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Human Proximal Tubular Epithelium Actively Secretes but Does Not Retain Rosuvastatin

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ABSTRACT

Rosuvastatin is a potent HMG-CoA reductase inhibitor that has proven to be effective in the treatment of dyslipidemia. Rosuvastatin is cleared from the body by both biliary and renal clearance, the latter believed to be due to active tubular secretion. Whereas the mechanisms of hepatic clearance of rosuvastatin are well documented, those of renal clearance are not. Because rosuvastatin (and other statins) may alter proximal tubular function, this study aimed to characterize the mechanisms of tubular rosuvastatin secretion to define the factors that could influence the presence/concentration of rosuvastatin in proximal tubular cells. Hereto, polarized monolayers of primary human tubular cells were used. We found rosuvastatin net secretion across proximal tubule cells, which was saturable (K_{50} = 20.4 ± 4.1 μM). The basolateral uptake step was rate-limiting and mediated by OAT3. Rosuvastatin efflux at the apical membrane was mediated by MRP2/4 and ABCG2 together with a small contribution from MDR1 or P-glycoprotein. These data, obtained in an intact human tubule cell model, provide a detailed insight into rosuvastatin's renal handling and the possible factors influencing it.

HMG-CoA reductase inhibitors (statins) are widely used in the reduction of elevated LDL cholesterol and the prevention of atherosclerotic cardiovascular disease. The action of statins is localized primarily to the liver, where inhibition of HMG-CoA reductase results in a decrease in intracellular sterol concentrations, up-regulation in LDL receptor expression, and an increased receptor-mediated uptake of LDL by the liver. Rosuvastatin (for chemical structure, see McTaggart et al., 2001) is a highly effective HMG-CoA reductase inhibitor that has produced dose-dependent reductions in LDL-cholesterol of up to 65%. In comparative studies, rosuvastatin was more effective in lowering LDL cholesterol than atorvastatin, simvastatin, or pravastatin (Jones et al., 2003).

Rosuvastatin is cleared primarily by biliary excretion, but renal clearance also plays a role, accounting for ~28% of the plasma clearance (Martin et al., 2003). Several other statins are also subject to renal clearance including lovastatin (~10%), simvastatin (~13%), pravastatin (~40%), and atorvastatin (~2%) (Vickers et al., 1990; Hatanaka, 2000; White, 2002).

The proximal tubule plays a central role in the tubular secretion of xenobiotics. Tubular secretion can be considered a three-step process consisting of uptake across the basolateral membrane, intracellular accumulation, and efflux across the apical membrane. The uptake and efflux steps are mediated by a range of transport proteins located at the basolateral and apical membranes of proximal tubule cells (Wright and Dantzler, 2004). At the molecular level, we have a detailed understanding of the properties of the transporters present in the proximal tubule; however, because of a lack of a suitable experimental model, we have little knowledge of the integration and importance of these individual apical and basolateral transporters to overall xenobiotic secretion.

To try to understand the mechanisms of xenobiotic secre-

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ABBREVIATIONS: LDL, low-density lipoprotein; MRP, multidrug resistance-associated protein; MDR, multidrug resistance; PTC, proximal tubular cells; DTC, distal tubular/collecting duct cells; PAH, *para*-aminohippuric acid; LAP, leucine aminopeptidase; J_{ab}, apical to basolateral flux; J_{ba}, basolateral to apical flux; J_{net}, net to flux; E3S: estrone-3-sulfate; FTC, fumitremorgin C; BCRP, breast cancer research protein; MK571, (3-[[[3-(7-chloro-2-quino-linyl)ethenyl]-phenyl[[3-(dimethylamino-3-oxoprolyl]thio]-methyl]thio]propionic acid.

tion in human proximal tubules, we further characterized our mixed and pure primary cultures of human proximal (PTC) and distal tubule/collecting duct cells (DTC), grown as polarized monolayers on filter supports to use them for transport studies (C. D. A. Brown, R. Sayer, A. S. Windass, I. S. Haslam, M. E. DeBroe, P. C. D'Haese, and A. Verhulst, submitted). Characterization of the PTC revealed that they express mRNA for proximal tubular transporters including an inorganic phosphate transporter, Na^+-dependent glucose transporter 2, organic anion transporters 1 and 3 (OAT1 and OAT3), multidrug resistant protein-2 (MRP2), MDR1 (p-glycoprotein), ATP-binding-cassette-G2 (or BCRP), organic cation transporter 2, and organic cation transporter N2, which were absent in DTC. At the protein level, we have found the expression of several of these transporters at the appropriate membrane. At the functional level, the basolateral-apical secretion of molecules such as *para*-aminohippuric acid (PAH) and creatinine and the net absorption of the glucose analog 3-*O*-methyl-D-glucose and albumin were demonstrated (Verhulst et al., 2004; C. D. A. Brown, R. Sayer, A. S. Windass, I. S. Haslam, M. E. DeBroe, P. C. D'Haese, and A. Verhulst, submitted). In this study, we used these monolayers of primary human mixed and pure PTC and DTC to investigate the renal handling of rosuvastatin.

Materials and Methods

Primary Human Tubular Kidney Cell Cultures. Human tubular epithelial cells were isolated as described previously (Van der Biest et al., 1994; Helbert et al., 1997, 1999, 2001). In brief, normal human kidney tissue, which became available from nephrectomies performed for oncological reasons, was collected in sterile RPMI 1640 media supplemented with 5% fetal bovine serum and 2% penicillin/streptomycin at 4°C. Under sterile conditions, macroscopically normal tissue was decapsulated, and the cortex and outer stripe of the outer medulla (if present) were dissected, cut into pieces of approximately 1 mm^3, and digested in collagenase D solution (Roche, Ottweiler, Germany) to a final concentration of 0.67 mg/ml in RPMI 1640 media. The suspension was shaken vigorously for 2 h at 37°C and then passed through a 120-μm sieve. The resulting cell suspension was loaded on top of a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient made up in RPMI 1640 media with densities of 1.04 and 1.07 g/ml. After centrifugation (3000 rpm, 25 min, 4°C) in a 4 × 200-ml swing-out rotor, cells from the intersection were carefully aspirated, washed, and brought into culture as a mixed population of PTC and DTC seeded directly onto 6.5-mm 0.4-μm pore size permeable (polycarbonate) filter supports (Costar, New York, NY) at a density of 5×10^4 cells per filter.

