Camenisch, 2012). Frozen hepatocytes were thawed and directly suspended in *InVitroGRO*TM HT medium. After centrifugation (537g, 5 min, low brakes), the supernatant was aspirated and the cells were immediately suspended in 1 mL of prewarmed Krebs-Henseleit-Buffer (KHB). Subsequently, cells were counted and the suspension was adjusted to a concentration of $1.0 - 1.5 \cdot 10^5$ viable cells/mL (viability: 83-95%).

Hepatocyte uptake studies were initiated by adding 50 μL of hepatocyte suspension to 100 μL of substrate solution (KHB containing a mixture of radio-labeled and non-labeled study compound at specific concentration). All incubations were carried out at 37°C and at 4°C following preincubation times of 5 min and 15 min at 37°C and 4°C, respectively. At designated time-points incubations were terminated transferring the sample to a mineral oil/NaOH containing tube [Hepatocyte Transporter Suspension Assay Kit (BD Biosciences, Woburn MA)], followed by immediate centrifugation (10'000 rpm; 1 min). The tubes were cut and the radioactivity in the cell pellets as well as in the supernatants (for mass balance studies) was analyzed by liquid scintillation counting (LSC; Packard Tri-Carb 2700TR; PerkinElmer Inc, Waltham MA).

All hepatocyte incubations were performed for 90 s. In time-dependent uptake experiments (1, 2, 3, 5 min) for E3S (0.1 μ M), pitavastatin (0.5 μ M), CCK8 (0.5 μ M), and rosuvastatin (0.5 μ M), the rate of uptake was found to lie in a time-linear uptake phase and was consequently applied to all statin incubations. For concentration-dependent kinetic studies, a broad concentration range was defined for all substrates (0.01 – 300 μ M; 5-10 concentration points). To demonstrate uptake transporter activity, E3S (0.03 μ M) uptake was measured in the absence and presence of an OATP inhibitor cocktail [a combination of atorvastatin (10 μ M) and rifamycin (20 μ M)] in all hepatocyte studies.

Uptake studies in human HEK293-OATP1B1 and HEK293-OATP1B3 expressing cell lines

Cellular uptake studies using plated HEK293-OATP1B1, HEK293-OATP1B3, and HEK293 parental cells, were performed as previously described (Kunze et al., 2012). Uptake studies at 37°C were initiated by incubating the cells with the substrate solution at the respective concentrations (mix of radiolabeled and unlabeled study compound in KHB). To determine mass balances of the studied compounds, aliquots of the incubation solution were taken from each well prior to the termination of the incubation. Afterwards, the incubation was terminated by aspirating the remaining incubation solution followed by washing the cells twice with ice-cold PBS. Subsequently, the cells were lysed in NaOH (0.2 N). The protein contents of the solubilized cells were determined as described above. The amount of radio-labeled compound in the cell samples and in the incubation solution was quantified using LSC as described in the previous section.

Time-dependent experiments in OATP1B1 and OATP1B3-expressing HEK293 cells were performed to define the time-linear range for subsequent concentration-dependent studies (0.01-300 μM ; 6-9 concentration points). An incubation time of 3 min was chosen for all cell lines and compounds, except for E3S (1 min). Uptake of each study compound (0.01 μM) was measured in the absence and presence of an OATP inhibitor cocktail [a combination of atorvastatin (10 μM) and rifamycin (20 μM)]. Functional activity of OATP1B1 and OATP1B3 in the recombinant cell lines was confirmed in each experiment as previously described (Kunze et al., 2012).

Data analysis

The uptake kinetics of the investigated compounds were calculated by normalizing the measured radioactivity to the incubation time and protein content. Consequently, these uptake rates (V_{app} ; $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) were divided by the applied substrate concentrations to obtain the apparent uptake clearances (PS_{app} ; $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$). Results are presented as uptake clearances throughout the whole manuscript to simplify the visualization of kinetic data.

As previously described, PS_{app} values determined in suspended hepatocytes are potentially affected by nonspecific binding (NSB) of the compound to plastic surfaces of the assay device or to cellular structures (Umehara and Camenisch, 2012). To account for plastic binding, total compound recoveries were calculated for all incubations and PS_{app} values were multiplied by a respective correction factor (total theoretical recovery divided by obtained recovery). In a second step, control incubations were performed at 4°C to correct PS_{app} obtained at 37°C

($PS_{app,tot,37^{\circ}C}$) for saturable NSB processes to cells (Umehara and Camenisch, 2012):

$$PS_{tot} = PS_{app,tot,37^{\circ}C} - (PS_{app,tot,4^{\circ}C,Cmin} - PS_{app,tot,4^{\circ}C,Cmax}) \quad \text{Eq. 1}$$

where the difference between the apparent uptake clearances determined from 4°C incubations at the lowest and highest substrate concentrations ($PS_{app,tot,4^{\circ}C,Cmin}$ and $PS_{app,tot,4^{\circ}C,Cmax}$, respectively) was used to describe saturable nonspecific cell binding. For incubations with OATP1B1 and OATP1B3 expressing HEK293 cells, none of the studied compounds showed significant NSB to plastic (recoveries > 90%). Moreover, no relevant saturable NSB to cells was observed in control incubations using HEK293 parental cells. Consequently, no correction of uptake clearances was required for studies with cell lines and PS_{tot} equals PS_{app} . The different experimental setups between HEK293 cells (plated) and hepatocytes (suspension) and the resulting difference in cell surface exposed to the incubation medium might explain the lack of saturable NSB in HEK293 parental cells. Moreover, as discussed previously, a difference in NSB between HEK293 cells and hepatocytes could be a result of different cell membrane compositions (e.g. lipids and protein contents), between HEK293 cells and hepatocytes (Mateus et al., 2013).

