Comparative Hepatic and Intestinal Efflux Transport of Statins

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ABSTRACT

Previous studies have shown that lipid-lowering statins are transported by various ATP-binding cassette (ABC) transporters. However, because of varying methods, it is difficult to compare the transport profiles of statins. Therefore, we investigated the transport of 10 statins or statin metabolites by six ABC transporters using human embryonic kidney cell-derived membrane vesicles. The transporter protein expression levels in the vesicles were quantified with liquid chromatography–tandem mass spectrometry and used to scale the measured clearances to tissue levels. In our study, apically expressed breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp) transported atorvastatin, fluvastatin, pitavastatin, pitavastatin, and rosuvastatin. Multidrug resistance-associated protein 3 (MRP3) transported atorvastatin, fluvastatin, pitavastatin, and, to a smaller extent, pravastatin. MRP4 transported fluvastatin and rosuvastatin. The scaled clearances suggest that BCRP contributes to 87%–91% and 84% of the total active efflux of rosuvastatin in the small intestine and the liver, respectively. For atorvastatin, the corresponding values for P-gp–mediated efflux were 43%–79% and 66%, respectively, MRP3, on the other hand, may contribute to 23%–26% and 25%–37% of total active efflux of atorvastatin, fluvastatin, and pitavastatin in jejunal enterocytes and liver hepatocytes, respectively. These data indicate that BCRP may play an important role in limiting the intestinal absorption and facilitating the biliary excretion of rosuvastatin and that P-gp may restrict the intestinal absorption and mediate the biliary excretion of atorvastatin. Moreover, the basolateral MRP3 may enhance the intestinal absorption and sinusoidal hepatic efflux of several statins. Taken together, the data show that statins differ considerably in their efflux transport profiles.

SIGNIFICANCE STATEMENT

This study characterized and compared the transport of atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin acid and four atorvastatin metabolites by six ABC transporters (BCRP, MRP2, MRP3, MRP4, MRP8, P-gp). Based on in vitro findings and protein abundance data, the study concludes that BCRP, MRP3, and P-gp have a major impact in the efflux of various statins. Together with in vitro metabolism, uptake transport, and clinical data, our findings are applicable for use in comparative systems pharmacology modeling of statins.

Introduction

Cardiovascular diseases are among the most common causes of death, accounting for approximately 17.9 million deaths worldwide in 2015 (Roth et al., 2017). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also known as statins, are first-line drugs for primary and secondary prevention of cardiovascular diseases. Statins inhibit mevalonate production mediated by 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is a key step in cholesterol biosynthesis. Inhibition of this enzyme leads to a reduced cholesterol production and increased expression of low-density lipoprotein cholesterol receptors in the liver (Slater and MacDonald, 1988). Ultimately, this results in a reduction in low-density lipoprotein cholesterol and triglyceride levels accompanied by decreased mortality and coronary events (Maron et al., 2000). Statins may also exert beneficial effects through a cholesterol-independent, pleiotropic manner by reducing systemic inflammation and platelet hyper-reactivity and improving endothelial function (Liao and Laufs, 2005). Although statins are widely used and generally accepted as efficient and safe (Yebyo et al., 2019), they may cause muscle toxicity ranging from mild and relatively common myalgia to rare but life-threatening rhabdomyolysis (Harper and Jacobson, 2007).

Drug transporters play a key role in regulating drug levels in systemic circulation and various tissues (Giacomini et al., 2010). These proteins are located on the plasma membranes of cells, where they either pump their substrates into the cytosol or out of the cell. ATP-binding cassette
Materials. Atorvastatin, atorvastatin-d5, 2-hydroxyatorvastatin, 2-hydroxyatorvastatin-d5, 2-hydroxyatorvastatin lactone, 2-hydroxyatorvastatin lactone-d5, 4-hydroxyatorvastatin, 4-hydroxyatorvastatin-d5, 4-hydroxyatorvastatin lactone, 4-hydroxyatorvastatin lactone-d5, fluvastatin-d8, pitavastatin-d5, pravastatin, pravastatin-d9, rosuvastatin, rosuvastatin-d6, and simvastatin acid-d6 were purchased from Toronto Research Chemicals (Toronto, Canada). Solvents used in assays and analytical methods were of analytical quality and purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water for assays and analyses was purified using Milli-Q water purification system (Merck Millipore, Burlington, MA).