To obtain pure cultures of PTC and DTC, a subconfluent mixed culture was trypsinized and purified by flow cytometric sorting. Cells were incubated for 30 min at 4°C with an anti-human leucine aminopeptidase (LAP) monoclonal antibody. LAP was identified previously in our laboratory as a marker of proximal tubular cells (Helbert et al., 1999). Subsequently, phycoerythrin-labeled rabbit $F(ab')_2$ anti-mouse (Dako Denmark A/S, Glostrup, Denmark) secondary antibody was added to the cell suspension. Labeled cells were sorted using a FACSvantage flow cytometer (BD Biosciences, San Diego, CA) into distinct PTC (LAP^+) and DTC (LAP^-) populations.

Mixed and pure cultures of PTC and DTC were grown until confluence (8–12 days) on semipermeable filter supports in α-MEM (Invitrogen, Carlsbad, CA) modified according to Gibson d'Ambrosio et al. (1987) supplemented with 10% fetal calf serum. Cell cultures grown on permeable filter supports were allowed to polarize and had a separated apical and basolateral compartment. Cell culture medium was replaced only once before performing the experiments (7–9 days after culturing of the cells). Cell culture medium was replaced by Krebs' solution for transport studies.

Transport Measurements. Bidirectional transepithelial flux measurements of substrates across monolayers of human tubular epithelial cells were made at steady state, essentially as described previously (Simmons, 1990). Cell monolayers grown on permeable filter supports were extensively washed four times in a modified Krebs' buffer: 140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 0.3 mM KH_2PO_4, 0.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES buffered to pH 7.4 at 37°C with Tris base. Filters were then placed in 12-well plastic plates, each well containing 1 ml of prewarmed Krebs' buffer with unlabeled substrate (rosuvastatin; PAH or mannitol) with a further 0.5 ml of identical solution added to the apical chamber. Monolayers were preincubated for 1 h at 37°C to allow for a steady state to be achieved. Apical to basolateral (J_{ab}) and basolateral to apical (J_{ba}) fluxes of rosuvastatin, PAH, creatinine, and mannitol were measured in paired resistance matched monolayers. Monolayers were paired according to their transepithelial resistance. In addition, monolayers were excluded if the transepithelial resistance of the monolayer corrected for the resistance of the filter was less than 60 $\Omega \cdot cm^2$. Flux was initiated by adding [^{3}H]rosuvastatin (1 μCi/ml), [^{3}H]PAH (1 μCi/ml), [^{14}C]creatinine, and either [^{3}H]mannitol or [^{14}C]mannitol (0.1 μCi/ml) to either the apical or basolateral chamber. Samples of 250 and 500 μl were removed from the apical and basolateral chamber, respectively, after a 60-min flux period. To generate a time course, samples were taken from either compartment at 30-min intervals, after which the volume was replaced with Krebs' buffer containing unlabeled substrate. ^{3}H or ^{14}C activity in the samples was determined by liquid scintillation spectrophotometry using a Beckman liquid scintillation counter. Fluxes across the monolayers are expressed as nanomoles per squared centimeter per hour. At the end of the flux period, the remaining solutions were aspirated off, and the filters were washed four times in a 500-ml volume of ice-cold Krebs' buffer at pH 7.4 to remove extracellular isotope. The cell monolayers were then excised from the filter insert, and the cell-associated isotope was determined by liquid scintillation counting. Cellular accumulation of either rosuvastatin or creatinine is expressed as cell/media ratio. Cell volume was determined using a geometric approach in which cell volume (V_c) = $((\pi r^2 h) \times 0.7)$, where πr^2 is the area of the insert, and h is the average cell height calculated from confocal images of confluent cell monolayers. Cell height was measured in series of cell monolayers derived from four separate kidneys, and an estimated value of 14.8 μm was used in subsequent calculations of cell volume. A correction factor of 0.7 was used as an estimate of extracellular volume. (Thwaites et al., 1993; Sun and Pang, 2008).

Initial rates of rosuvastatin uptake across the basolateral membrane were measured in an almost identical manner except that cell monolayers were preincubated for 1 h in Krebs' buffer rather than in Krebs' buffer plus unlabeled substrate. Uptake was initiated by the replacement of Krebs' buffer in the basolateral chamber with buffer containing [^{3}H]rosuvastatin (0.5 μCi/ml) and [^{14}C]mannitol (0.25 μCi/ml) and competitive substrates as denoted in the figure legend. At the end of the uptake period, cell monolayers were washed four times in a 500-ml volume of ice-cold Krebs' buffer at pH 7.4 to remove extracellular isotope. The cell monolayers were then excised from the filter insert, and the cell-associated isotope was determined by liquid scintillation counting.

Study Set-Up and Statistics. Because of the interindividual variation in transport rates in cell monolayers derived from individual kidney samples (Fig. 3), data are represented as single representative experiments. In each experiment, at least three sets of paired monolayers were used to generate the data. Each experiment was repeated at least three times on monolayers derived from at least three individual kidneys (average age of all kidney donors was 63 years, range 44–89 years). Data are presented as mean ± S.E.M. Statistical comparison of mean values was made using a Student's t test. For multiple comparisons, a one-way analysis of variance test

was used, and significance was assigned using a Dunnett post test. Differences in the mean values were considered to be significant when $p \leq 0.05$. Nonlinear regression analysis of the data was carried out using Prism 3.0 software (GraphPad Software Inc., San Diego, CA).

Materials. [*N*-methyl-^{3}H]Rosuvastatin (specific activity, 79 Ci/mmol) was a gift from AstraZeneca (Alderley Park, Cheshire, UK). *p*-[Glycyl-2-^{3}H]-aminohippurate (specific activity, 4.5 Ci/mmol), [^{14}C]- and [^{3}H]mannitol (45–60 mCi/mmol) were from PerkinElmer Life and Analytical Sciences (Beaconsfield, Bucks, UK). [^{14}C]Creatinine was purchased from American Radiochemical (St. Louis, MO).

Rosuvastatin (Ca^{2+} salt) was a gift from AstraZeneca. Estrone-3-sulfate, PAH, creatinine, vinblastine, and MK571 were from Sigma-Aldrich (Poole, Dorset, UK). Fumitremorgin C (FTC) was from Calbiochem (Nottingham, UK). All other chemicals were of the highest analytical quality available.

