A number of ATP-binding cassette transporters (ABC) are expressed at the canalicular membranes of hepatocytes and the brush-border membranes of enterocytes including the P-glycoprotein (Pgp) (MDR1/ABCB1), the multidrug resistance protein 2 (MRP2/ABCC2), and the breast cancer resistance protein (BCRP/ABCG2), which is also known as the mitoxantrone resistance protein (MXR). These efflux transporters have distinct, but overlapping, substrate specificity and have been shown to be involved in biliary excretion of many drugs (Sasabe et al., 1998; Jonker et al., 2000). MRP2 has been shown to transport many glutathione-(S-glucuronide and sulfate conjugates. In addition to conjugates, MRP2 transports some unconjugated organic anions including methotrexate, irinotecan, and ampicillin (Gerkm and Vore, 2002). The BCRP gene has been cloned and the protein product was found to be a half-transporter, consisting of a single 70-kDa, six-transmembrane peptide. Structurally diverse compounds such as anthracyclines, mitoxantrone, methotrexate, the camptothecins, and estrone-3-sulfate have been identified as BCRP substrates (Sarkadi et al., 2004).}

Rosuvastatin is a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor for the treatment of patients with dyslipidemia, is primarily excreted via bile as unchanged drug. The present study was designed to determine whether rosuvastatin is transported by MDR1, MRP2, and BCRP. The apparent permeability value for rosuvastatin across MDR1-Madin-Darby canine kidney cells was low (8 nm/s), and no directional transport was observed. Rosuvastatin uptake into control SF9 membranes and membranes expressing MRP2 was similar in the presence or absence of GSH. In contrast, ATP dramatically stimulated rosuvastatin uptake into membranes expressing BCRP, but not control membranes. Rosuvastatin transport occurred into an osmotically sensitive space and was saturable. An Eadie-Hofstee analysis suggested that there were two transport sites in BCRP, with an apparent $K_m$ of 10.8 μM for the high affinity site and 307 μM for the low affinity site. These data demonstrate that rosuvastatin is transported efficiently by BCRP and suggest that BCRP plays a significant role in the disposition of rosuvastatin.

**ABBREVIATIONS:** ABC, ATP-binding cassette transporter; BCRP, breast cancer resistance protein; E$_{17}$G, estradiol-17β-o-glucuronide; GSH, glutathione; MDR1, multidrug resistance protein 1; MDR1-MDCK, Madin-Darby canine kidney cells transfected with the human MDR1 gene; MRP2, multidrug resistance protein 2; MXR, mitoxantrone resistance protein; $P_{app}$, apparent permeability; Pgp, P-glycoprotein; OATP, organic anion-transporting polypeptide; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; CTRL, control; A→B apical to basolateral; B→A, basolateral to apical.
Materials and Methods

Materials. Rosuvastatin was supplied by AstraZeneca Pharmaceuticals LP. GF120918 was obtained from GlaxoSmithKline (Research Triangle Park, NC) under a material transfer agreement. [3H]Estradiol-17β-d-glucuronidase (E2-17γ) was purchased from PerkinElmer (Boston, MA). [3H]Rosuvastatin was supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Madin-Darby canine kidney cells transfected with human MDR1 (MDR1-MDCK) were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands). Transwells (12-well, 11-mm diameter, 0.4-µm pores) were purchased from Corning Costar (Cambridge, MA).

MRP2-S9-VT membranes (membranes isolated from Sf9 cells expressing MRP2; ATP-dependent transport of 200 nM [3H]leukotriene C4 was 20.8 pmol/mg protein/min), CTRL-Sf9 membranes (control membranes isolated from Sf9 cells expressing β-galactosidase), MXR-M-VT membranes (membranes isolated from mammalian cells expressing BCRP; ATP-dependent transport of 100 μM [3H]methotrexate was 172.6 pmol/mg protein/min), and CTRL-M membranes (control membranes isolated from mammalian cells that have no BCRP expression) were purchased from Solvo Biotechnology (Budapest, Hungary).

Monolayer Efflux Studies. MDR1-MDCK cells were cultured and transport experiments were conducted as described by Polli et al. (2001). Briefly, cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in the absence of any antibiotics or selection agents. Cells were seeded onto Transwells at a density of 300,000 cells/cm², and medium was replaced daily and 2 h before transport experiments were performed. Monolayers were used for transport studies 3 days after seeding. Compounds were dissolved in dimethyl sulfoxide and then diluted in Hank’s balanced transport buffer (pH 7.4) (Mediatech, Herndon, VA). The amount of dimethyl sulfoxide in the final transport solution was less than 0.5% (v/v).