Vesicular Transport Assay. Vesicular transport assay was used to examine statin transport. The assays were performed essentially as described previously (Lehtisalo et al., 2020). In brief, transporter-expressing membrane vesicles (7.5 μg) were preincubated at 37°C for 10 minutes in transport assay buffer (PharmTox, Radboud UMC, Nijmegen, The Netherlands). Solvents used in assays and analytical methods were of analytical quality and purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water for assays and analyses was purified using Milli-Q water purification system (Merck Millipore, Burlington, MA).

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Mannheim, Germany) and incubated for 20 minutes at 60°C. After cooling down, 10 μl iodoacetamide (400 mM, Sigma-Aldrich) were added, and the samples were incubated in a darkened water bath for 15 minutes at 37°C. For protein digestion, 10 μl trypsin (trypsin/protein ratio: 1:40, Promega) was added, and samples were incubated in a water bath for 16 hours at 37°C. Digestion was stopped by addition of 20 μl formic acid (10% v/v, Sigma-Aldrich). Afterward, the samples were centrifuged one more time for 15 minutes at 16,000 g and 4°C. Then 50 μl of the supernatant was mixed with 25 μl isotope-labeled internal standard peptide mix (10 nM of each labeled peptide, Thermo Fisher Scientific). All sample preparation and digestion steps were performed using Protein LoBind tubes (Eppendorf, Hamburg, Germany). Protein quantification was conducted on a 5500 QTRAP triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) coupled to an Agilent Technologies 1260 Infinity system (Agilent Technologies, Waldbronn, Germany). Transporter proteins were simultaneously quantified using proteospecific peptides. Final protein abundance data (picomoles per milligram) were calculated by normalization to the total protein content of the isolated membrane fraction as determined by the BCA assay.

Data and Statistical Analysis. ATP-dependent transport was calculated by subtracting the statin uptake into vesicles in the absence of ATP from the statin uptake into vesicles in the presence of ATP. Uptake ratio was determined by uptake of investigated statin into vesicles in the presence of ATP divided by that in the absence of ATP. The ATP-dependent transport and uptake ratios in screening and time-dependent transport studies were compared with those in control vesicles using one-way ANOVA and Fisher’s least significant difference analysis (GraphPad Software version 8.4, San Diego, CA). A P value below 0.05 was considered statistically significant.

The kinetic parameters of statin transport were determined with GraphPad Prism 8.4. For these calculations, the mean ATP-dependent transport values of each concentration point from separate experiments were pooled, and these values were fitted to the Michaelis-Menten equation (eq. 2), wherein v stands for the velocity of ATP-dependent transport, Vmax stands for the maximal transport rate, [S] stands for the substrate concentration, and Km stands for the Michaelis-Menten constant.

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]  

The in vitro statin clearance was calculated from Michaelis-Menten parameters as shown in eq. 3.

\[ CL_{\text{in vitro}} = \frac{V_{\text{max}}}{K_m} \]  

The in vitro statin clearance was adjusted with vesicle protein expression as shown in eq. 4,

\[ CL_{\text{adj}} = \frac{CL_{\text{in vitro}}}{\text{Protein expression}_{\text{vesicle}} \cdot f_{\text{inverted}}} \]  

in which CLadj stands for expression-adjusted clearance, Protein expressionvesicle stands for transporter abundance measured in vesicles, and finverted stands for the fraction of membrane vesicles that is inverted. The clearance was further scaled to the tissue level by multiplying CLadj with published transporter abundance data (eq. 5).

\[ CL_{\text{tissue}} = CL_{\text{adj}} \cdot \text{Protein expression}_{\text{tissue}} \]  

In eq. 5, CLtissue is the estimated tissue level efflux statin clearance, and Protein expression_{tissue} is the abundance of the efflux transporter in the tissue of interest (Burt et al., 2016; Drozdzik et al., 2019).