Results

Time-Dependence of Rosuvastatin Fluxes across Human Tubular Cell Monolayers. Steady-state unidirectional fluxes of [^{3}H]rosuvastatin (10 μM) were measured at three time points (30, 60, and 90 min) in mixed monolayers of PTC and DTC. Over this time period, both flux in the absorptive direction (apical to basolateral, J_{ab}) and flux in the secretory direction (basolateral to apical, J_{ba}) were linear (r^2 = 0.97 and 0.99) (Fig. 1). At all time points, J_{ba} was significantly higher ($p < 0.0001$) than J_{ab}, resulting in a time-dependent net secretion (J_{net}) of rosuvastatin across the monolayer from the basolateral toward the apical (luminal) side of the cells. On the basis of the linear relationship between flux and time up to 90 min, a 60-min time point was adopted as standard for subsequent measurements.

Rosuvastatin Secretion Is Restricted to Proximal Tubule Cells. In vivo, rosuvastatin secretion is believed to result from tubular secretion of rosuvastatin in the proximal tubule. To identify the cellular location of rosuvastatin secretion in human tubular cell monolayers, the steady-state flux of rosuvastatin was measured in monolayers of either purified PTC or DTC. The data shown in Fig. 2 clearly demonstrate that the net secretion of rosuvastatin is confined to monolayers of PTC. J_{ab} and J_{ba} values of rosuvastatin measured in PTC monolayers differed significantly ($p < 0.05$) resulting in a J_{net} of 1037.0 ± 512.0 pmol/cm^2/h (n = 4), whereas in monolayers of DTC, rosuvastatin J_{ab} and J_{ba} showed no asymmetry (p = 0.99) and thus no net secretion of rosuvastatin.

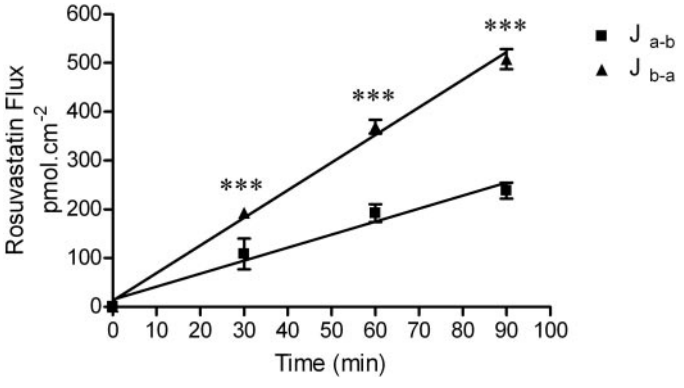

Fig. 1. Time dependence of unidirectional fluxes of rosuvastatin. Unidirectional fluxes of rosuvastatin (10 μM) were measured at steady state. Under these conditions, both apical to basolateral (J_{a-b}) and basolateral to apical (J_{b-a}) fluxes were linear over the time course measured (r^2 = 0.97 and 0.99, respectively). At all time points, J_{b-a} is significantly higher (***, $p < 0.0001$) than J_{ab}. The data are expressed as the mean ± S.E.M. of four monolayers per time point from a single experiment representative of three separate experiments.

Interindividual Variation in Rosuvastatin Net Flux across Human Tubular Cell Monolayers. To gain an estimate of the variability of rosuvastatin net fluxes between individual kidneys, the magnitude of rosuvastatin J_{net} was compared in mixed monolayers of PTC and DTC from 20 different kidney specimens. The results in Fig. 3 show that net secretion of rosuvastatin, found in 100% of the cell monolayers, however, varied from 2558.2 ± 513.5 to 130.00 ± 43.9 pmol/cm^2/h (mean = 603.2 ± 188.35 pmol/cm^2/h).

Determination of the Magnitude of the Transcellular Component of Rosuvastatin Secretion. To distinguish the transcellular flux of rosuvastatin from the paracellular component of rosuvastatin transport, steady-state unidirectional rosuvastatin fluxes were compared with the unidirectional mannitol fluxes in mixed monolayers of PTC and DTC. Mannitol is universally used as a measure of paracellular transport. Consistent with a transcellular component of rosuvastatin secretory flux, the J_{ba} value of rosuvastatin was 10-fold higher than the J_{ba} value of mannitol (460.8 ± 112.0 versus 45.1 ± 1.3, p = 0.02, n = 3). In contrast, the J_{ab} value of rosuvastatin was only 2-fold greater than the J_{ab} value of mannitol (86.5 ± 22.8 versus 33.8 ± 3.5, p = 0.2, n = 3) (Fig. 4). These data suggest that there is a substantial transcellular secretion of rosuvastatin and that rosuvastatin flux in the absorptive direction is significantly lower in magnitude than in the secretory direction.

Comparison of Cell/Media Concentration Ratios of Rosuvastatin and Creatinine. To estimate the relative rates of rosuvastatin uptake across the basolateral membrane and efflux across the apical membrane, the intracellular concentration of rosuvastatin was measured at steady state in mixed monolayers of PTC and DTC (Fig. 5). After exposure of the monolayer to rosuvastatin (10 μM) for 120 min, the cell/media ratio for rosuvastatin never exceeded 0.75. In contrast, similar measurement of cell/media concentration ratios for creatinine showed a marked asymmetry (Fig. 5). Indeed, after exposure to creatinine (10 μM) for 120 min at the basolateral membrane, the intracellular creatinine concentration was 3-fold higher than the culture medium concentration, consistent with a concentrative uptake of creatinine across the basolateral mem-

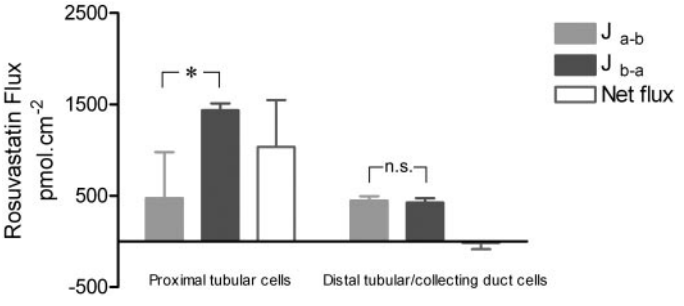

Fig. 2. The secretion of rosuvastatin is a function of proximal tubule cells. Comparison of the magnitude of J_{ab}, J_{ba}, and J_{net} fluxes of rosuvastatin (10 μM) in PTC monolayers versus DTC monolayers. Net secretion of rosuvastatin is confined to the PTC (J_{ab} and J_{ba} differ significantly, *, $p < 0.05$). In DCT monolayers, J_{ab} and J_{ba} show no asymmetry (n.s., not significant). The data are expressed as the mean ± S.E.M. of four monolayers per time point from a single experiment representative of three separate experiments.

brane. Similar to rosuvastatin, apical exposure to creatinine results in no cellular accumulation of creatinine. These data suggest that the influx of rosuvastatin at the basolateral membrane is the rate-limiting step for rosuvastatin secretion and that, in contrast, the rate-limiting step for creatinine lies in the efflux step at the apical membrane.