Rosuvastatin concentrations were analyzed by liquid chromatography-tandem mass spectrometry using a Sciex API 3000 with Turbolon spray source (Applied Biosystems/MDS Scies, South San Francisco, CA) (Schneck et al., 2004). Erythromycin and propranolol concentrations were determined by liquid chromatography-tandem mass spectrometry using a Micromass Quattro Ultima (Micromass UK Ltd, Cheshire, UK) equipped with an electrospray ionization interface. The samples were loaded (injection volume 10–20 µl) on columns by means of an HTS Pal autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatography was performed on columns of Synergi MAX-RP 30 × 2.0 mm, 4 µm (Phenomenex, Torrance, CA) at a flow rate of 1.5 ml/min. The mobile phase consisted of two solvents: 0.1% formic acid in water (A) and 10% methanol in acetonitrile (B). The gradient profile was set to 0.3 min 0% B, 0.3 to 1.3 min linear gradient to 95% B, 1.3 to 1.6 min 95% B, and 1.6 to 2 min 0% B.

The apparent permeability (P_app) values were calculated using the equation $P_{app} = (1/AC_m) \times (dQ/dt)$, where $A$ is the membrane surface area, $C_m$ is the donor drug concentration at $t=0$, and $Q/dt$ is the amount of drug transported within a given time period. Flux ratio $= P_{app} (B \rightarrow A)/P_{app} (A \rightarrow B)$.

Rosuvastatin Transport in Membrane Vesicles. ATP-dependent transport of [3H]rosuvastatin ([3H]E17γ) was assayed in a potassium-substrate for MRP2, was measured using a rapid filtration technique (Huang et al., 2000). Briefly, membranes were quickly thawed at 37°C. Transport was initiated by adding membranes (30–66 µg) to the preincubation buffer (50 mM Tris, 50 mM mannitol, and 10 mM MgCl₂, pH 7.5) containing 5 mM ATP or AMP and [3H]rosuvastatin or [3H]E17γ. Transport was terminated by the addition of 2.5 ml of ice-cold transport buffer, and vesicle-associated [3H]rosuvastatin was separated from free [3H]rosuvastatin by rapid filtration through a GF/F filter (Whatman, Florham Park, NJ). Filters were further washed twice with 2.5 ml of ice-cold buffer and assayed for radioactivity in 10 ml of liquid scintillation cocktail. Because nonspecific binding to the filter was relatively minimal, the data were not corrected for the binding to the filter unless stated otherwise in the figure legend. ATP-dependent transport was determined as the difference in uptake between 5 mM ATP and 5 mM AMP.

Statistics. Statistical differences ($p < 0.05$) were determined by one-way analysis of variance, followed by a multiple comparison test or unpaired Student’s t test. Prism 3.0 software (GraphPad Software Inc., San Diego, CA) was used to produce the best-fitting curves.

Results

Pgp-Mediated Transport of Rosuvastatin. Rosuvastatin transport across MDR1-MDCK monolayers was measured at concentrations of 10 and 50 μM in the presence or absence of 2 μM GF120918, a potent inhibitor for both Pgp and BCRP. Rosuvastatin transport in both B→A and A→B directions was similar, with a $P_{app}$ value of ~8 nm/s, and was not affected by GF120918. In contrast, transport of the Pgp substrate erythromycin (10 μM) in the B→A direction was 35-fold greater than that in the A→B direction (Table 1).

MXP2-Mediated Transport of Rosuvastatin. No significant ATP-dependent transport of rosuvastatin was observed in either CTRL-S9-(control) or MRP2-S9-membranes in the absence of glucathione (GSH) (Fig. 1A). Significant ATP-dependent transport of rosuvastatin was observed in the presence of 5 mM GSH in both MRP2- and control-membrane vesicles (Fig. 1B). However, ATP-dependent transport of rosuvastatin between control and MRP2-S9-membranes was not significantly different ($p > 0.05$).

