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Human proximal tubular epithelium actively secretes but does not retain rosuvastatin *

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Running title: Renal tubular rosuvastatin handling

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Abbreviations

HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryl-CoenzymA reductase
OAT: organic anion transporter
OCT: organic cation transporter
MRP: multi-drug resistance associated protein
MDR: multi-drug resistance
ABC: ATP-binding-cassette
PTC: proximal tubular cells
DTC: distal tubular/collecting duct cells
PAH: para-aminohippuric acid
LAP: leucine aminopeptidase
PE: phyco-erythrine
J_{ab}: apical to basolateral flux
J_{ba}: basolateral to apical flux
J_{net}: netto flux
E3S: estrone-3-sulphate
ANOVA: analysis of variance
FTC: fumitremorgin C

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Abstract

Rosuvastatin is a potent HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoenzymA) reductase inhibitor that has proven to be effective in the treatment of dyslipidemia. Rosuvastatin is cleared from the body both by biliary and renal clearance, the latter thought 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 characterise the mechanisms of tubular rosuvastatin secretion in order to define the factors that could influence the presence/concentration of rosuvastatin in proximal tubular cells. Hereto polarised monolayers of primary human tubular cells were used. We found rosuvastatin net secretion across proximal tubule cells, which was saturable (K_{50}=20.4±4.1μM). The basolateral uptake step was rate limiting, and mediated by OAT3 (organic aniontransporter-3). Rosuvastatin efflux at the apical membrane was mediated by MRP2/4 (Multidrug resistance associated protein-2/4) and ABCG2 (ATP binding cassette-G2) together with a small contribution from MDR1 (Multidrug resistance-1 or P-glycoprotein). These data, obtained in an intact human tubule cell model, provide a detailed insight into rosuvastatins renal handling and the possible factors influencing it.

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HMG-CoA reductase inhibitors (statins) are widely employed in the reduction of elevated LDL-cholesterol and prevention of atherosclerotic cardiovascular disease. The action of statins is localised 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 (chemical structure see Mc Taggart *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 3-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.

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To try to understand the mechanisms of xenobiotic secretion in human proximal tubules, we further characterised our mixed and pure primary cultures of human proximal (PTC) and distal tubule/collecting duct cells (DTC), grown as polarised monolayers on filter supports in order to use them for transport studies (Brown et al. 2008, Characterisation of Human Tubular Cell Monolayers As a Model of Proximal Tubular Xenobiotic Handling, Submitted manuscript). Characterisation of the PTC revealed that they express mRNA for proximal tubular transporters including NaPiIIa (inorganic phosphate transporter), SGLT2 (Na^+-dependent glucose transporter 2), OAT1, OAT3 (organic anion transporters 1 and 3), MRP2 multidrug resistant protein (Multidrug resistance associated protein 2), MDR1 (p-glycoprotein), ABCG2 (ATP-binding-cassette-G2 or BCRP), OCT2 and OCTN2 (organic cation transporters), which were absent in DTC. At the protein level we have found 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 analogue 3-O-methyl-D-glucose and albumin were demonstrated (Brown et al. 2008, Characterisation of Human Tubular Cell Monolayers As a Model of Proximal Tubular Xenobiotic Handling, Submitted manuscript) (Verhulst *et al.*, 2004).

In this study we have used these monolayers of primary human mixed and pure PTC and DTC to investigate the renal handling of rosuvastatin.

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Materials and methods

Primary human tubular kidney cell cultures

Human tubular epithelial cells were isolated as previously described (Van der Biest *et al.*, 1994;Helbert *et al.*, 1997;Helbert *et al.*, 1999;Helbert *et al.*, 2001). Briefly, normal human kidney tissue, that 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 cortex and outer stripe of outer medulla (if present) were dissected, cut into pieces of about 1 mm^3 and digested in collagenase D solution (Roche, Ottweiler, Germany), final concentration 0.67mg/ml, in RPMI 1640 media. The suspension was shaken vigorously for 2 h at 37°C then passed through a 120µm 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 (3000rpm, 25 min, 4°C) in a 4x200ml 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 6.5mm 0.4µm pore size polycarbonate permeable (polycarbonate) filter supports (Costar, New York, NY, USA) at a density of 50 000 cells/filter

To obtain pure cultures of PTC and DTC a subconfluent mixed culture was trypsinized and purified by flowcytometric sorting. Cells were incubated for 30 min at 4°C with an anti-human leucine aminopeptidase (LAP) monoclonal antibody. LAP was previously identified in our lab as a marker of proximal tubular cells (Helbert *et al.*, 1999). Subsequently, phyco-erythrin (PE) labeled rabbit $F(ab')_2$ anti-mouse (DAKO, Glostrup, Denmark) secondary antibody was added to the cell suspension.

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Labelled cells were sorted using a FACSvantage flowcytometer (Becton Dickinson, San Diego, CA, USA) into distinct PTC (LAP^+) and DTC (LAP^-) populations.