Determination of the Concentration Dependence of Rosuvastatin Secretion. To determine the concentration-dependence of transcellular rosuvastatin secretion, the J_{net} value of rosuvastatin was measured over a range of rosuvastatin concentrations (0–50 μM) in mixed monolayers of PTC and DTC (Fig. 6). Data indicate the net secretion of rosuvastatin to be saturable. Fitting the data with a least squares nonlinear regression analysis gave an apparent K_{50} value (half-saturation constant) of 20.4 ± 4.1 μM and an apparent V_{max} value of 4951.0 ± 542.0 pmol/cm^2/h (Fig. 6).

Comparison of Rosuvastatin and PAH Acid Fluxes across Human Tubular Cell Monolayers. Proximal tubular PAH handling is well characterized. To better understand the mechanism of rosuvastatin transport in human PTC, the transepithelial fluxes of rosuvastatin and PAH were measured in pure PTC and DTC.

Similar to rosuvastatin, there was a net secretory flux (410.0 ± 294.0 pmol/cm^2/h) of PAH in PTC monolayers (Fig. 7a). Furthermore, the secretion of PAH was limited to the PTC because there was no net flux of PAH in purified DTC monolayers (Fig. 7a). To investigate whether rosuvastatin shared transport pathways with PAH, the unidirectional fluxes of rosuvastatin (10 μM) were measured in the absence and presence of 50 μM PAH. In the presence of PAH in the basolateral compartment, J_{ba} of rosuvastatin reduced in magnitude. Although this change did not reach statistical significance ($p > 0.05$), the addition of PAH to the basolateral chamber resulted in an abolition of net secretion (Fig. 7b). Consistent with the lack of transcellular PAH and rosuvastatin transport in the DTC monolayers, there was no effect of adding PAH on either J_{ab}, J_{ba}, or J_{net} in these cells.

Rosuvastatin Uptake across the Basolateral Membrane of Human Tubular Cell Monolayers Is Mediated by OAT3. An inhibition of the secretory flux of rosuvastatin by PAH is consistent with competition for a common uptake pathway at the basolateral membrane. It is well recognized that PAH is a substrate for both OAT1 and OAT3 found on the basolateral membrane and OAT4 found on the apical membrane of PTC (Kusuhara et al., 1999; Inui et al., 2000; Van Aubel et al., 2000; Wright and Dantzler, 2004). Previous studies in *Xenopus laevis* oocytes suggested that rosuvastatin is a substrate for OAT3 but not OAT1 (Windass et al., 2007). To elucidate the importance of OAT3 in the basolateral handling of rosuvastatin, the effect of estrone-3-sulfate (E3S), a selective substrate of OAT3 (Kusuhara et al., 1999; Rizwan and Burckhardt, 2007), upon initial rates of rosuvastatin uptake were measured in mixed monolayers of PTC and DTC (Fig. 8). At the basolateral membrane, the addition of 100 μM E3S significantly reduced the uptake of rosuvastatin into the cells (192.6 ± 4.3 versus 23.4±.4.8, $p < 0.0001$, $n = 6$). In contrast, the addition of E3S to the apical membrane had no significant effect on the relatively small magnitude of rosuvastatin uptake at that membrane.

Pharmacology of Rosuvastatin Fluxes and Intracellular Concentration across Human Tubular Cell Monolayers. Figure 9a shows the effects of vinblastine and probenecid on the unidirectional fluxes of rosuvastatin across mixed monolayers of PTC and DTC. Both vinblastine (50 μM) and probenecid (50 μM) had no significant effect on the magnitude of the absorptive flux of rosuvastatin. In contrast, the addition of either vinblastine or probenecid to the cell monolayers significantly inhibited the magnitude of the secretory flux of rosuvastatin [control flux of 4354.0 ± 451.2 versus 3340.0 ± 100.0 ($p < 0.02$) and 1948.0 ± 228.8 ($p < 0.01$), respectively]. The inhibition of the secretory flux resulted in a significant ~20% net flux reduction in the presence of vinblastine and a total abolition of net flux in the presence of probenecid. The effects of both vinblastine and probenecid on fluxes of rosuvastatin were reflected in changes in intracellular concentrations of rosuvastatin at steady state (Fig. 9b). The most marked effect was found with vinblastine (intracellular rosuvastatin concentration at least 2-fold greater than under control conditions), consistent with the inhibition of rosuvastatin efflux across the apical membrane by a vinblastine-sensitive transporter (MDR1-MRP2/4). In the presence of probenecid, there was no significant