Results

Transport of Known Substrates of MRP2, MRP3, MRP4, MRP8, and P-gp. The functionalities of the vesicles were verified using estradiol-17-glucuronide (50 μM for MRP2; 5 μM for MRP3, MRP4, MRP8, and control vesicles) and N-methyl-quindoline (5 μM for P-gp) as positive controls. The uptake ratios of these probe substrates in MRP2, MRP3, MRP4, MRP8, and P-gp vesicles were 79, 38, 15, 2.8, and 2.4, respectively, verifying the functionality of the vesicles and vesicular transport assay (Supplemental Fig. S1).

Screening of Statin Efflux Transport. The screening of statin transport was conducted by incubating 10 μM of statins (or 1 μM in the case of simvastatin acid) with BCRP, MRP2, MRP3, MRP4, MRP8, P-gp, and control vesicles (Fig. 1; Supplemental Table S2). Atorvastatin was significantly transported by MRP3 (uptake ratio 2.2 ± 0.6) and P-gp (3.1 ± 0.6), and the uptake ratio of transport differed significantly from control (MRP3, P = 0.0102; P-gp, P < 0.0001). 3R,5S-fluvastatin was significantly transported by BCRP (3.8 ± 0.9, P < 0.0001) and P-gp (2.8 ± 0.3, P = 0.0078), whereas 3S,5R-fluvastatin was significantly transported by BCRP (3.1 ± 1.6, P = 0.0497), MRP3 (3.2 ± 1.0, P = 0.0261), and MRP4 (3.1 ± 0.8, P = 0.0458). Pitavastatin was clearly transported by BCRP (4.6 ± 0.6, P = 0.0006) and P-gp (3.3 ± 1.0, P = 0.0306), and rosuvastatin was efficiently transported by BCRP (8.4 ± 3.1, P = 0.0002). For pravastatin and simvastatin acid, none of the uptake ratios differed significantly from the control.

In addition, we tested the transport of 10 μM atorvastatin metabolites: 2-hydroxyatorvastatin, 4-hydroxyatorvastatin, 2-hydroxyatorvastatin lactone, and 4-hydroxyatorvastatin lactone in BCRP, MRP2, MRP3, MRP4, P-gp, and control vesicles. 2-Hydroxyatorvastatin was taken up in BCRP (uptake ratio 3.5 ± 0.9, P = 0.0279) and MRP3 (3.3 ± 0.6, P = 0.0492) vesicles significantly more than in control vesicles (uptake ratio 1.9 ± 0.7) (Fig. 1; Supplemental Fig. S3). The transport of 4-hydroxyatorvastatin in MRP3 (4.4 ± 1.5, P = 0.0028) and P-gp (4.0 ± 1.1, P = 0.0076) vesicles differed significantly from control vesicles (1.3 ± 0.7). The 2- and 4-hydroxyatorvastatin lactones showed no transport in any vesicles (Supplemental Table S3).

Time-Dependent Transport. The transport of statins by selected transporters was further investigated by studying the time-dependent transport (Supplemental Figs. S2-S7; Supplemental Tables S4-S5). In most cases, when notable transport was observed, the ATP-dependent transport plateaued already at 5 minutes as the statin uptake into vesicles and the escape of the statins due to passive diffusion reached equilibrium.

Atorvastatin transport was already prominent at 5 minutes in BCRP, MRP3, and P-gp vesicles with uptake ratios of 2.5 (P = 0.0012), 2.2 (P = 0.0167), and 2.5 (P = 0.0019), respectively, which were significantly higher than that in control vesicles (Supplemental Fig. S2). On the other hand, the ATP-dependent transport and uptake ratio in MRP2 and MRP4 vesicles did not differ from the control vesicles, except for MRP2 at 10-minute time point.