Mixed and pure cultures of PTC and DTC were grown until confluence (8 to 12 days) on semi-permeable filter supports in α-MEM (Life Technologies, Rockville, MA, USA) modified according to Gibson d'Ambrosio (Gibson d'Ambrosio *et al.*, 1983), supplemented with 10 % fetal calf serum. Cell cultures grown on permeable filter supports are allowed to polarise and have a separated apical and basolateral compartment. Cell culture medium was replaced only once before performing experiments (7 to 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 previously described (Simmons, 1990). Cell monolayers grown on permeable filter supports were extensively washed 4x in a modified Krebs' buffer (mM): NaCl 140; KCl 5.4; $MgSO_4$ 1.2; KH_2PO_4 0.3; NaH_2PO_4 0.3; $CaCl_2$ 2; glucose 5; Hepes 10 buffered to pH 7.4 at 37^0C with Tris base. Filters were then placed in 12-well plastic plates, each well containing 1ml of prewarmed Krebs with unlabelled substrate (rosuvastatin; PAH or mannitol) with a further 0.5ml of identical solution added to the apical chamber. Monolayers were preincubated for 1 hour 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. Additionally monolayers were excluded if the transepithelial resistance of the monolayer corrected for the resistance of the filter was less than 60 $\Omega.cm^2$.

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Flux was initiated by adding [^{3}H]-rosuvastatin (1µCi/ml), [^{3}H]-para-aminohippuric acid (PAH) (1µCi/ml), [^{14}C]- creatinine and either [^{3}H] or [^{14}C] mannitol (0.1µCi/ml) to either the apical or basolateral chamber. A 250ul sample and a 500ul sample were removed from the apical and basolateral chamber respectively after a 60min flux period. To generate a time course, samples were taken from either compartment at 30-minute 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 nmol/cm^2/hr. At the end of the flux period, the remaining solutions were aspirated off and the filters washed 4x in a 500ml 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 determined by liquid scintillation counting. Cellular accumulation of either rosuvastatin or creatinine is expressed as cell/media (c/m) ratio. Cell volume was determined using a geometric approach in which cell volume (V_c) = (($\pi r^2 h$)x 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 4 separate kidneys and an estimated value of 14.8µm 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 uptake of rosuvastatin uptake across the basolateral membrane were measured in almost an identical manner except that cell monolayers were preincubated for 1 hour in Krebs buffer rather than in Krebs buffer + unlabeled substrate. Uptake was initiated by the replacement of Krebs buffer in the basolateral chamber with buffer containing [^{3}H]-rosuvastatin (0.5µCi/ml) and [^{14}C]-

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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 4x in a 500ml 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 determined by liquid scintillation counting.

Study set-up and statistics

Due to the inter-individual variation in transport rates in cell monolayers derived from individual kidney samples (figure 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, range 44 to 89). Data are presented as mean ± SEM. Statistical comparison of mean values was made using a Student's t-test. For multiple comparisons, a one-way analysis of variance (ANOVA) test was employed and significance assigned using a Dunnett post test. Differences in the mean values were considered to be significant when $p \leq 0.05$. Non-linear regression analysis of the data was carried out using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA, USA).

Materials

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

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Rosuvastatin (Ca^{2+} salt) was a gift from AstraZeneca. Estrone-3-sulfate, *para*-aminohippurate (PAH), creatinine, vinblastine, MRK 571were from Sigma-Aldrich (Poole, Dorset, UK). Fumitremorgin C (FTC) was from Calbiochem (Nottingham, UK). All other chemicals were of the highest analytical quality available.

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Results

Time-dependence of rosuvastatin fluxes across human tubular cell monolayers

Steady state unidirectional fluxes of [^{3}H]-rosuvastatin (10µM) were measured at 3 time-points (30, 60 and 90 minutes) 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) (figure 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 towards the apical (luminal) side of the cells. On the basis of the linear relationship between flux and time up to 90 minutes, a 60-minute time point was adopted as standard for subsequent measurements.

Rosuvastatin secretion is restricted to proximal tubule cells

In vivo, rosuvastatin secretion is thought 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 figure 2 clearly demonstrate that the net secretion of rosuvastatin is confined to monolayers of PTC. J_{ab} and J_{ba} of rosuvastatin measured in PTC monolayers differed significantly ($p<0.05$) resulting in a J_{net} of 1037.0±512.0 pmoles/cm^2/h (n=4), while in monolayers of DTC rosuvastatin J_{ab} and J_{ba} showed no asymmetry (p=0.99) and thus no net secretion of rosuvastatin.

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Inter-individual 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 figure 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 pmoles/cm^2/h (mean=603.2±188,35 pmoles/cm^2/h).

Determination of the magnitude of the transcellular component of rosuvastatin secretion

In order to distinguish the transcellular flux of rosuvastatin from the paracellular component of rosuvastatin transport, steady state unidirectional rosuvastatin fluxes were compared to 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 flux secretory flux, J_{ba} of rosuvastatin was 10-fold higher than J_{ba} of mannitol (460.8±112.0 vs 45.1±1.3, p=0.02, n=3). In contrast, the J_{ab} of rosuvastatin was only 2-fold greater than J_{ab} of mannitol (86.5±22.8 vs 33.8±3.5, p=0.2, n=3) (figure 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.