BCRP-Mediated Transport of Rosuvastatin. ATP dramatically stimulated rosuvastatin uptake in MXR-M-VT membranes expressing high levels of BCRP in a time-dependent manner (Fig. 2). An initial uptake rate of ATP-dependent transport of rosuvastatin (5 μM) was too rapid to be determined accurately, and the transport at 30 s was 239.7 pmol/mg protein. In contrast, no ATP-dependent transport of rosuvastatin was observed in CTRL-M membranes that did not express BCRP. The difference in the uptake between ATP and AMP was sensitive to extravesicular osmolarity. As the concentration of extravesicular sucrose increased, rosuvastatin uptake decreased in the presence of ATP ($p < 0.05$), whereas the uptake did not change significantly in the presence of AMP ($p > 0.05$) (Fig. 3).

Concentrations of rosuvastatin ranging from 0.2 to 1000 μM were used to determine whether BCRP-mediated transport of rosuvastatin was saturable (Fig. 4). An Eadie-Hofstee analysis suggested that there were two binding sites in BCRP for rosuvastatin, with an apparent $K_m$ of 10.8 ± 1.1 μM for the high affinity site, and 307 ± 89.4 μM for the low affinity site.

GF120918 (2 μM), an inhibitor of Pgp and BCRP, inhibited ATP-dependent transport of rosuvastatin by 89% ($p < 0.05$) (Fig. 5). Pravastatin up to 250 μM inhibited rosuvastatin transport by only 30%.

Discussion

The purpose of this study was to identify and characterize ATP-dependent transporters for rosuvastatin. Our results provide the direct evidence that rosuvastatin is a substrate for BCRP. Rosuvastatin transport in MXR-M-VT membranes was dramatically stimulated by...
ATP and was saturable, with an apparent $K_m$ of 10.8 $\mu$M for the high affinity binding or transport site. This value is comparable to the $K_i$ (15.4 ± 2.4 $\mu$M) for inhibiting [$^{3}$H]estrone-3-sulfate uptake by human BCRP-membrane vesicles (Hirano et al., 2005), suggesting that rosuvastatin and estrone-3-sulfate may share the same transport or binding site. The uptake in the presence of ATP, but not in the presence of AMP, was decreased as the intravesicular space was decreased by increasing extravesicular sucrose concentration (osmolarity). These data indicate that rosuvastatin transport occurred into the intravesicular space, and the observed difference in uptake between ATP and AMP was mainly due to transport, not binding (Vore et al., 1996).

Western blot analysis showed that high levels of BCRP, moderate levels of MRP4 and MRP5, very low levels of MRP1, and undetectable levels of Pgp, MRP2, and MRP3 were expressed in MXR-M-VT; and total MRPs expressed in MXR-M-VT were much lower than that in control membranes (personal communication with the chief scientist Dr. Peter Krajsci in Solvo Biotechnology). Moreover, rosuvastatin transport in MXR-M-VT membranes was inhibited by GF120918. Thus, the observed ATP-dependent transport in MXR-M-VT membranes is probably mediated by BCRP. In an attempt to determine whether pravastatin is also transported by BCRP, the ability of pravastatin to inhibit BCRP-mediated transport of rosuvastatin was evaluated. However, pravastatin up to 250 $\mu$M inhibited rosuvastatin transport (1 $\mu$M) by only 30%, suggesting that pravastatin is not transported by BCRP, or possibly binds to a different site. These data suggest that the transporter mechanisms for the first-pass and biliary excretion between pravastatin and rosuvastatin may be different.

The Eadie-Hofstee analysis suggested that there were two binding or transport sites for rosuvastatin in MXR-M-VT membranes. Although multiple binding sites in BCRP have not been reported previously, and the presence of unknown transporters for rosuvastatin in these membranes cannot be excluded, our results are consistent with the findings of multiple binding sites in other ABC transporters. It has been proposed that MDR1 has three binding sites, including two transport sites and an allosteric site. Pgp-mediated drug transport was stimulated by prazosin and progesterone (Shapiro et al., 1999). Similarly, two transport or binding sites have been proposed for MRP2. Zelcer et al. (2003) postulated two MRP2 transport sites with positive
and represent means ± S.D. of triplicate determinations from a single experiment. * p < 0.05.

Based on the fact that rosuvastatin is an organic anion and that pravastatin is an MRP2 substrate, we had speculated that rosuvastatin was an MRP2 substrate. However, there was no ATP-dependent transport of rosuvastatin in MRP2-Sf9-membranes or control membranes in the absence of GSH, although significant ATP-dependent transport of rosuvastatin in MRP2-Sf9 membranes or control membranes was an MRP2 substrate. However, there was no ATP-dependent accumulation to stimulate MRP2-mediated transport.