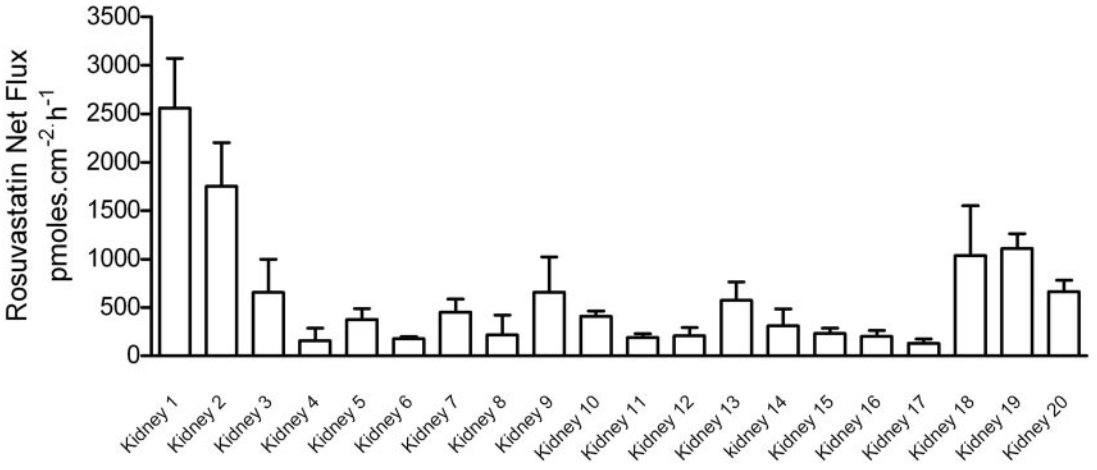

Fig. 3. Variation in rosuvastatin secretion across cell monolayers derived from 20 human kidney samples. Steady-state net flux of rosuvastatin (10 μM) was measured across cell monolayers generated from 20 different kidney specimens from the Belgium, United Kingdom, and The Netherlands. In all monolayers, a net secretion of rosuvastatin was observed but the absolute magnitude of J_{net} varied (range, 130 to 2558.2 pmol/cm^2/h; mean, 603.2 pmol/cm^2/h). The data are expressed as the mean± S.E.M. of four monolayers per kidney sample.

effect on intracellular rosuvastatin concentration, consistent with an inhibition of both uptake mediated by OAT3 and efflux mediated by a probenecid-sensitive transporter (MDR1-MRP2/4). To try to dissect out the apical efflux route in more detail, the effect of the relatively specific inhibitors MK571 (MRP2/4), fumetrimorgin C (FTC; BCRP), and vinblastine (MDR1) on the secretory flux of rosuvastatin was tested. The results (Fig. 10a) show that in the presence of MK571 (50 μM), the secretory flux of rosuvastatin (5 μM) was reduced to 38.0 $\pm$ 6.7% of the control flux ($p < 0.001$); equally in the presence of FTC (50 μM), the secretory flux of rosuvastatin was 33 $\pm$ 4.4% of the flux in the absence of FTC ($p < 0.001$). In the presence of vinblastine (50 μM), the secretory flux was reduced to 68.7 $\pm$ 7.6% of control values ($p < 0.01$). These data suggest that the apical exit of rosuvastatin is mediated largely by BCRP and MRP2/4 and, to a lesser extent, by MDR1. Consistent with the inhibitory effects of these agents on rosuvastatin exit across the apical membrane, intracellular concentrations of rosuvastatin were significantly higher in the presence of MK571 (12.6 $\pm$ 1.8 μM, $p < 0.01$), FTC (14.1 $\pm$ 2.3 μM, $p < 0.01$), or vinblastine (7.8 $\pm$ 0.2 μM $p < 0.05$) versus no inhibitor (2.8 $\pm$ 0.1 μM).

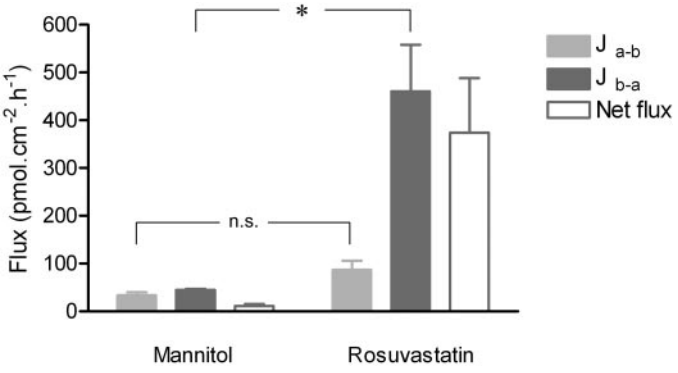

Fig. 4. Comparison of the magnitude of the steady-state flux of rosuvastatin and mannitol. To delineate between passive paracellular and transporter-mediated transcellular transport of rosuvastatin, the unidirectional fluxes of both mannitol and rosuvastatin were measured across tubular cell monolayers. Consistent with a transcellular component of rosuvastatin secretory flux (J_{ba}), J_{ba} of rosuvastatin was 10-fold higher than the mannitol flux in that direction (*, $p < 0.05$). In contrast, the apical to basolateral transport of rosuvastatin (J_{ab}) was only 2-fold greater than the flux of mannitol in the absorptive direction and did not statistically differ (n.s., not significant). These data suggest that there is a substantial transcellular secretion of rosuvastatin. The data are expressed as the mean $\pm$ S.E.M. of 3 monolayers per time point from a single experiment representative of 3 separate experiments

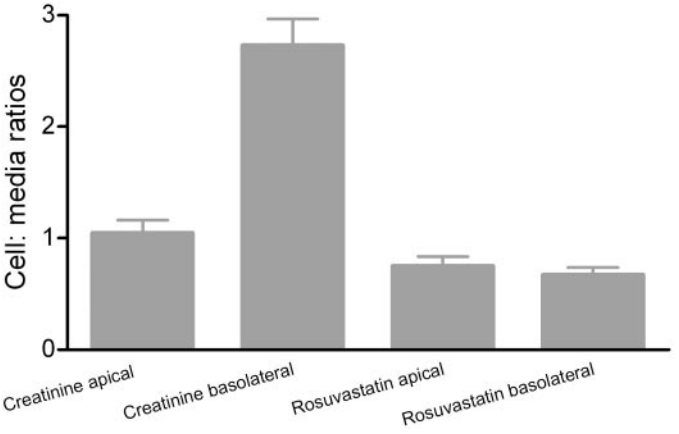

Fig. 5. Comparison of steady-state cell/media ratios for creatinine and rosuvastatin. Steady-state cell/media ratios were calculated from cells exposed to either 10 μM creatinine or rosuvastatin after a 120-min uptake period. Cell/media ratios were calculated for both apical and basolateral uptake of creatinine and rosuvastatin. In contrast to creatinine, the cell/media concentration ratio for rosuvastatin never exceeded unity. The data are expressed as the mean $\pm$ S.E.M. of four monolayers per condition from a single experiment representative of three separate experiments.