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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 (figure 5). After exposure of the monolayer to rosuvastatin (10µM) for 120 minutes 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 (figure 5). Indeed, after exposure to creatinine (10µM) for 120 minutes 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 membrane. 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, J_{net} of rosuvastatin was measured over a range of rosuvastatin concentrations (0-50µM) in mixed monolayers of PTC and DTC (figure 6). Data indicate the net secretion of rosuvastatin to be saturable. Fitting the data with a least squares non linear regression analysis gave an apparent K_{50} (half-saturation constant) of 20.4±4.1 µM and an apparent V_{max} 4951.0±542.0 pmol/cm^2/h (figure 6).

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Comparison of rosuvastatin and PAH acid fluxes across human tubular cell monolayers

Proximal tubular PAH handling is well characterised. In order 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 (figure 7a). Furthermore, the secretion of PAH was limited to the PTC as there was net flux of PAH in purified DTC monolayers (figure 7a). To investigate whether rosuvastatin shared transport pathways with PAH, the unidirectional fluxes of rosuvastatin (10μM) were measured in the absence and presence of 50μM PAH. In the presence of PAH in the basolateral compartment, J_{ba} of rosuvastatin reduced in magnitude. Although this change did not reach statistically significance ($p>0.05$), addition of PAH to the basolateral chamber resulted in an abolition of net secretion (figure 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 oocytes suggested that rosuvastatin is a substrate for OAT3 but not OAT1 (Windass *et al.*, 2007). In order to elucidate the importance of

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OAT3 in the basolateral handling of rosuvastatin, the effect of estrone-3-sulphate (E3S), a selective substrate of OAT3 (Rizwan A, 2007;Kusuhara *et al.*, 1999), upon initial rates of rosuvastatin uptake were measured in mixed monolayers of PTC and DTC (figure 8). At the basolateral membrane, addition of 100μM E3S significantly reduced the uptake of rosuvastatin into the cells (192.6±4.3 vs 23.4±.4.8, $p<0.0001$, $n=6$). In contrast, addition of E3S to the apical membrane had no significant effect upon 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 upon the unidirectional fluxes of rosuvastatin across mixed monolayers of PTC and DTC. Both vinblastine (50μM) and probenecid (50μM) had no significant effect upon the magnitude of the absorptive flux of rosuvastatin. In contrast, 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 vs 3340.0±100.0 ($p<0.02$) and 1948.0±228.8 ($p<0.01$), respectively). 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 upon fluxes of rosuvastatin were reflected in changes in intracellular concentrations of rosuvastatin at steady state (figure 9b). The most marked effect was found with vinblastine (intracellular rosuvastatin concentration at least 2-fold greater than under control conditions), consistent with 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 effect upon 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) upon the secretory flux of rosuvastatin was tested. The results (figure 10a) show that in the presence of MK571 (50μM) the secretory flux of rosuvastatin (5μM) was reduced to 38.0±6.7% of the control flux ($p<0.001$), equally in the presence of FTC (50μM) the secretory flux of rosuvastatin was 33±4.4% of the flux in the absence of FTC ($p<0.001$). In the presence of vinblastine (50μM) the secretory flux was reduced to 68.7±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 MDR1. Consistent with the inhibitory effects of these agents upon rosuvastatin exit across the apical membrane, intracellular concentrations of rosuvastatin were significantly higher in the presence of, MK571 (12.6±1.8μM, $p<0.01$), FTC (14.1±2.3μM, $p<0.01$) or vinblastine (7.8±0.2 μM $p<0.05$) versus no inhibitor (2.8 ±0.1 μM).

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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 polarised 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 xeniobiotics (Brown et al. 2008, Characterisation of Human Tubular Cell Monolayers As a Model of Proximal Tubular Xenobiotic Handling, Submitted manuscript) 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}) on average ~3-4-fold greater than the apical to basolateral flux (J_{ab}). This is consistent with data on tubular secretion of rosuvastatin in humans (White, 2002). As expected, given that each batch of cell monolayers was derived from an individual kidney, there was some inter-individual variability in the magnitude of rosuvastatin fluxes. However, significant net secretion of rosuvastatin was demonstrated in 100% of kidney samples tested. Mean net secretion of rosuvastatin from 20 kidneys was 603.2±188.4 pmoles/cm^2/h.