The total uptake clearance (PS_{tot}) is the sum of the active uptake clearance for saturable, transporter-mediated and passive permeation processes (PS_{act} and PS_{pas} , respectively):

$$PS_{tot} = PS_{act} + PS_{pas} = \frac{PS_{act,max} \cdot K_m}{K_m + S} + PS_{pas} \quad \text{Eq. 2}$$

where the active transporter-mediated process is following Michaelis-Menten kinetics with K_m and S representing the Michaelis-Menten constant (μM) and the substrate concentration (μM), respectively. For initial uptake rates and clearances at very low substrate concentration ($S \ll K_m$), PS_{act} can be approximated with $PS_{\text{act,max}}$ representing a measure of the intrinsic transporter activity. Kinetic parameters were estimated by fitting Equation 2 to the measured data using a nonlinear least square method.

Estimation of OATP contribution in human hepatocytes

The relative contribution of OATP1B1 and OATP1B3 to the hepatic uptake of compounds can be assessed by determining a relative activity factor (RAF) or a relative expression factor (REF) for a specific transporter. In brief, RAFs for OATP1B1 and OATP1B3 were determined by comparing the transporter activities of transporter specific reference substrates (E3S for OATP1B1; CCK8 for OATP1B3) in recombinant cell lines and in hepatocytes, as described by Hirano et al., 2004 and Kimoto et al., 2012:

$$RAF_{1B1} = \frac{PS_{\text{act,max,ES_HEP}}}{PS_{\text{act,max,ES_HEK OATP1B1}}} \quad \text{Eq. 3}$$

$$RAF_{1B3} = \frac{PS_{\text{act,max,CCK8_HEP}}}{PS_{\text{act,max,CCK8_HEK OATP1B3}}} \quad \text{Eq. 4}$$

Subsequently, RAF_{1B1} and RAF_{1B3} were applied to predict the combined uptake transporter activity of OATP1B1 and OATP1B3 co-substrates in hepatocytes

($PS_{act,max,HEP}$) based on the transporter activities measured in recombinant cells

($PS_{act,max,1B1;1B3}$):

$$PS_{act,max,HEP} = PS_{act,max,HEK\ OATP1B1} \cdot RAF_{1B1} + PS_{act,max,HEK\ OATP1B3} \cdot RAF_{1B3} \quad \text{Eq. 5}$$

Alternatively, we used absolute OATP1B1 and OATP1B3 protein expression data in hepatocytes and recombinant HEK293 cells to derive the REFs for OATP1B1 and OATP1B3:

$$REF_{1B1} = \frac{\exp_{OATP1B1,HEP}}{\exp_{OATP1B1,HEK\ OATP1B1}} \quad \text{Eq. 6}$$

$$REF_{1B3} = \frac{\exp_{OATP1B3,HEP}}{\exp_{OATP1B3,HEK\ OATP1B3}} \quad \text{Eq. 7}$$

where $\exp$ represents the specific transporter expression [fmol/(μ g prot)] determined as described in section “Determination of absolute transporter protein abundance”. In analogy to equation 5, the net transporter activity of compounds into hepatocytes was calculated from recombinant cells using the REF values:

$$PS_{act,max,HEP} = PS_{act,max,HEK\ OATP1B1} \cdot REF_{1B1} + PS_{act,max,HEK\ OATP1B3} \cdot REF_{1B3} \quad \text{Eq. 8}$$

Statistics

All incubations for kinetic studies were performed in triplicates ($n = 3$) where values are given as the mean and standard deviation. Statistical significance for the differences in uptake clearances obtained in incubations in the presence and absence of transporter inhibitors was assessed by unpaired, two-tailed Student's *t*-test. Differences were considered to be statistically significant for *p*-values below 0.05. For parameter estimation based on data fitting the coefficients of

determination (R^2) were determined to assess the goodness of fit. Moreover, fold-error deviations between the observed and predicted hepatic $PS_{act,max}$ values were calculated to assess the performance of the REF and RAF-based prediction methods.

Results

Transporter abundance

The transporter protein abundance in plasma membrane fractions of HEK293-OATP1B1 and HEK293-OATP1B3 cells and pooled human cryopreserved hepatocytes (lot PQP) was determined by the QTAP method. Figure 1 and Table 1 show the measured abundances of the uptake transporters OATP1B1, OATP1B3, OATP2B1, NTCP, OAT2, and OCT1 in cryopreserved human hepatocytes. Significant differences in expression levels were found between the specific transporters, ranging from 0.35 fmol/($\mu\text{g prot}_{\text{mem}}$) for OATP1B3 to 15.85 fmol/($\mu\text{g prot}_{\text{mem}}$) for OATP1B1. OATP2B1, NTCP, and OAT2 showed similar expression levels [1.03 to 2.62 fmol/($\mu\text{g prot}_{\text{mem}}$)] while the expression of OCT1 was comparatively higher [6.94 fmol/($\mu\text{g prot}_{\text{mem}}$)]. In plasma membranes of recombinant HEK293 cells, the protein expression levels of OATP1B1 and OATP1B3 were measured at 23.97 fmol/($\mu\text{g prot}_{\text{mem}}$) and 1.44 fmol/($\mu\text{g prot}_{\text{mem}}$) (Table 1). Transporter protein expression levels, shown in Table 1, are either normalized to the amount of total plasma membrane ($\mu\text{g prot}_{\text{mem}}$) or to the total sample protein ($\mu\text{g prot}$).