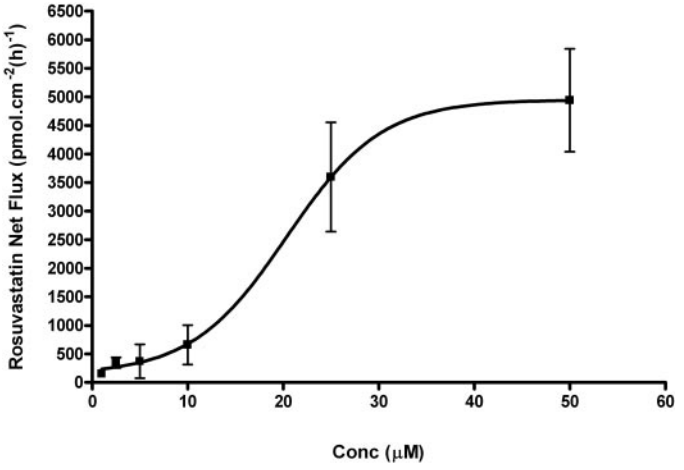

Fig. 6. The kinetics of the net secretion of rosuvastatin. The concentration-dependence of the net secretion of rosuvastatin J_{net} was measured over a range of rosuvastatin concentrations (0–50 μM). At each concentration, J_{net} was calculated by the subtraction of J_{ab} from J_{ba}. Nonlinear least squares regression analysis of the data generated an apparent K_m and V_{max} value of 20.4 $\pm$ 4.1 μM and 4951 $\pm$ 542 pmol/cm^2/h, respectively. The data are expressed as the mean $\pm$ S.E.M. of three monolayers per concentration from a single experiment representative of three separate experiments.

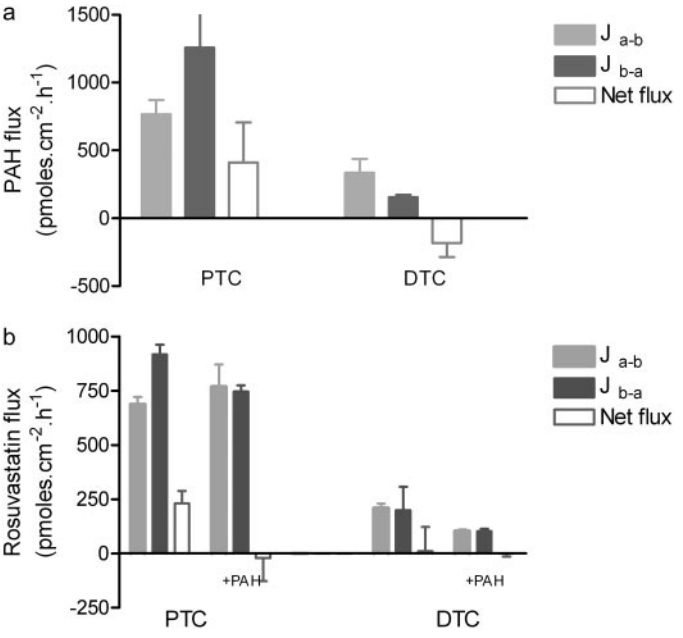

Fig. 7. Comparison of rosuvastatin and PAH fluxes in PCT and DCT cell monolayers. A, the steady-state fluxes of PAH (10 μM) in proximal (PTC) versus distal DTC cell monolayers. The data are expressed as the mean $\pm$ S.E.M. of four monolayers per condition from a single experiment representative of three separate experiments. B, the impact of the addition of PAH (50 μM) on rosuvastatin steady-state fluxes (10 μM) in PTC and DTC cell monolayers. PAH reduced J_{ba} of rosuvastatin. Although this change in unidirectional flux did not reach statistical significance ($p > 0.05$), PAH abolished net secretion. In the DTC monolayers, there was no effect of PAH on either J_{ab}, J_{ba}, or J_{net}. The data are expressed as the mean $\pm$ S.E.M. of four monolayers per condition from a single experiment.

Discussion

Rosuvastatin is cleared primarily by biliary excretion but also by a significant renal clearance. The aim of this investigation was to use primary cultures of human tubular cells grown as polarized monolayers to assess the importance of individual transporters at each membrane to the renal secretion of rosuvastatin. Human tubule cell monolayers remain differentiated in culture and express, at the mRNA and protein level, a range of organic anion transporters involved in the secretion of xenobiotics (C. D. A. Brown, R. Sayer, A. S. Windass, I. S. Haslam, M. E. DeBroe, P. C. D'Haese, and A. Verhulst, submitted), making them a powerful tool to investigate the renal handling of rosuvastatin.

We were able to demonstrate a net secretion of rosuvastatin across monolayers of human tubule cells with the basolateral to apical flux (J_{ba}) value on average ~3- to 4-fold greater than the apical to basolateral flux value (J_{ab}). This is consistent with data on tubular secretion of rosuvastatin in

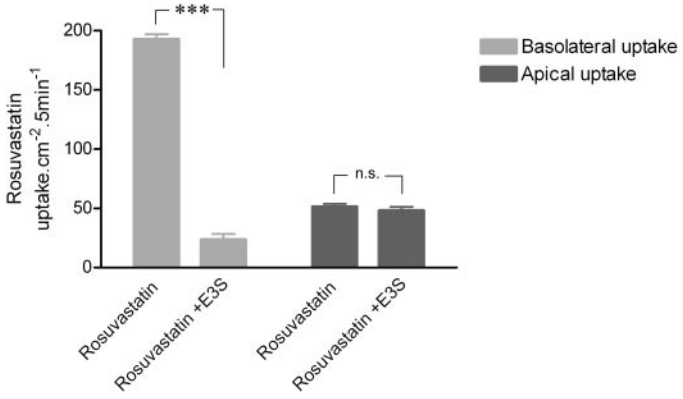

Fig. 8. Rosuvastatin uptake at the basolateral membrane is mediated by OAT3. To differentiate between the contributions of OAT1 and OAT3 to the basolateral uptake of rosuvastatin, the effect of estrone-3-sulfate (100 μM), a specific substrate for OAT3, upon the initial uptake rate of rosuvastatin (10 μM) was measured. Estrone-3-sulfate significantly reduced the uptake of rosuvastatin (***, $p < 0.0001$) at the basolateral membrane. In contrast, at the apical membrane, there was no significant effect of estrone-3-sulfate on the relatively low rosuvastatin uptake. The data are expressed as the mean ± S.E.M. of four monolayers per condition from a single experiment representative of three separate experiments. n.s., not significant.