Using immuno-separated 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 and OAT3 and BCRP, thought to play a role in rosuvastatin

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secretion. Importantly, 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 immuno-separated 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} of 20.4±4.1μM. 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. Addition of probenecid at the basolateral membrane resulted in abolition of net rosuvastatin secretion. This was mediated by a significant inhibition of rosuvastatin basolateral to apical flux. Probenecid had no significant impact upon rosuvastatin apical to basolateral fluxes. These data are consistent with inhibition of a probenecid sensitive uptake mechanism at the basolateral membrane presumably OAT1 or OAT3 (Rizwan A, 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 Xenopus 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

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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 (figure 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 (Matsushima *et al.*, 2005;Hirano *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 3-5 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

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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 ~ 2.5-2.8. The peak plasma concentration (C_{max}) after a 40mg dose of rosuvastatin is around 40-65nM (Lee *et al.*, 2005;Schneck *et al.*, 2004) several orders of magnitude less than the apparent K_{50} for rosuvastatin uptake by OAT3 (7.4μM) (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 phaseIII studies of rosuvastatin, which included comparisons with placebo, a transient dipstick positive proteinuria was observed in 12% and 4% of subjects taking 80 (above the currently recommended dose of 5 to 40) and 40 mg rosuvastatin, respectively, compared to 3% in placebo (Brewer, 2003) and see slide 24 in 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, inhibit protein uptake by the proximal nephron via inhibition of HMG-CoA reductase and reduced prenylation of proteins involved in endocytosis (Verhulst *et al.*, 2004;Sidaway *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 upon 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 makes 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)

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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 regulation of plasma statin levels is dominated by hepatic clearance of statins (Bergman *et al.*, 2006;Ho *et al.*, 2006).

In summary, we have investigated the handling of rosuvastatin in polarised 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. Characterisation 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 appeared 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 understanding of xenobiotic secretion in an intact human system.

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Footnotes

*

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Figure legends

Figure 1: Time dependence of unidirectional fluxes of rosuvastatin

Unidirectional fluxes of rosuvastatin (10µM) 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±SEM of 4 monolayers per time point from a single experiment representative of 3 separate experiments.

Figure 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 (10µM) in pure proximal tubule cell (PTC) monolayers versus distal tubule/collecting duct cell (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±SEM of 4 monolayers per time point from a single experiment representative of 3 separate experiments.

Figure 3: Variation in rosuvastatin secretion across cell monolayers derived from 20 human kidney samples

Steady state net flux of rosuvastatin (10µM) was measured across cell monolayers generated from 20 different kidney specimens from the Belgium, UK 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 pmoles/cm^2/h, mean: 603.2 pmoles/cm^2/h). The data are expressed as the mean±SEM of 4 monolayers per kidney sample.

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Figure 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.). These data suggest that there is a substantial transcellular secretion of rosuvastatin. The data are expressed as the mean±SEM of 3 monolayers per time point from a single experiment representative of 3 separate experiments

Figure 5: Comparison of steady state cell/media ratios for creatinine and rosuvastatin.

Steady state cell/media ratios were calculated from cells exposed to either 10μM creatinine or rosuvastatin after a 120-minute 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±SEM of 4 monolayers per condition from a single experiment representative of 3 separate experiments.

Figure 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 (0-75μM). At each

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concentration J_{net} was calculated by the subtraction of J_{ab} from J_{ba}. Non linear least squares regression analysis of the data generated an apparent K_m and V_{max} of 20.4±4.1 µM and 4951±542 pmol/cm^2/h, respectively. The data are expressed as the mean±SEM of 3 monolayers per concentration from a single experiment representative of 3 separate experiments.

Figure 7: Comparison of rosuvastatin and PAH fluxes in PCT and DCT cell monolayers. **(A)** The steady state fluxes of PAH (10µM) in proximal (PTC) v distal DTC cell monolayers. The data are expressed as the mean ± SEM of 4 monolayers per condition from a single experiment representative of 3 separate experiments **(B)** The impact of the addition of PAH (50µM) on rosuvastatin steady state fluxes (10µM) in PTC and DTC cell monolayers. PAH reduced J_{ba} of rosuvastatin. Although this change in unidirectional flux did not reach statistically 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±SEM of 4 monolayers per condition from a single experiment.

Figure 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 (100µM), a specific substrate for OAT3, upon the initial uptake rate of rosuvastatin (10µM) 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 upon the relatively low rosuvastatin uptake.

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The data are expressed as the mean±SEM of 4 monolayers per condition from a single experiment representative of 3 separate experiments.

Figure 9: Impact of probenecid and vinblastine upon rosuvastatin fluxes and cell/media ratios (A) The effect of probenecid (50 µM) and vinblastine (50µM) upon steady state fluxes of rosuvastatin (10µM). Both vinblastine and probenecid, significantly inhibit the magnitude of the secretory flux of rosuvastatin (*$p<0.05$ and **$p<0.01$ v control flux). **(B)** Impact of vinblastine (50 µM) and probenecid (50 µM) upon the steady state intracellular concentration of rosuvastatin. The data are expressed as the mean±SEM of 3 monolayers per condition from a single experiment representative of 3 separate experiments.