Determination of pharmacokinetic parameters

We performed concentration-dependent incubations in HEK293-OATP1B1 and OATP1B3 over-expressing cells, and in suspended hepatocytes to subsequently estimate pharmacokinetic parameters of our study compounds. Control

incubations in the presence of the OATP inhibitors atorvastatin and rifamycin resulted in a significant decrease in PS_{tot} values in recombinant cell lines and hepatocytes, confirming transporter functionality in all uptake experiments (data not shown).

Figure 2 shows the uptake clearances of the OATP1B1 and OATP1B3-specific reference compounds E3S and CCK8, respectively, into recombinant cell lines and suspended hepatocytes. E3S uptake clearances in HEK293-OATP1B1 were decreased in a concentration-dependent manner and PS_{tot} was significantly reduced in the presence of the OATP inhibitors. Also HEK293-OATP1B3 cells showed transport activity for E3S, which however, was significantly lower compared to HEK293-OATP1B1 cells (Fig 2A). For CCK8, a concentration-dependent decrease in uptake clearances and inhibition in presence of OATP inhibitors were found in recombinant HEK293-OATP1B3 but not in HEK293-OATP1B1 cells (Fig. 2B).

Compound recoveries in hepatocytes studies above 85% were obtained for E3S, CCK8, atorvastatin, lovastatin, pitavastatin, and rosuvastatin. In contrast, substantial concentration-dependent NSB to the experimental device was found for cerivastatin (69%), fluvastatin (79%), pravastatin (56%), and simvastatin (71%).

As illustrated in Figures 2C and 2D, experiments with E3S and CCK8 using human hepatocytes showed a concentration-dependent decrease in PS_{app} values at 4°C incubations. As previously described, such a profile indicates saturation of temperature-independent NSB of the compound to cellular structures. Consequently, uptake clearances determined from 37°C incubations

were corrected according to Equation 1. The resulting PS_{tot} values showed a concentration-dependent decrease for E3S and CCK8 indicating that both compounds were actively transported into hepatocytes. The observed PS_{tot} value for E3S was more than ten-fold higher than the value observed for CCK8.

Figure 3 shows representative kinetic profiles for rosuvastatin and cerivastatin. For both compounds, OATP1B1 or OATP1B3 uptake clearances were significantly reduced in the presence of the OATPs inhibitors (Figure 3A and 3B). Moreover, saturation of transporter activities at high concentrations of both statins were observed in HEK293-OATP1B1 and HEK293-OATP1B3 cells. However, in both cell lines the concentration-dependent decrease in PS_{tot} was more pronounced for rosuvastatin than for cerivastatin, probably due to the comparatively high passive uptake clearance obtained for the latter compound.

Atorvastatin, fluvastatin, pravastatin and pitavastatin were also actively transported by OATP1B1 and OATP1B3, while lovastatin was found to be a substrate for only OATP1B1 but not for OATP1B3. The simvastatin uptake clearances decreased in a concentration-dependent manner in OATP1B1 and OATP1B3 cells but co-incubation with the OATP inhibitors did not affect the total uptake clearance in either cell line.

Hepatocyte uptake profiles of rosuvastatin and cerivastatin are shown in Figure 3C and 3D. The uptake clearances of both compounds were decreased in a concentration-dependent manner. Compared to the PS_{tot} value of rosuvastatin observed at initial concentrations, a very high PS_{tot} of cerivastatin was obtained. However, at high substrate concentrations, PS_{tot} of cerivastatin was significantly higher compared to the value determined for rosuvastatin, indicating a high

contribution of the passive uptake clearance to the total cerivastatin uptake clearance.

Table 2 summarizes the estimated maximal activities ($PS_{act,max}$), the ratios between active to passive compound clearances, and the global goodness of fit (R^2) for parameter estimations according to equation 2. In recombinant cell lines highest $PS_{act,max}$ values were obtained for E3S and CCK8 in HEK293-OATP1B1 and OATP1B3-expressing cells, respectively. While E3S also exhibited the highest $PS_{act,max}$ value in hepatocytes, active CCK8 transport was comparatively low. For the statins, comparable activities ($PS_{act,max}$) of OATP1B1 and OATP1B3 were derived from recombinant cells while higher activities were generally obtained in human hepatocytes. The ratios between active and passive uptake clearances ($PS_{act,max}/PS_{pas}$) represent a measure of the contribution of the transporter-mediated process to the total uptake clearance. E3S exhibited the highest ratios in HEK293-OATP1B1 cells and in hepatocytes. Among the statins, the highest ratio was measured for pravastatin in hepatocytes. Together with atorvastatin and rosuvastatin, pravastatin also showed highest ratios in recombinant cell lines. Ratios below one were obtained for simvastatin, fluvastatin, and cerivastatin in all expression system, indicating an extensive contribution of passive permeation to the total uptake clearance for these compounds.