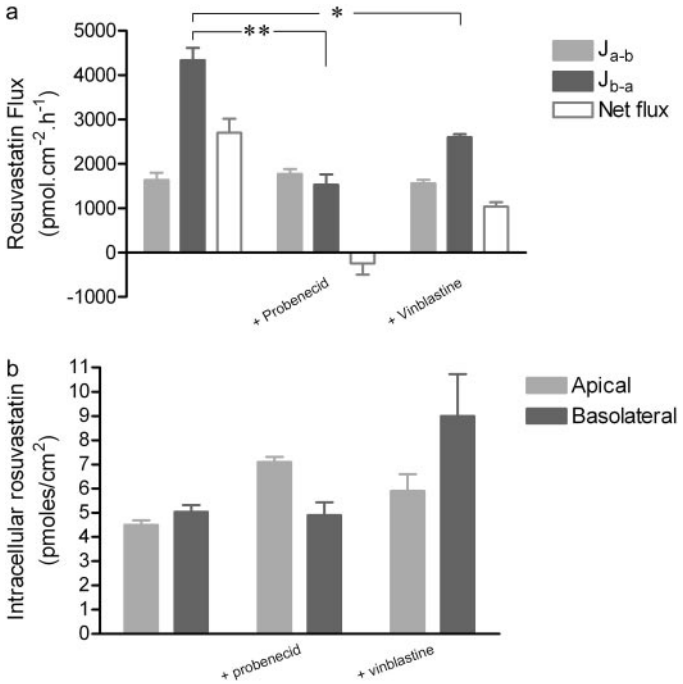

Fig. 9. The impact of probenecid and vinblastine on rosuvastatin fluxes and cell/media ratios. A, the effect of probenecid (50 μM) and vinblastine (50 μM) on steady-state fluxes of rosuvastatin (10 μM). Both vinblastine and probenecid significantly inhibit the magnitude of the secretory flux of rosuvastatin (*, $p < 0.05$, and **, $p < 0.01$ versus control flux). B, the impact of vinblastine (50 μM) and probenecid (50 μM) on the steady-state intracellular concentration of rosuvastatin. The data are expressed as the mean ± S.E.M. of three monolayers per condition from a single experiment representative of three separate experiments.

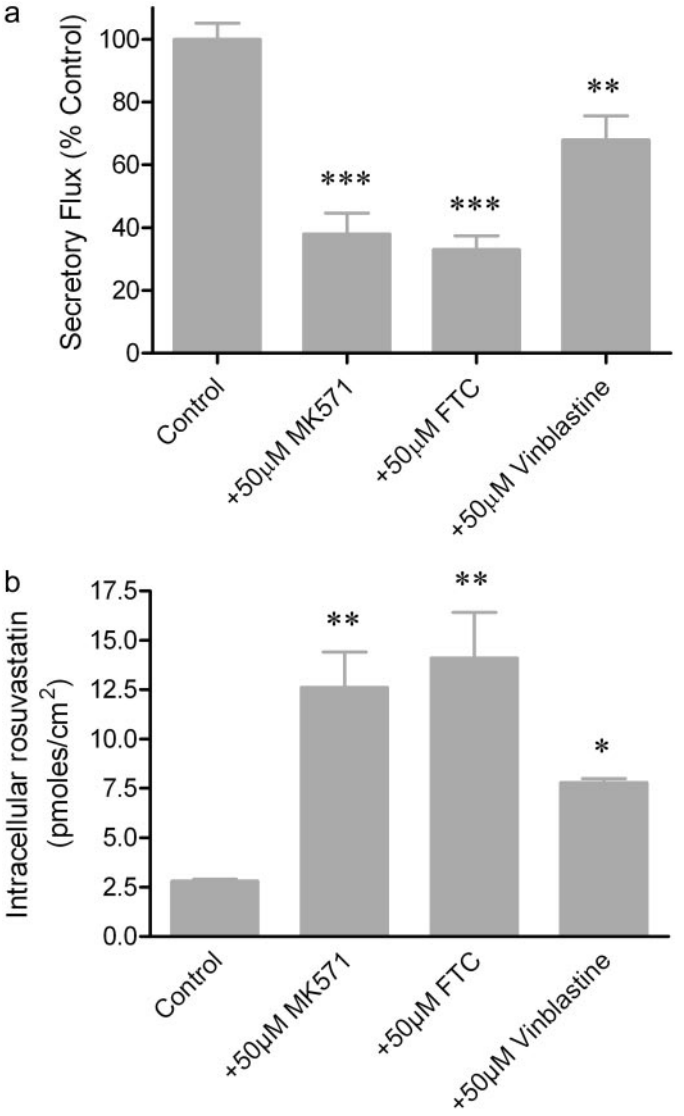

Fig. 10. Pharmacology of the efflux of rosuvastatin across the apical membrane of human tubular cell monolayers. A, the impact of three ATP binding cassette transport inhibitors (50 μM) on the secretory flux of rosuvastatin: all three molecules—MK571 (MRP2), FTC (BCRP), and vinblastine (MDR1/MRP)—significantly inhibited (***, $p < 0.001$; ***, $p < 0.001$, and **, $p < 0.01$, respectively) the secretory flux of rosuvastatin (5 μM). B, the impact of MK571 (50 μM), FTC (50 μM), and vinblastine (50 μM) on steady-state intracellular concentration of rosuvastatin (5 μM). All three increased significantly (**, $p < 0.01$, **, $p < 0.01$, and *, $p < 0.05$, respectively) the intracellular concentration of rosuvastatin. The data are expressed as the mean ± S.E.M. of three monolayers per condition from a single experiment representative of three separate experiments.

humans (White, 2002). As expected, given that each batch of cell monolayers was derived from an individual kidney, there was some interindividual variability in the magnitude of rosuvastatin fluxes. However, significant net secretion of rosuvastatin was demonstrated in 100% of kidney samples tested. The mean net secretion of rosuvastatin from 20 kidneys was 603.2 ± 188.4 pmol/cm^2/h.

Using immunoseparated pure cultures of PTC and DTC, we were able to demonstrate that secretion of rosuvastatin was a function of PTC and not of DTC. These functional data fitted with the proximal tubule location of transport proteins, such as OAT1, OAT3, and BCRP, believed to play a role in rosuvastatin secretion. It is noteworthy that the lack of rosuvastatin transport by DTC had the important consequence that we could use mixed cultures of tubule cells to investigate rosuvastatin transport specifically by proximal cells. Using mixed cultures rather than immunoseparated PTC cultures significantly increased the cell yield and number of monolayers available from each kidney.