Figure 10: Pharmacology of the efflux of rosuvastatin across the apical membrane of human tubular cell monolayers (A) The impact of three ABC binding cassette transport inhibitors (50µM) upon the secretory flux of rosuvastatin: all three molecules MK571 (MRP2); Fumetrimorgin C (FTC) (BCRP) and vinblastine (MDR1/MRP) significantly inhibited (***$p<0.001$, ***$p<0.001$ and $p**<0.01$, respectively) the secretory flux of rosuvastatin (5µM). **(B)** Impact of MK571 (50µM); FTC (50µM) and vinblastine (50µM) upon steady state intracellular concentration of rosuvastatin (5µM). 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 ± SEM of 3 monolayers per condition from a single experiment representative of 3 separate experiments.

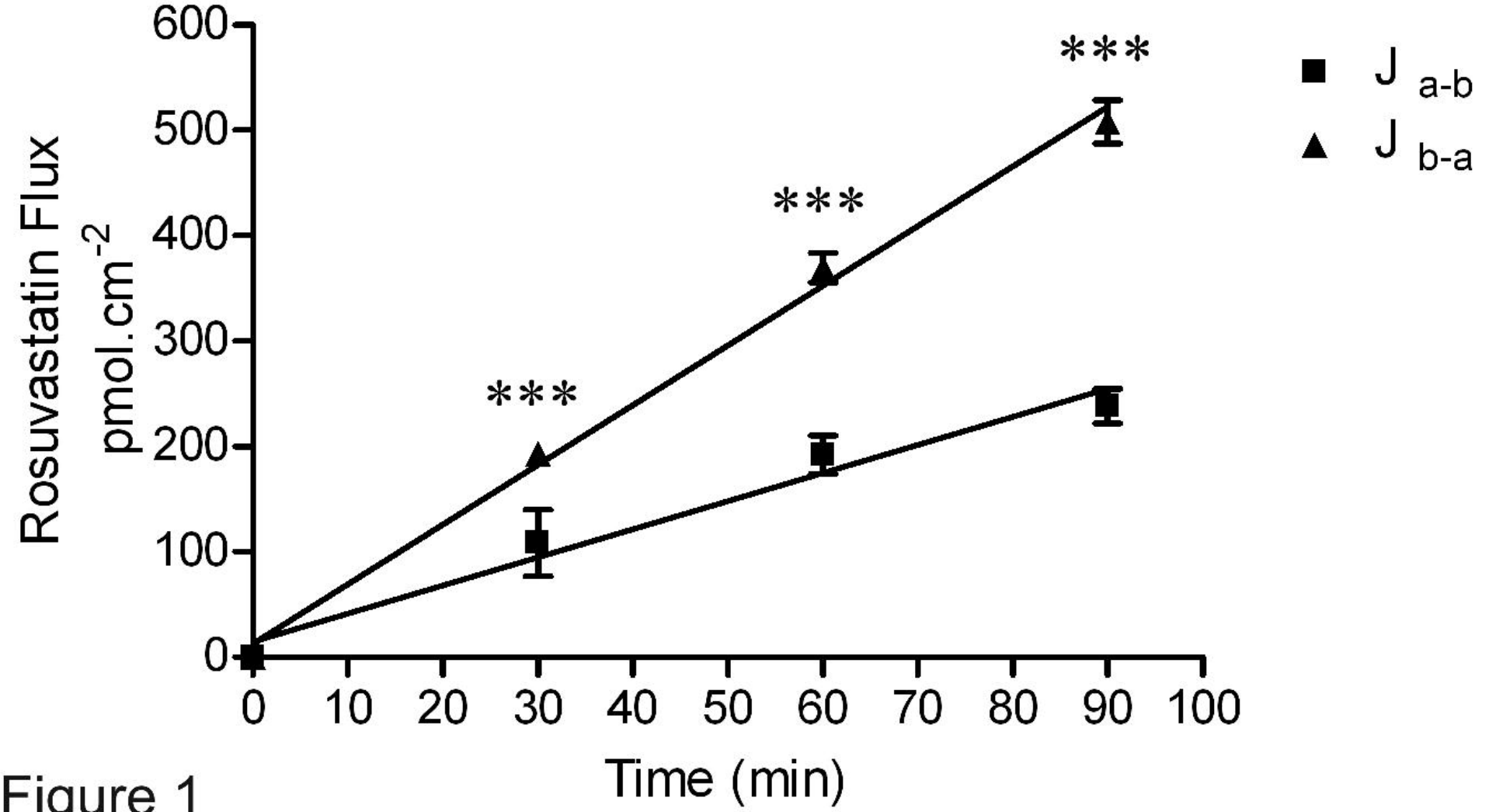
600
500
400
300
200
100
0
Rosuvastatin Flux
pmol.cm-2
***
***
***
J a-b
J b-a
0
10
20
30
40
50
60
70
80
90
100
Time (min)