REF and RAF based prediction of compound uptake in suspended hepatocytes

Table 3 lists the observed and predicted hepatic $PS_{act,max}$ values as well as the determined scaling factors. We obtained very similar transporter specific scaling factors with values of 0.853 and 1.112 for REF_{1B1} and RAF_{1B1} (1.3-fold deviation), respectively, and of 0.181 and 0.113 for REF_{1B3} and RAF_{1B3} (1.6-fold deviation), respectively. Consequently, the $PS_{act,max}$ values predicted from the RAF and REF-methods were highly comparable. Given the high similarity between the two scaling methods, only the results for REF-based scaling are discussed in the following.

$PS_{act,max}$ values in hepatocytes were predicted from the extrapolated sum of OATP1B1 and OATP1B3 activities (Equation 8). A good resulting prediction accuracy between the observed and predicted values for E3S, CCK8, atorvastatin, pravastatin, rosuvastatin, simvastatin was obtained with errors below two-fold. In contrast, poor predictability was observed for cerivastatin, fluvastatin, lovastatin, and pitavastatin with errors between two to six-fold, thus indicating a significant under-prediction.

Figure 4 illustrates the contribution of OATP1B1 and OATP1B3 to the observed active uptake clearance of statins in suspended hepatocytes predicted from REF-scaling. For all statins, OATP1B1-mediated uptake into hepatocytes was significantly higher than active uptake by OATP1B3. Moreover, the contribution of OATP1B1 and OATP1B3 to the hepatic uptake clearance of atorvastatin, pravastatin, rosuvastatin, and simvastatin was above 50% indicating that both isoforms were the major contributors to the hepatic uptake of these compounds. In contrast, OATP1B1 and OATP1B3-mediated uptake of cerivastatin, fluvastatin, lovastatin, and pitavastatin was not the major

determinant of the active hepatic uptake clearance determined in hepatocyte lot
PQP.

Discussion

In the present study we determined the OATP1B1 and OATP1B3-mediated uptake clearances of statins in single-transporter expressing HEK293 cells. Based on relative transporter protein expression data, we used the transporter activities to estimate the OATP1B1 and OATP1B3-mediated uptake of statins into hepatocytes.

Recently, QTAP methods were established to quantify low abundant proteins, and first expression levels in cryopreserved hepatocytes and liver samples were reported for the major hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, and NTCP (Bi et al., 2012; Kimoto et al., 2012; Ohtsuki et al., 2012; Bi et al., 2013). Literature data on OATP transporter expression show high inter-individual differences. For OATP1B1 values between 2-12 fmol/($\mu\text{g prot}_{\text{mem}}$) in human liver samples and 2-7 fmol/($\mu\text{g prot}_{\text{mem}}$) in cryopreserved hepatocytes are reported (Karlgrén et al., 2012; Kimoto et al., 2012; Ohtsuki et al., 2012; Vildhede et al., 2014).

For OATP1B3 reported values vary between 1-6 fmol/($\mu\text{g prot}_{\text{mem}}$) in human liver samples and 1-2 fmol/($\mu\text{g prot}_{\text{mem}}$) in cryopreserved hepatocytes (Karlgrén et al., 2012; Ohtsuki et al., 2012; Vildhede et al., 2014). Reported values for other uptake transporters in human liver tissues vary between 1-4 (OATP2B1), 1-10 (NTCP), 1-3 (OAT2), and 3-15 fmol/($\mu\text{g prot}_{\text{mem}}$) (OCT1) (Karlgrén et al., 2012; Kimoto et al., 2012; Ohtsuki et al., 2012; Vildhede et al., 2014).

In the present study, transporter protein expression was quantified for pooled (20 donors) human cryopreserved hepatocytes. Measured protein expression levels

were within the range of reported values, with 16 (OATP1B1), 0.4 (OATP1B3), 2 (OATP2B1), 3 (NTCP), 1 (OAT2), and 7 fmol/($\mu\text{g prot}_{\text{mem}}$) for OCT1 (Table 1). In the tested lot of human hepatocytes, OATP1B1 protein was found to be expressed at a substantially higher level than OATP1B3. Such a pronounced OATP1B1 abundance with a concomitant low OATP1B3 expression has not yet been reported for protein levels in cryopreserved hepatocytes. In contrast, comparatively high differences in OATP1B1 and OATP1B3 expression levels were observed in human liver samples (Vildhede et al., 2014). Thus, the level of OATP1B1 protein expression is in agreement with reported values in human liver samples while generally lower values are reported in human cryopreserved hepatocytes.

On a functional level, high variability in active uptake values determined in different cryopreserved hepatocytes is shown for statins and our reference substrates (Hirano et al., 2004; Watanabe et al., 2010; Kimoto et al., 2012). Hirano et al. reported maximum transporter activities between 36-84 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ for E3S and 1-5 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ for CCK8 in three different cryopreserved human hepatocyte lots (Hirano et al., 2004). While our CCK8 results were comparable, we obtained a significantly higher maximal activity for E3S uptake in hepatocytes. These findings are in-line with the high OATP1B1 protein expression level obtained in the hepatocyte lot PQP.