The transport rates of both fluvastatin enantiomers in BCRP, MRP3, and P-gp vesicles were significantly higher than those in control vesicles at any given time, and the ATP-dependent transport was nearly plateaued 5 minutes after initiation of transport (Supplemental Figs. S3 and S4). The 3R,5S-fluvastatin transport rate in MRP2 and MRP4 vesicles was higher than that in control vesicles at 5 and 10 minutes. The 3S,5R-fluvastatin transport rate in MRP2 and MRP4 vesicles was higher than that in control vesicles only at 10 minutes. The transport rates of 3R,5S-fluvastatin and 3S,5R-fluvastatin in MRP8 vesicles differed significantly from those of control vesicles only at 5- and 10-minute time points, respectively.

Pitavastatin was transported by BCRP with high efficiency: The transport rates in BCRP vesicles were three times higher than those in MRP3 and P-gp vesicles (Supplemental Fig. S5). The transport rates in MRP3 vesicles were significantly higher than those of control at 5- and 15-minute time points, but only at 15 minutes the uptake ratio of 2.3 was significantly greater than in control vesicles (P = 0.0011). P-gp vesicles showed significantly higher transport rate (P = 0.0002) and the uptake ratio (3.6, P = 0.0004) at 5-minute time point than control vesicles.
In the screening, the uptake ratios of pravastatin were relatively high in BCRP, MRP3, and MRP4 vesicles, although they did not differ significantly from that of control vesicles. For this reason, the time-dependent transport of pravastatin was further studied. MRP3 exhibited low yet significant transport rates at 5-minute \((P = 0.0050)\) and 15-minute \((P = 0.0115)\) time points despite notable variation (Supplemental Fig. S6). In addition, the uptake ratio at 5 minutes \((2.2, P = 0.0018)\) was significantly greater than that of control. Pravastatin transport rates in BCRP, MRP2, and MRP4 vesicles did not differ significantly from control, except for MRP2 at 15 minutes \((P = 0.0473)\).

Rosuvastatin was transported by BCRP with high rate and uptake ratio at any given time, although the ATP-dependent transport plateaued already at 5 minutes (Supplemental Fig. S7). Although the MRP4 and P-gp transported rosuvastatin to some extent, the transport rates did not differ significantly from control vesicles and were approximately an order of magnitude lower than those in BCRP vesicles. Only the transport rate at 10 minutes in MRP4 vesicles was significantly greater than that in control vesicles \((P = 0.0410)\). The high uptake ratio of rosuvastatin in MRP8 vesicles in screening studies was not replicated in the time-dependence studies.

The time-dependent transport of simvastatin acid was not further investigated, as the uptake ratios of simvastatin acid transport in the screening were relatively close to that in control vesicles.

**Concentration-Dependent Transport (Transporter Kinetics).**

Based on atorvastatin screening and time-dependence studies, BCRP, MRP3, and P-gp were selected for kinetic measurements (Fig. 2). Atorvastatin had apparent affinities of 82.4, 31.5, and 10.7 \(\mu\)M in BCRP, MRP3, and P-gp, respectively (Table 1). The maximum transport rate in the investigated transporters followed the same order, with the rate being the greatest for BCRP and the lowest for P-gp.