Figure 1

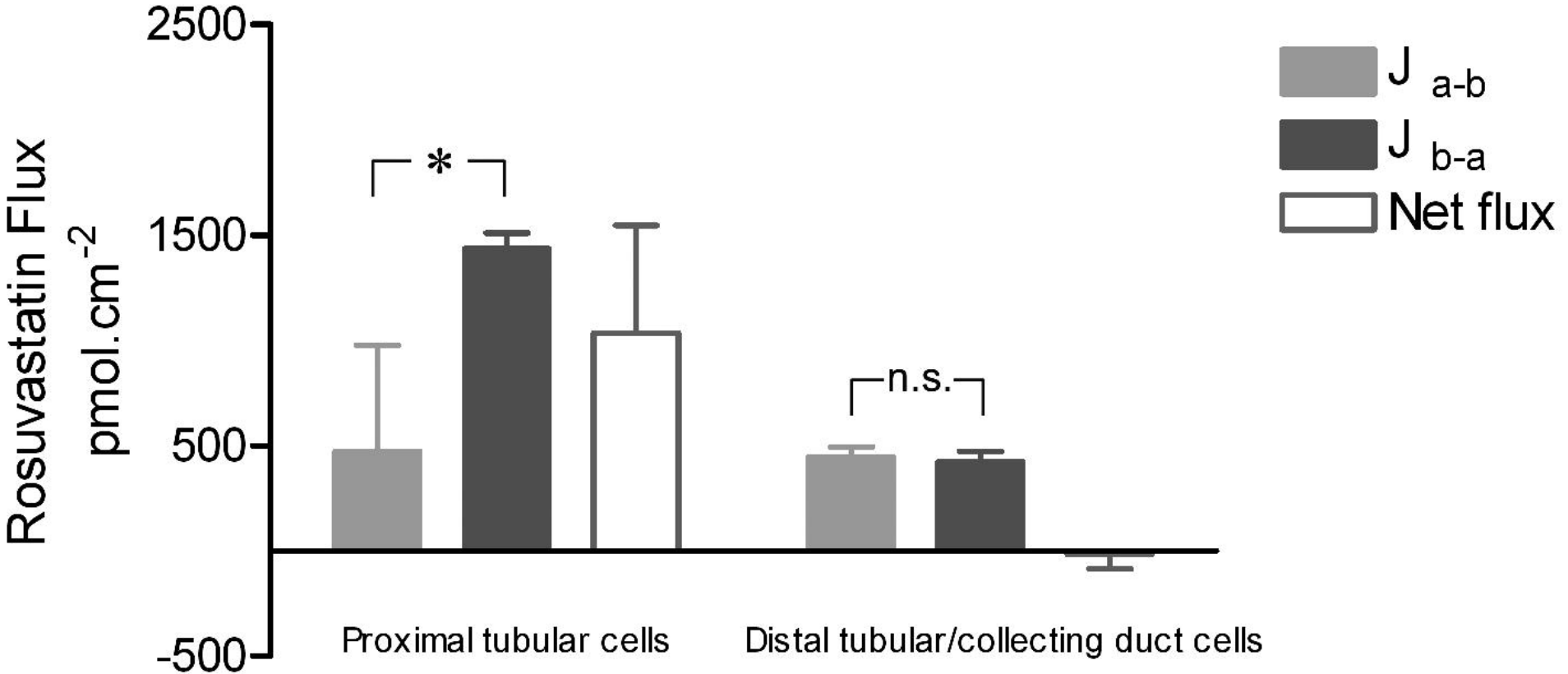
2500
1500
500
-500
Rosuvastatin Flux
pmol.cm-2
*
n.s.
J a-b
J b-a
Net flux
Proximal tubular cells
Distal tubular/collecting duct cells

Figure 2

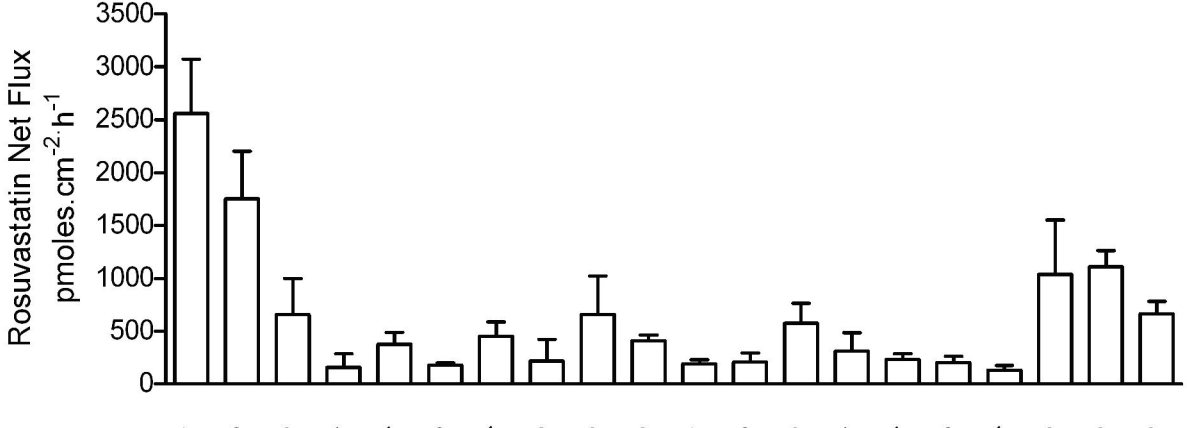
Rosuvastatin Net Flux
pmoles.cm-2.h-1
3500
3000
2500
2000
1500
1000
500
0
Kidney 1
Kidney 2
Kidney 3
Kidney 4
Kidney 5
Kidney 6
Kidney 7
Kidney 8
Kidney 9
Kidney 10
Kidney 11
Kidney 12
Kidney 13
kidney 14
Kidney 15
Kidney 16
Kidney 17
Kidney 18
Kidney 19
Kidney 20