The secretion of rosuvastatin was saturable with an apparent K_{50} value of 20.4 ± 4.1 μM. This K_{50} value is a composite of affinities for both the uptake step at the basolateral membrane and the exit step at the apical membrane. The addition of probenecid at the basolateral membrane resulted in the abolition of net rosuvastatin secretion. This was mediated by a significant inhibition of rosuvastatin basolateral to apical flux. Probenecid had no significant impact on rosuvastatin apical to basolateral fluxes. These data are consistent with the inhibition of a probenecid-sensitive uptake mechanism at the basolateral membrane, presumably OAT1 or OAT3 (Rizwan and Burckhardt, 2007). The inhibition of rosuvastatin uptake at the basolateral membrane in the presence of estrone-3-sulfate, a substrate of OAT3 but not OAT1, suggests that OAT3, not OAT1, is responsible for the uptake of rosuvastatin across the basolateral membrane. These data are in agreement with our recent demonstration of rosuvastatin uptake by OAT3 but not by OAT1 expressed in *X. laevis* oocytes (Windass et al., 2007) and the observation that pravastatin is a substrate for both rOat3 expressed in renal LLC-PK1 cells (Hasegawa et al., 2002) and for human OAT3 expressed in mouse PTC (Takeda et al., 2004). It is also in line with the fact that PAH, which is an OAT1/3 but by far preferable OAT1 substrate, inhibited rosuvastatin basolateral to apical flux only partially. It is important to note that in addition to inhibition of rosuvastatin uptake mediated by OAT3, PAH may also have inhibited an MRP4-mediated efflux of rosuvastatin at the apical membrane (Smeets et al., 2004). Taken together, these data strongly support the conclusion that OAT3 plays a key role in the uptake of rosuvastatin at the basolateral membrane of PTC.

At the apical membrane, the net rosuvastatin secretory flux was substantially reduced in the presence of inhibitors of MRP2/4 (MK571, ~60% inhibition) (Rius et al., 2003), BCRP (FTC, ~70% inhibition), or MDR1 (vinblastine, ~30% inhibition). Furthermore, inhibition of the secretory flux was accompanied by a concomitant proportional increase in intracellular rosuvastatin concentration consistent with inhibition of rosuvastatin efflux across the apical membrane. The inhibition of each efflux component was not additive (Fig. 10), perhaps illustrating the difficulties of dissecting out the contribution of each component pharmacologically. Despite this caveat, it is clear that rosuvastatin exit across the apical membrane is primarily mediated by both MRP2/4 and BCRP and that MDR1 contributes only a minor role in rosuvastatin efflux. This conclusion is similar to that proposed for a range of statins, including pravastatin and pitavastatin, in which evidence supports a key role for both BCRP and MRP2 in the clearance of hydrophilic statins and a minor role for MDR1, particularly in the handling of more lipophilic statins (Hirano et al., 2005; Matsushima et al., 2005; Huang et al., 2006). The intracellular concentration of organic anions within PTC is determined by a number of factors, including the relative rates of uptake across the basolateral membrane and efflux across the apical membrane and their intracellular distribution and binding. For PAH and fluorescein, cell/media ratios of 3 to 5 have been reported, but many drugs do not exceed a cell/media ratio greater than unity (Masereeuw et al., 1994). In human tubule cell monolayers, in the absence of inhibitors/competitors, the cell/media ratio of rosuvastatin did not exceed 0.75, suggesting that the rate-limiting step for rosuvastatin was the basolateral uptake step. This was supported by the observation that inhibition of the apical exit step, for example by FTC or MK571, resulted in a significant increase in cell/media ratio from ~0.75 to ~2.5 to 2.8. The peak plasma concentration (C_{max}) after a 40-mg dose of rosuvastatin is approximately 40 to 65 nM (Schneck et al., 2004; Lee et al., 2005), several orders of magnitude less than the apparent K_{50} value for rosuvastatin uptake by OAT3 (7.4 μM) (Windass et al., 2007).

An understanding of the intracellular concentration of rosuvastatin and how competition at either apical or basolateral membrane may modulate intracellular concentration of rosuvastatin and other statins is important given that there is evidence that statins have functional effects in the proximal tubule. During phase III studies of rosuvastatin, which included comparisons with placebo, a transient dipstick-positive proteinuria was observed in 12 and 4% of subjects taking 80 (greater than the currently recommended dose of 5–40 mg) and 40 mg of rosuvastatin, respectively, compared with 3% in placebo (Brewer, 2003; and see slide 24 available at http://www.fda.gov/ohrms/dockets/ac/03/slides/3968S1_01_C-AstraZeneca-Safety_files/frame.htm).

Studies using both opossum (OK cells) and human cells revealed that statins, in the absence of cellular toxicity, inhibited protein uptake by the proximal nephron via inhibition of HMG-CoA reductase and reduced prenylation of proteins involved in endocytosis (Sidaway et al., 2004; Verhulst et al., 2004). In this context, competition between drug molecules and statins for a common route of exit at the apical membrane might in theory result in an increased proximal tubule exposure to statins with an impact on intracellular prenylation of proteins. The fact that rosuvastatin efflux at the apical membrane is a function of at least three transporters and supposing that these three transporters are expressed in the same cells of the proximal tubule make it rather unlikely that a particular drug would be able to completely inhibit its efflux. It may be for this reason that there have been so far no reports of adverse drug interactions occurring with rosuvastatin (or any other statin) in human kidneys such as has been the case for sirolimus and cyclosporine in which the former drug inhibited the MDR-1-mediated efflux of cyclosporine from proximal tubule cells with resultant nephrotoxicity (Anglicheau et al., 2006).

Drug-statin interactions at the OAT3-mediated uptake

step at the basolateral membrane are unlikely to increase plasma exposure to statins given that the regulation of plasma statin levels is dominated by the hepatic clearance of statins (Bergman et al., 2006; Ho et al., 2006).

In summary, we have investigated the handling of rosuvastatin in polarized monolayers of human PTC and DTC. We have shown that rosuvastatin is secreted by monolayers of PTC but is not handled by monolayers of DTC. Characterization of the transport proteins involved in rosuvastatin secretion identified a key role for OAT3 in the basolateral uptake and for MRP2/4 and BCRP in the efflux across the apical membrane of tubular monolayers. At the apical membrane, MDR1 seemed to play only a minor role in rosuvastatin efflux. Taken together, these data suggest that primary cultures of human tubular cells will prove to be an important model with which to gain an understanding of xenobiotic secretion in an intact human system.

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