Racemic fluvastatin was employed in kinetic studies, since the enantiomers exhibited no distinct selectivity in the investigated transporters, and racemic mixture is more readily available. Racemic fluvastatin was well transported by BCRP and MRP3 with similarly high affinities (Km...
25.8 and 48.9 μM, respectively), although the transport rate in BCRP was three times higher than that in MRP3 (Fig. 3; Table 1). In addition, MRP2, MRP4, MRP8, and P-gp vesicles were also capable of accumulating racemic fluvastatin in a concentration-dependent manner. Large deviation was observed in experiments despite the numerous replications, and this reflects the reliability of estimated Michaelis-Menten parameters. Therefore, only the curve fit and parameters of BCRP can be considered reliable. Nevertheless, the ATP-dependent transport was notably lower in control vesicles than in other vesicles. Furthermore, the uptake ratio in three control experiments was on average 1.26 ± 0.44 and 2.39 ± 0.15, whereas the uptake ratio of MRP2 and P-gp, which had lowest ratio and highest deviation, respectively, were 1.81 ± 0.44 and 2.39 ± 1.09 (Supplemental Table S6).

The apparent affinities of pitavastatin in BCRP, MRP3 and P-gp vesicles were high and relatively similar across the transporters (16, 9.0, and 37 μM, respectively, Fig. 4). The maximum rate of transport in BCRP vesicles was four times higher than in other vesicles (Table 1). The MRP3-mediated transport of pitavastatin was less clear as the uptake ratio of 12 μM pitavastatin in MRP3 vesicles remained below two (1.8 ± 0.2).

Pravastatin was transported in MRP3 vesicles only (Fig. 5). In the kinetic studies, pravastatin transport exhibited nonsaturable transport over the concentration range. Furthermore, the clearance of pravastatin was notably lower compared with the clearance of other statins in MRP3 (Table 1).

Rosuvastatin is a well established BCRP substrate with high transport rate and apparent affinity of transport. This was confirmed in our study with a single experiment, which concluded that Km of rosuvastatin transport in BCRP was approximately 4.2 μM (Fig. 6). Meanwhile the apparent affinities of rosuvastatin in MRP4 and P-gp vesicles were 10-fold lower (Km of 39.3 and 46.2 μM, respectively). However, their transport rates in the presence of high concentrations of rosuvastatin showed large variability, which leads to the unreliable estimation of Michaelis-Menten parameters.

**5'-Nucleotidase Activity and the Fraction of Inverted Membrane Vesicles.** The fraction of inverted membrane vesicles was determined using the 5'-nucleotidase activity assay (Supplemental Fig. S8). The f<sub>inverted</sub> of BCRP, MRP2, MRP4, MRP8, and P-gp vesicles were similar (36%–52%), whereas the f<sub>inverted</sub> of MRP3 vesicles was higher (65%).

**Proteomic Measurements of Membrane Vesicles and the Estimation of Tissue-Specific Active Efflux Clearance.** The transporter abundance in vesicle preparations was measured with a quantitative proteomic technique based on LC-MS/MS. The absolute abundances in membrane vesicles were 146, 85, 54, 44, and 59 pmol/mg of protein, respectively (Supplemental Table S7).

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**TABLE 1**  
The summary of kinetic parameters and expression-adjusted clearance of atorvastatin, racemic fluvastatin, pitavastatin, pravastatin, and rosuvastatin transport in BCRP, MRP2, MRP3, MRP4, MRP8, and P-gp vesicles. The 95% confidence intervals (95% CI) for the derived parameters are presented in parentheses.