Figure 3

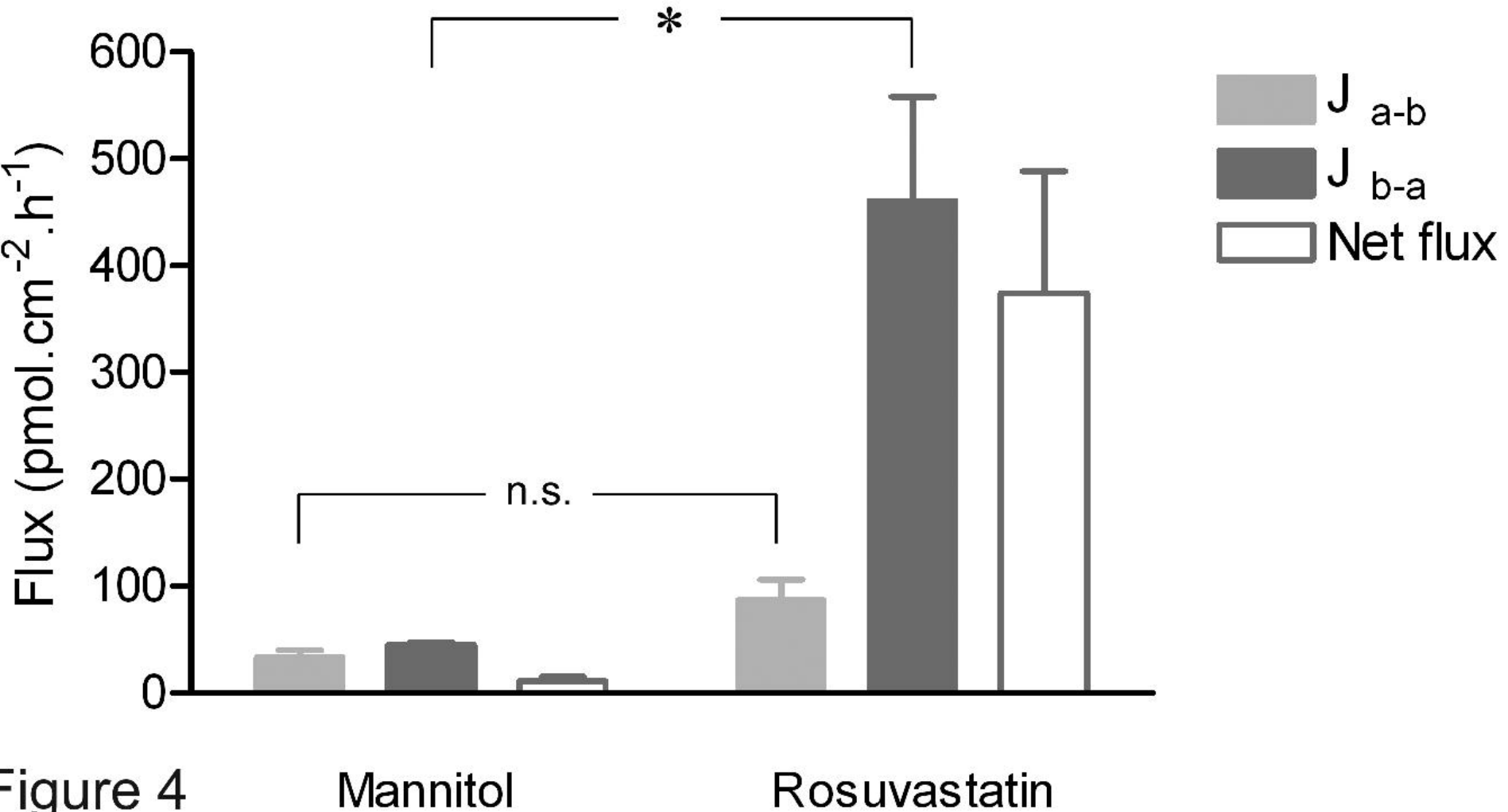
*
n.s.
600
500
400
300
200
100
0
Flux (pmol.cm-2.h-1)
J a-b
J b-a
Net flux
Mannitol
Rosuvastatin