Differences in reported transporter activities and protein expression levels might be a result of substantial inter-individual variation in transporter protein abundances observed in human liver samples (Nies et al., 2013; Vildhede et al., 2014). Furthermore, differences in hepatocyte isolation or transporter protein

quantification procedures, as well as in the selection of reference peptides for QTAP analysis were attributed to impact determined transporter protein abundances (Balogh et al., 2012; Lundquist et al., 2014). All studied statins are reported substrates of OATP1B1, and fluvastatin, rosuvastatin, pravastatin, and pitavastatin are substrates of OATP1B3 (Hirano et al., 2004; Neuvonen et al., 2006; Noe et al., 2007; Kalliokoski and Niemi, 2009). Our results confirmed that all statins were substrates of OATP1B1. Only lovastatin was not identified as an OATP1B3 substrate. Simvastatin demonstrated concentration-dependent uptake kinetics in OATP1B1 and OATP1B3 cells but no significant inhibition was observed upon co-administration with OATP inhibitors. We assume that its extensive passive permeation might have masked the contribution of the active transport process.

To investigate the correlation between transporter activity and expression level, we predicted the OATP1B1 and OATP1B3 activities in hepatocytes from studies in cell lines expressing the respective transporter, using RAF and REF-based methods. In the ideal case, where measured activity of a specific transporter is directly proportional to its protein expression level, the transporter-specific REF, would be equal to the respective RAF. Our determined REF and RAF values for OATP1B1 (0.853 and 1.112) and for OATP1B3 (0.181 and 0.113) showed a high similarity indicating a direct correlation between expression levels and activity for OATP1B1 and OATP1B3. These results should neither be affected by individual variation in transporter protein expression, nor by variation in the hepatocyte preparation as absolute protein abundance measurements, as well as kinetic experiments, were performed for the same batch of human

hepatocytes. Thus, even if transporter protein expression in cryopreserved hepatocytes would not represent transporter expression levels in freshly-isolated or human liver samples, as indicated in studies by Kimoto et al. and Lundquist et al., the correlation between transporter protein expression and activity should not be affected as protein abundances and transporter activities were determined for the same lot of hepatocytes in the present study (Kimoto et al., 2012; Lundquist et al., 2014). In contrast, given the substantial variation in reported transporter protein abundances between different hepatocyte lots or liver samples, it is crucial to characterize transporter expression in hepatocytes in order to compare with the respective activities.

Using protein expression based REF-scaling, the observed hepatic $PS_{act,max}$ for the reference compounds E3S and CCK8 were predicted within two-fold error. For statins, the REF-based predicted $PS_{act,max}$ values were in good agreement with the observed values with fold error deviation below two for atorvastatin, pravastatin, rosuvastatin, and simvastatin, indicating that their active hepatic uptake was mainly described by OATP1B1 and OATP1B3 mediated transport. In contrast, the active hepatic uptake of cerivastatin, fluvastatin, lovastatin, and pitavastatin was under-estimated.

Within the scope of this study, the hepatic uptake activities were only extrapolated using OATP1B1 and OATP1B3 values. Therefore, a potential explanation could be the involvement of other transporters in the hepatic uptake of these compounds. Recently, Bi et al. showed that NTCP is significantly involved in the hepatic uptake of fluvastatin, pitavastatin, and rosuvastatin (Bi et al., 2013). NTCP protein abundance in our hepatocyte pool was about two-fold

higher than the reported value by Bi et al. Hence a significant contribution of NTCP to the net hepatic uptake of the respective statin is likely to be expected for our investigated hepatocyte lot. Moreover, atorvastatin, fluvastatin, pravastatin and rosuvastatin, as well as E3S, were shown to be substrates of OATP2B1 (Noe et al., 2007; Kalliokoski and Niemi, 2009; Knauer et al., 2010). OATP2B1 was found to be expressed at a comparable level to reported human liver data (Kimoto et al., 2012; Vildhede et al., 2014). Therefore, we assumed that OATP2B1 might have contributed to the uptake of statins in the studied hepatocyte lot. Thus, comparing REF-based extrapolation of specific hepatic transporter activities with measured hepatic net uptake provides information about the potential involvement of other transporters in the net hepatic uptake activity.

It is expected that REF-based scaling will represent a powerful tool for *in vitro-in vivo* extrapolation of OATP1B1 and OATP1B3 activities to subsequently predict their contribution to the net hepatic uptake clearance and to assess the impact of OATP1B1 and OATP1B3-mediated DDIs. Recently Karlgren et al. and Vildhede et al. predicted the contribution of OATP1B1, OATP1B3, OATP2B1, and NTCP to the atorvastatin uptake clearance based on protein expression data determined for human liver samples and recombinant cell lines by an LC-MS method. Subsequently, the impact on isoform-specific inhibition to the atorvastatin clearance was assessed (Karlgren et al., 2012; Vildhede et al., 2014). Based on the substantial variation in transporter protein abundance among the investigated liver samples (twelve donors), the predicted transporter-specific uptake clearances showed high inter-individual variability ranging between 26-89% and

1.8-60% for OATP1B1 and OATP1B3, respectively. Moreover, Nies et al., demonstrated that genetic polymorphism significantly contributed to variation in OATP1B1 protein expression and functionality, observed among 82 individuals (Nies et al., 2013). The high inter-individual variability in transporter-protein expression observed in these studies clearly needs to be considered when REF-based methods are used for *in vitro-in vitro* extrapolation of transporter activities. This aspect becomes especially important when results from DDI studies with relatively small numbers of subjects are compared with IVIVE approaches.