<table>
<thead>
<tr>
<th>Statin</th>
<th>Transporter</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (95% CI) [pmol/min/mg]</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (95% CI) [μM]</th>
<th>CL&lt;sub&gt;in vitro&lt;/sub&gt; [nl/min/pmol of transporter]</th>
<th>CL&lt;sub&gt;adj&lt;/sub&gt; [nl/min/pmol of transporter]</th>
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</thead>
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<tr>
<td>Atorvastatin</td>
<td>BCRP</td>
<td>135 (92.3–258)</td>
<td>82.4 (34.05–249)</td>
<td>1.6</td>
<td>30.8</td>
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<td></td>
<td>MRP3</td>
<td>77.0 (57.7–114)</td>
<td>31.5 (12.3–84.2)</td>
<td>2.4</td>
<td>68.7</td>
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<tr>
<td></td>
<td>P-gp</td>
<td>32.9 (27.3–40.0)</td>
<td>10.7 (4.34–23.1)</td>
<td>3.1</td>
<td>133</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>BCRP</td>
<td>408 (313–580)</td>
<td>25.8 (9.81–68.4)</td>
<td>15.8</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>MRP2</td>
<td>105 (45.2–infinity)</td>
<td>54.0 (1.92–infinity)</td>
<td>1.9</td>
<td>49.9</td>
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<tr>
<td></td>
<td>MRP3</td>
<td>170 (77.1–infinity)</td>
<td>48.9 (2.12–infinity)</td>
<td>3.5</td>
<td>97.6</td>
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<td>MRP4</td>
<td>54.7 (23.4–4053)</td>
<td>31.8 (0–infinity)</td>
<td>1.7</td>
<td>76.4</td>
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<td></td>
<td>MRP8</td>
<td>74.5 (41.6–580)</td>
<td>73.0 (10.5–1524)</td>
<td>1.0</td>
<td>n/a</td>
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<td></td>
<td>P-gp</td>
<td>169 (51.6–infinity)</td>
<td>282 (21.3–infinity)</td>
<td>0.6</td>
<td>25.8</td>
</tr>
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<td>Pitavastatin</td>
<td>BCRP</td>
<td>115 (98.1–137)</td>
<td>15.8 (6.53–32.4)</td>
<td>7.3</td>
<td>138</td>
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<td></td>
<td>MRP3</td>
<td>17.2 (10.7–29.0)</td>
<td>8.97 (0–73.3)</td>
<td>1.9</td>
<td>54.1</td>
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<td></td>
<td>P-gp</td>
<td>31.8 (18.3–97.4)</td>
<td>36.6 (3.64–316)</td>
<td>0.9</td>
<td>37.4</td>
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<td>Pravastatin</td>
<td>MRP3</td>
<td>n/d</td>
<td>n/d</td>
<td>0.2</td>
<td>5.6</td>
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<td>Rosuvastatin</td>
<td>BCRP</td>
<td>86.5 (71.4–106)</td>
<td>4.24 (2.02–8.44)</td>
<td>20.4</td>
<td>385</td>
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<td></td>
<td>MRP4</td>
<td>12.3 (5.13–infinity)</td>
<td>39.3 (0.28–infinity)</td>
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<td>13.9</td>
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<td></td>
<td>P-gp</td>
<td>19.1 (11.3–51.8)</td>
<td>46.2 (5.7–344)</td>
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Based on the in vitro results, 5'-nucleotidase activity data, proteomics measurements, and published transporter abundance data from literature, we calculated the expression-adjusted clearance, CL_{adj}, (Table 1), and the individual contribution of efflux transporters to the total active efflux clearance in various tissues (Fig. 7; Supplemental Table S8). For atorvastatin, fluvastatin, and pitavastatin, basolateral pumps were estimated to

Fig. 3. Concentration-dependent transport of fluvastatin in BCRP, MRP2, MRP3, MRP4, MRP8, P-gp, and control vesicles. The time of incubation and vesicle amount were 5 minutes and 7.5 μg, respectively. Results are presented as mean ± S.D. transport, which is calculated from the means of each separate experiment. In total, three separate experiments were performed for each transporter with triplicate samples in each experiment.

Fig. 4. Concentration-dependent transport of pitavastatin in BCRP, MRP3, P-gp, and control vesicles. The time of incubation and vesicle amount were 5 minutes and 7.5 μg, respectively. Results are presented as mean ± S.D. transport, which is calculated from the means of each separate experiment. In total, three separate experiments (two for control vesicles) were performed for each transporter with triplicate samples in each experiment.
MRP2 transported atorvastatin, which, in the vesicular transport assay, could be due to high passive permeability, which may result in a false negative result. This could be due to high passive permeability, which may result in a false negative result in the vesicular transport assay. Furthermore, kinetic parameters from membrane vesicle assays combined with protein expression data, allowed the scaling of the obtained findings to tissue level. Taken together, our data indicate that statins differ in their efflux transport profiles.