Cooperativity to explain the ability of sulfinpyrazone and indomethacin to stimulate MRP2-mediated transport.

Based on the fact that rosuvastatin is an organic anion and that pravastatin is an MRP2 substrate, we had speculated that rosuvastatin was an MRP2 substrate. However, there was no ATP-dependent transport of rosuvastatin in MRP2-Sf9-membranes or control membranes in the absence of GSH, although significant ATP-dependent transport of E217G, a known MRP2 substrate, was clearly observed. Uptake of E217G measured over 5 min was 839 ± 64 and 146 ± 4 (pmol/mg protein) in the presence of 5 mM ATP and AMP, respectively. The high expression level of MRP2 in these membranes, but not in control membranes, was confirmed by Western blot analysis (data not shown). It has been shown that GSH stimulates MRP-mediated transport of vincristine (Loe et al., 1998). In the presence of 5 mM GSH, significant ATP-dependent transport of rosuvastatin was observed in both control and MRP2-S9-VT membranes. However, there was no significant difference in ATP-dependent transport of rosuvastatin between control and MRP2-S9-VT membranes, suggesting that endogenous transporters in control Sf9 cells transport rosuvastatin in the presence of GSH. These data do not support our speculation that rosuvastatin is an MRP2 substrate. However, the possibility that MRP2 transports rosuvastatin in the presence of GSH cannot be excluded. It is also possible that the expression levels of endogenous MRP-like proteins in Sf9 cells expressing MRP2 are lower than that in control cells, and MRP2-mediated transport is masked by the high background transport in control membranes.

The role of Pgp in rosuvastatin efflux was assessed using MDR1-MDCK cells, a cell line widely used to identify Pgp substrates. No significant directional transport of rosuvastatin across MDR1-MDCK cells was observed. The apparent permeability of rosuvastatin was low, and was similar to that of mannitol, a marker for paracellular diffusion, suggesting that rosuvastatin may not be able to penetrate into cells without the activity of uptake transporters. The lack of directional transport of rosuvastatin across MDR1-MDCK cells may reflect its inability to access Pgp rather than it not being transported by Pgp. Therefore, rosuvastatin uptake into membrane vesicles isolated from wild-type and MDR1-MDCK cells was further examined. Marginal ATP-dependent transport of rosuvastatin was observed in membranes isolated from both wild-type and MDR1-MDCK cells. The ATP-dependent transport at 1 min was 11 and 9 (pmol/mg protein) for wild-type and MDR1-MDCK membranes, respectively (p > 0.05) (data not shown). These data do not support the idea that rosuvastatin is a Pgp substrate.

Recently, Tiberg et al. (2004) reported that rosuvastatin transport in the serosal to the mucosal (secretory) direction was higher than that in the mucosal to the serosal (absorptive) direction across rat intestinal segments mounted in Ussing chambers, with the highest efflux in the ileum. The efflux ratios were 8.2, 7.2, 15, and 1.7 across duodenum, jejunum, ileum, and colon segments, respectively, which is consistent with the expression pattern of Bcrp in rat intestine (Tanaka et al., 2005). Bcrp mRNA levels are high throughout the intestinal tract, with the highest levels in the ileum, which differs from the limited expression of Mrp2 in proximal segments of rat small intestine (Motino et al., 2000). Mrp2 protein is present mainly in brush-border membranes of the proximal segments and gradually decreases from jejunum to the distal ileum. It has been shown that Bcrp plays an important role in biliary excretion and oral absorption of topotecan (Jonker et al., 2000). The present data clearly demonstrated that rosuvastatin is a substrate for Bcrp, and this transporter probably contributes to its biliary excretion. However, it is not known whether Bcrp plays a significant role in limiting oral absorption of rosuvastatin. Rosuvastatin has poor permeability, and its flux across cell monolayers in vitro is mainly through the paracellular pathway. If this is the case in vivo, rosuvastatin may not be able to access Bcrp for efflux without the involvement of uptake transporters. Recently, it was found that OATP-B is expressed at the apical membrane of human intestinal epithelial cells and may involve pH-dependent absorption of pravastatin (Kobayashi et al., 2003). An uptake transport system for rosuvastatin at the apical membranes of the intestines may exist and, therefore, Bcrp may limit its oral absorption.

In summary, the present studies demonstrate that rosuvastatin is transported efficiently by Bcrp in vitro. These data suggest that Bcrp may play a significant role in the disposition of rosuvastatin.

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References


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