In summary we demonstrated as a proof-of-concept that the OATP1B1 and OATP1B3 activity in hepatocytes can be extrapolated from recombinant cell lines based on absolute transporter protein measurements. We further assessed the relative contribution of OATP1B1 and OATP1B3 to the total hepatic uptake. Moreover, in contrast to RAF-based scaling methods, this approach is expected to allow scaling of transporter activities from *in vitro* incubations in recombinant cell lines to any tissues, given the respective transporter abundance is known. Therefore, future research will be required to strengthen the evidence that scaling of transporter activities based on absolute protein abundance data represents a powerful tool to predict transporter-mediated *in vivo* clearance processes and DDI effects.

Acknowledgements

The authors wish to acknowledge the many Novartis Drug Metabolism and Pharmacokinetic scientists of Basel Switzerland who have supported this work. Special thanks go to Francis Heitz for technical assistance and Joel Krauser for critical evaluation of this manuscript.

Authorship Contributions

Participated in research design: Kunze, Huwyler, Camenisch, Poller

Conducted experiments: Kunze

Performed data analysis: Kunze, Poller

Wrote or contributed to the writing of the manuscript: Kunze, Huwyler,
Camenisch, Poller

References

- Balogh LM, Kimoto E, Chupka J, Zhang H, and Lai Y (2012) Membrane Protein Quantification by Peptide-Based Mass Spectrometry Approaches: Studies on the Organic Anion-Transporting Polypeptide Family. *J Proteomics Bioinform* **S4**.
- Bi YA, Kimoto E, Sevidal S, Jones HM, Barton HA, Kempshall S, Whalen KM, Zhang H, Ji C, Fenner KS, El-Kattan AF, and Lai Y (2012) In vitro evaluation of hepatic transporter-mediated clinical drug-drug interactions: hepatocyte model optimization and retrospective investigation. *Drug metabolism and disposition: the biological fate of chemicals* **40**:1085-1092.
- Bi YA, Qiu X, Rotter CJ, Kimoto E, Piotrowski M, Varma MV, El-Kattan AF, and Lai Y (2013) Quantitative assessment of the contribution of sodium-dependent taurocholate co-transporting polypeptide (NTCP) to the hepatic uptake of rosuvastatin, pitavastatin and fluvastatin. *Biopharmaceutics & drug disposition* **34**:452-461.
- Hirano M, Maeda K, Shitara Y, and Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *The Journal of pharmacology and experimental therapeutics* **311**:139-146.
- Hirano M, Maeda K, Shitara Y, and Sugiyama Y (2006) Drug-drug interaction between pitavastatin and various drugs via OATP1B1. *Drug metabolism and disposition: the biological fate of chemicals* **34**:1229-1236.

- Kalliokoski A and Niemi M (2009) Impact of OATP transporters on pharmacokinetics. *British journal of pharmacology* **158**:693-705.
- Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, and Terasaki T (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharmaceutical research* **25**:1469-1483.
- Karlgren M, Vildhede A, Norinder U, Wisniewski JR, Kimoto E, Lai Y, Haglund U, and Artursson P (2012) Classification of inhibitors of hepatic organic anion transporting polypeptides (OATPs): influence of protein expression on drug-drug interactions. *Journal of medicinal chemistry* **55**:4740-4763.
- Kimoto E, Yoshida K, Balogh LM, Bi YA, Maeda K, El-Kattan A, Sugiyama Y, and Lai Y (2012) Characterization of Organic Anion Transporting Polypeptide (OATP) Expression and Its Functional Contribution to the Uptake of Substrates in Human Hepatocytes. *Molecular pharmaceuticals* **9**:3535-3542.
- Kitamura S, Maeda K, Wang Y, and Sugiyama Y (2008) Involvement of multiple transporters in the hepatobiliary transport of rosuvastatin. *Drug metabolism and disposition: the biological fate of chemicals* **36**:2014-2023.
- Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BF, Kim RB, and Tirona RG (2010) Human skeletal muscle drug transporters determine local exposure and toxicity of statins. *Circulation research* **106**:297-306.

- König J, Cui Y, Nies AT, and Keppler D (2000) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *American journal of physiology Gastrointestinal and liver physiology* **278**:G156-164.
- Kunze A, Huwyler J, Camenisch G, and Gutmann H (2012) Interaction of the antiviral drug telaprevir with renal and hepatic drug transporters. *Biochemical pharmacology* **84**:1096-1102.
- Lundquist P, Loof J, Sohlenius-Sternbeck AK, Floby E, Johansson J, Bylund J, Hoogstraate J, Afzelius L, and Andersson TB (2014) The Impact of Solute Carrier (SLC) Drug Uptake Transporter Loss in Human and Rat Cryopreserved Hepatocytes on Clearance Predictions. *Drug metabolism and disposition: the biological fate of chemicals* **42**:469-480.
- Mateus A, Matsson P, and Artursson P (2013) Rapid measurement of intracellular unbound drug concentrations. *Molecular pharmaceutics* **10**:2467-2478.
- Neuvonen PJ, Niemi M, and Backman JT (2006) Drug interactions with lipid-lowering drugs: mechanisms and clinical relevance. *Clinical pharmacology and therapeutics* **80**:565-581.
- Nies AT, Niemi M, Burk O, Winter S, Zanger UM, Stieger B, Schwab M, and Schaeffeler E (2013) Genetics is a major determinant of expression of the human hepatic uptake transporter OATP1B1, but not of OATP1B3 and OATP2B1. *Genome medicine* **5**:1.
- Noe J, Portmann R, Brun ME, and Funk C (2007) Substrate-dependent drug-drug interactions between gemfibrozil, fluvastatin and other organic anion-

transporting peptide (OATP) substrates on OATP1B1, OATP2B1, and OATP1B3. *Drug metabolism and disposition: the biological fate of chemicals* **35**:1308-1314.