BCRP, MRP2, and P-gp are expressed on the apical plasma membranes in tissues important for pharmacokinetics. There, they limit the absorption and facilitate the excretion of their substrates. Previous pharmacogenetic studies have suggested that BCRP restricts the intestinal absorption of atorvastatin, fluvastatin, and rosuvastatin (Keskitalo et al., 2009b,c). Indeed, we observed extensive BCRP-mediated transport of fluvastatin, pitavastatin, and rosuvastatin, and minor but significant transport of atorvastatin. The more lipophilic pitavastatin and especially atorvastatin were good P-gp substrates, whereas the hydrophilic rosuvastatin was poorly transported by P-gp. Interestingly, all the statins transported by P-gp contain at least one nitrogen atom, which could be beneficial when interacting with the negatively charged binding cavity of P-gp (Deng et al., 2020).

MRP3 and MRP4 are expressed on the basolateral plasma membrane in small intestine and liver (Drozdzik et al., 2019). According to a recent meta-analysis, the hepatic abundance of MRP3 in healthy Caucasian adults is similar to that of P-gp and 40% lower than that of MRP2 (Burt et al., 2016). Furthermore, when all the ethnicities and disease states were included in the meta-analysis, the hepatic abundance of MRP4 was 70% lower than that of MRP3 and similar to the level of BCRP. In the enterocytes, they may facilitate the vectorial movement of their substrate drugs and metabolites by flipping them from the entero-ocyte to mesenteric blood (van de Wetering et al., 2009; Kitamura et al., 2010a; Kitamura et al., 2010b; Proctor et al., 2016). Our estimations of active efflux clearance suggest that this basolateral pathway may be important for the absorption of several statins (Fig. 7). In the liver, statins are actively taken into the hepatocytes by OATP1B1, OATP1B3, and OATP2B1 (Bi et al., 2019). MRP3 and MRP4 may partly counteract this by returning statins from hepatocytes back to the sinusoidal blood. We hypothesize that basolateral transporters may additionally promote statin elimination and pharmacological activity by distributing statins and their metabolites more evenly in the hepatocytes along the sinusoids. This hepatocyte hopping could prevent the saturation of the pharmacological target, metabolic enzymes, and biliary efflux transporters (Iusuf et al., 2012). Based on our scaled clearance values, MRP3 could affect the intracellular statin efflux and enable hepatocyte shuffle for statins.

Atorvastatin is a lipophilic statin with an oral bioavailability of 14%, which is explained by first-pass hepatic extraction (Lennernäs, 2003). Here, the transport kinetics of atorvastatin and its metabolites were determined for the first time using membrane vesicles. The highest affinity and abundance-scaled clearance of atorvastatin were observed

**Discussion**

In the present study, we evaluated the transport of six statins by six efflux transporters relevant for drug absorption, distribution, and elimination. This study is unique in that the transport was studied using uniform methods in a single laboratory, thus allowing comparison of statin efflux profiles. Of the apical efflux transporters tested, BCRP and P-gp transported atorvastatin, fluvastatin, pitavastatin, and rosuvastatin, and MRP2 transported fluvastatin. Interestingly, atorvastatin, fluvastatin, pitavastatin, and pravastatin were transported by the basolaterally expressed MRP3 and fluvastatin and rosuvastatin were expressed by MRP4. Simvastatin acid was not transported by any of the investigated transporters. This could be due to high passive permeability, which may result in a false negative result in the vesicular transport assay.
in P-gp vesicles. The estimated tissue-specific efflux clearances indicate that P-gp, and BCRP to a smaller extent, could regulate atorvastatin plasma and tissue levels in humans. In line with this, previous studies have demonstrated increased systemic exposure to atorvastatin in association with genetic variants, which reduce the function or expression of the P-gp and BCRP (Keskitalo et al., 2008; Keskitalo et al., 2009c; León-Cachón et al., 2016). Moreover, our findings suggest that MRP3 may facilitate the intestinal absorption of atorvastatin especially in the jejunum and the hepatocyte hopping of atorvastatin and its metabolites. Moreover, MRP3 could be an important basolateral pathway for 2-
Fluvastatin is a hydrophilic statin with a low bioavailability (18%) limited by acid-catalyzed biotransformation (Singhvi et al., 1990) and hepatic uptake (Niemi et al., 2006b). This is the first study to demonstrate that pravastatin is a substrate of MRP3. In fact, MRP3 was the only transporter, which transported pravastatin in our study. Our data may explain how pravastatin is transferred from enterocytes to the mesenteric vein, and effluxed from hepatocytes back to the circulation. Surprisingly, we observed no pravastatin transport in either human embryonic kidney-MRP2 or S9-MRP2 vesicles (Supplemental Fig. S9), even though previous studies have suggested that MRP2 is a key canalicular transporter for pravastatin (Niemi et al., 2006a; Nakagomi-Hagihara et al., 2007; Elsby et al., 2011). In Mrp2-deficient rats, pravastatin Cmax and AUC were increased compared with wild-type rats (Kivistö et al., 2005), which might be explained by the increased hepatic Mrp3 expression. Instead of MRP2, bile salt export pump could mediate the biliary excretion of pravastatin (Hirano et al., 2005a).

Rosuvastatin is another hydrophilic statin with a low bioavailability (20%), minimal metabolism, and a long terminal half-life of 20 hours (Neuvonen et al., 2006). Of the statins tested in our study, BCRP transported rosvastatin with the highest affinity and clearance. In contrast, minimal transport of rosvastatin was observed in P-gp and MRP4 vesicles. Our findings suggesting an important role for BCRP in the hepatic and intestinal efflux of rosvastatin are consistent with clinical data showing over 2-fold increase in the Cmax and AUC of rosvastatin in healthy volunteers homozygous for a genetic defect in BCRP (Keskitalo et al., 2009c). The scaled clearance values imply that P-gp plays a limited role in rosvastatin efflux, a finding supported by clinical pharmacogenetic data (Keskitalo et al., 2009a; Bai et al., 2019). Because of its hydrophilic nature, rosvastatin may require basolateral efflux transporters in absorption and distribution. MRP4 could facilitate rosvastatin absorption in the duodenum, where it was estimated to contribute 10% of rosvastatin efflux clearance. Rat liver perfusion and SCHH studies have demonstrated significant basolateral efflux of rosvastatin in the liver (Pfeifer et al., 2013a,b), but in our estimations, the role of hepatic MRP4 was minimal because of low in vitro activity and hepatic expression (Burt et al., 2016).

In conclusion, we compared the transport of atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin acid in BCRP, MRP2, MRP3, MRP4, MRP8, and P-gp vesicles. Using the vesicular transport assay and LC-MS/MS-based protein abundance measurements, we demonstrated the different efflux transport profiles and estimated the efflux clearances of statins in small intestine and liver. Because of assay variability in kinetic studies for certain statins and transporters, the estimated relative contributions of the different transporters to tissue efflux clearance should be interpreted with caution. Moreover, polarized-cell-monolayer-based or other alternative methods may be useful to confirm our findings. Overall, the present data can be applied to physiologically based pharmacokinetic or systems pharmacology modeling of statins.

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Authorship Contributions

Participated in research design: Deng, Tuomi, Hirvensalo, Niemi.
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Contributed new reagents or analytic tools: Neuvonen.
Performed data analysis: Deng, Tuomi, Oswald, Niemi.
Wrote or contributed to the writing of the manuscript: Deng, Tuomi, Neuvonen, Hirvensalo, Kulju, Wenzel, Oswald, Filppula, Niemi.

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