Ohtsuki S, Schaefer O, Kawakami H, Inoue T, Liehner S, Saito A, Ishiguro N, Kishimoto W, Ludwig-Schwellinger E, Ebner T, and Terasaki T (2012) Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. *Drug metabolism and disposition: the biological fate of chemicals* **40**:83-92.

Sakamoto A, Matsumaru T, Ishiguro N, Schaefer O, Ohtsuki S, Inoue T, Kawakami H, and Terasaki T (2011) Reliability and robustness of simultaneous absolute quantification of drug transporters, cytochrome P450 enzymes, and Udp-glucuronosyltransferases in human liver tissue by multiplexed MRM/selected reaction monitoring mode tandem mass spectrometry with nano-liquid chromatography. *Journal of pharmaceutical sciences* **100**:4037-4043.

Schaefer O, Ohtsuki S, Kawakami H, Inoue T, Liehner S, Saito A, Sakamoto A, Ishiguro N, Matsumaru T, Terasaki T, and Ebner T (2012) Absolute quantification and differential expression of drug transporters, cytochrome P450 enzymes, and UDP-glucuronosyltransferases in cultured primary human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **40**:93-103.

- Shimizu M, Fuse K, Okudaira K, Nishigaki R, Maeda K, Kusuvara H, and Sugiyama Y (2005) Contribution of OATP (organic anion-transporting polypeptide) family transporters to the hepatic uptake of fexofenadine in humans. *Drug metabolism and disposition: the biological fate of chemicals* **33**:1477-1481.
- Shitara Y, Maeda K, Ikejiri K, Yoshida K, Horie T, and Sugiyama Y (2013) Clinical significance of organic anion transporting polypeptides (OATPs) in drug disposition: their roles in hepatic clearance and intestinal absorption. *Biopharmaceutics & drug disposition* **34**:45-78.
- Shitara Y and Sugiyama Y (2006) Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. *Pharmacology & therapeutics* **112**:71-105.
- Staffa JA, Chang J, and Green L (2002) Cerivastatin and reports of fatal rhabdomyolysis. *The New England journal of medicine* **346**:539-540.
- Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, and Terasaki T (2011) Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *Journal of neurochemistry* **117**:333-345.
- Umehara K and Camenisch G (2012) Novel in vitro-in vivo extrapolation (IVIVE) method to predict hepatic organ clearance in rat. *Pharmaceutical research* **29**:603-617.

- Vildhede A, Karlgren M, Svedberg EK, Wisniewski JR, Lai Y, Noren A, and Artursson P (2014) Hepatic uptake of atorvastatin: influence of variability in transporter expression on uptake clearance and drug-drug interactions. *Drug metabolism and disposition: the biological fate of chemicals* **42**:1210-1218.
- Watanabe T, Kusuhara H, Maeda K, Kanamaru H, Saito Y, Hu Z, and Sugiyama Y (2010) Investigation of the rate-determining process in the hepatic elimination of HMG-CoA reductase inhibitors in rats and humans. *Drug metabolism and disposition: the biological fate of chemicals* **38**:215-222.
- Williamson B, Soars AC, Owen A, White P, Riley RJ, and Soars MG (2013) Dissecting the relative contribution of OATP1B1-mediated uptake of xenobiotics into human hepatocytes using siRNA. *Xenobiotica; the fate of foreign compounds in biological systems* **43**:920-931.

Figure legends

Figure 1: Transporter protein expression in plasma membrane fractions of pooled cryopreserved human hepatocytes. The bars represent mean values of two independent measurements performed in triplicates and errorbars represent standard deviations.

Figure 2: Uptake kinetics of reference compounds in recombinant HEK293-OATP1B1 or HEK293-OATP1B3 cells (A, B) and human cryopreserved hepatocytes (C, D). The total uptake clearances (PS_{tot}) of E3S (A) and CCK8 (B) in recombinant HEK293 cells are shown in the presence and absence of OATP inhibitors atorvastatin and rifamycin (AR). For hepatocytes, PS_{tot} , as well as apparent uptake clearances determined from 37°C and 4°C incubations ($PS_{app,tot,37^{\circ}C}$ and $PS_{app,tot,4^{\circ}C}$, respectively) are shown for E3S (C) and CCK8 (D). Data are presented as mean values of triplicates with the error bars representing the standard deviation. Lines represent the fit to the data according to Equation 2.

Figure 3: Uptake kinetics of rosuvastatin and cerivastatin in recombinant HEK293-OATP1B1 or HEK293-OATP1B3 cells (A, B) and human cryopreserved hepatocytes (C, D). The total uptake clearance (PS_{tot}) of rosuvastatin (A) and cerivastatin (B) in recombinant cell lines are shown in the presence and absence of OATP inhibitors atorvastatin and rifamycin (AR). Data are presented as mean

values of triplicates with the error bars representing the standard deviation. Lines represent the fit to the data according to Equation 2.

Figure 4: Fractional contribution of OATP1B1 (gray) and OATP1B3 (black) to the observed maximal active uptake clearance of statins in suspended hepatocytes (lot PQP), where $PS_{act,max}$ measured in hepatocytes represents 100%. The transporter contributions were predicted with the REF-method based on measured transporter activities in recombinant HEK293-OATP1B1 and HEK293-OATP1B3 cells (Table 2).

Tables

Table 1: Absolute transporter protein expression.

expression system	transporter protein	protein expression	
		(fmol/ μ g prot _{mem})	(fmol/ μ g prot)
human hepatocytes	OATP1B1	15.85	0.230
	OATP1B3	0.35	0.005
	OATP2B1	1.57	0.022
	OAT2	1.03	0.014
	NTCP	2.62	0.036
	OCT1	6.94	0.095
HEK-OATP1B1	OATP1B1	23.97	0.269
HEK-OATP1B3	OATP1B3	1.44	0.028

Table notes: The absolute transporter protein expression was normalized to the amount of plasma membrane protein (fmol/ μ g prot_{mem}) or total protein (fmol/ μ g prot) in the respective expression system.

Table 2: Pharmacokinetic parameters of statins and reference compounds.

Compounds	HEK293-OATP1B1				HEK293-OATP1B3				Hepatocytes (lot PQP)			
	PS _{act,max}	SD	PS _{act,max} / PS _{pas}	R ²	PS _{act,max}	SD	PS _{act,max} / PS _{pas}	R ²	PS _{act,max}	SD	PS _{act,max} / PS _{pas}	R ²
	(μL/min/mg prot)		(-)	(-)	(μL/min/mg prot)		(-)	(-)	(μL/min/mg prot)		(-)	(-)
E3S	138.1	7.2	27.7	0.99	6.7	1.9	1.2	0.93	157.7	6.1	9.6	0.99
CCK8	no substrate				76.1	2.8	20.0	0.99	7.4	0.5	2.5	0.98
Atorvastatin	26.3	0.45	5.5	1.00	31.9	1.8	6.4	0.99	45.1	11.6	1.3	0.96
Cerivastatin	8.2	1.9	0.2	0.91	8.6	1.1	0.1	0.94	54.4	3.5	0.9	0.99
Fluvastatin	20.9	1.8	0.5	0.96	29.8	2.0	0.5	0.98	53.7	12.1	0.7	0.88
Lovastatin	8.9	1.0	0.4	0.92	no substrate				40.6	4.1	1.1	0.96
Pitavastatin	33.5	3.2	1.8	0.97	14.5	3.5	0.5	0.82	89.5	11.1	1.4	0.97
Pravastatin	10.6	3.2	6.3	0.96	2.2	0.3	1.3	0.93	13.4	1.5	7.4	0.93
Rosuvastatin	5.3	0.6	3.8	0.92	4.0	0.2	2.8	0.99	6.7	0.5	1.1	0.97
Simvastatin	14.4	5.0	0.3	0.75	14.3	5.4	0.3	0.65	28.5	3.1	0.4	0.98

Table notes: PS_{act,max} refers to the maximal transporter activity in the respective expression system; PS_{pas} refers to the passive permeation, PS_{act,max}/PS_{pas} describes the ratio of the active to passive uptake clearance, and R² determines the coefficient of determination.

Table 3: Observed and predicted hepatic $PS_{act,max}$ values.

	observed	REF-based scaling			RAF-based scaling		
	$PS_{act,max}$	$PS_{act,max}$			$PS_{act,max}$		
	total	1B1	1B3	1B1+1B3	1B1	1B3	1B1+1B3
	($\mu\text{L}/\text{min}/\text{mg}$)	($\mu\text{L}/\text{min}/\text{mg}$)			($\mu\text{L}/\text{min}/\text{mg}$)		
E3S	153.5 ^{a)}	117.8	1.2	119.0	n.a.	n.a.	n.a.
CCK8	8.6 ^{a)}	0.0	13.8	13.8	n.a.	n.a.	n.a.
Atorvastatin	45.1	22.4	5.8	28.2	29.2	3.6	32.8
Cerivastatin	54.4	7.0	1.6	8.6	9.1	1.0	10.1
Fluvastatin	53.7	17.5	5.4	22.8	22.8	3.4	26.1
Lovastatin	40.6	7.6	0.0	7.6	9.9	0.0	9.9
Pitavastatin	89.5	28.6	2.6	31.2	37.2	1.6	38.9
Pravastatin	13.4	8.8	0.4	9.2	11.5	0.3	11.8
Rosuvastatin	6.7	4.5	0.7	5.3	5.9	0.5	6.4
Simvastatin	28.5	12.3	2.6	14.9	16.0	1.6	17.6

Table notes: $PS_{act,max}$ refers to the observed or predicted maximal transporter activity in hepatocytes. 1B1 and 1B3 determine OATP1B1 and OATP1B3, respectively. According to Eqs 3-7, the following scaling factors were used: $REF_{1B1} = 0.853$; $REF_{1B3} = 0.181$; $RAF_{1B1} = 1.112$; $RAF_{1B3} = 0.113$.

^{a)}The value corresponds to the mean $PS_{act,max}$ derived from two independent experiments.

Figure 1

Figure 2

Figure 3

A

B

C

D

Figure 4