Figure 4

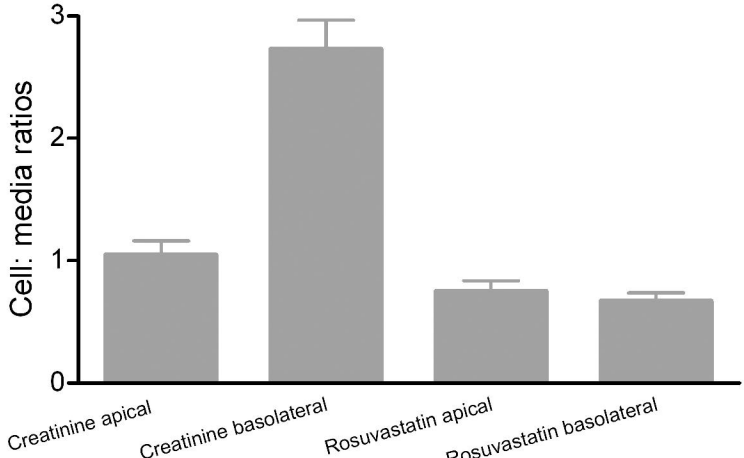
Cell: media ratios
3
2
1
0
Creatinine apical
Creatinine basolateral
Rosuvastatin apical
Rosuvastatin basolateral

Figure 5

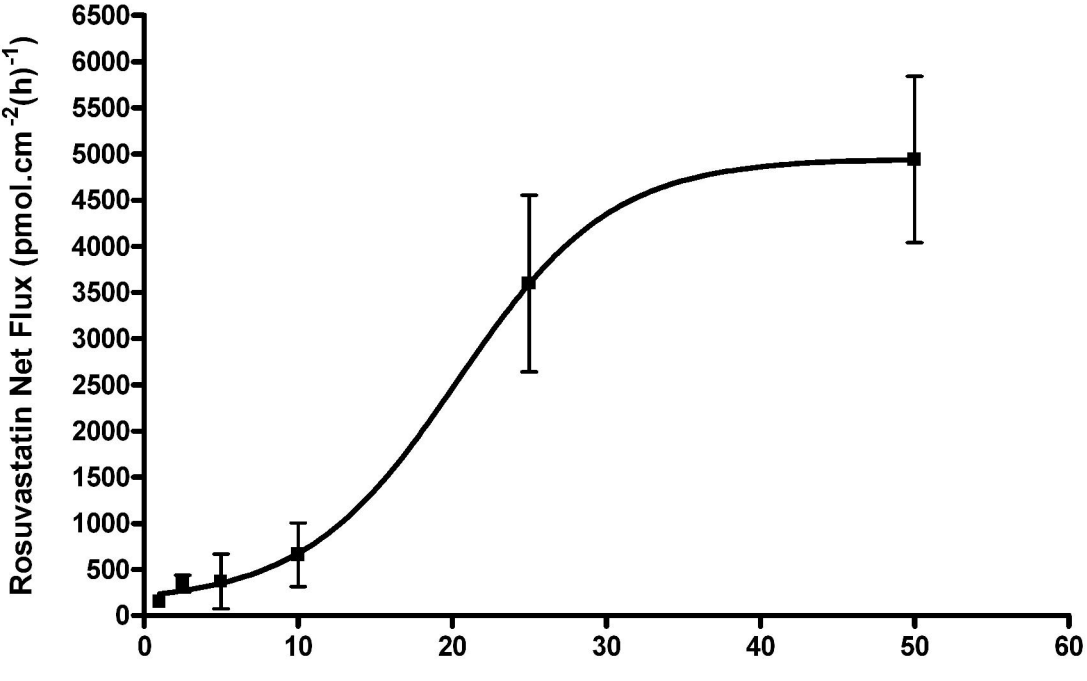
Rosuvastatin Net Flux (pmol.cm-2(h)-1)
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Conc (μM)

Figure 6

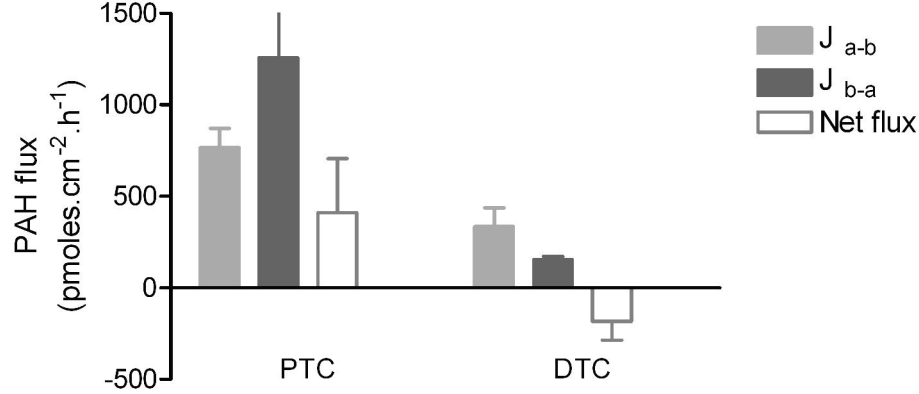

Figure 7a

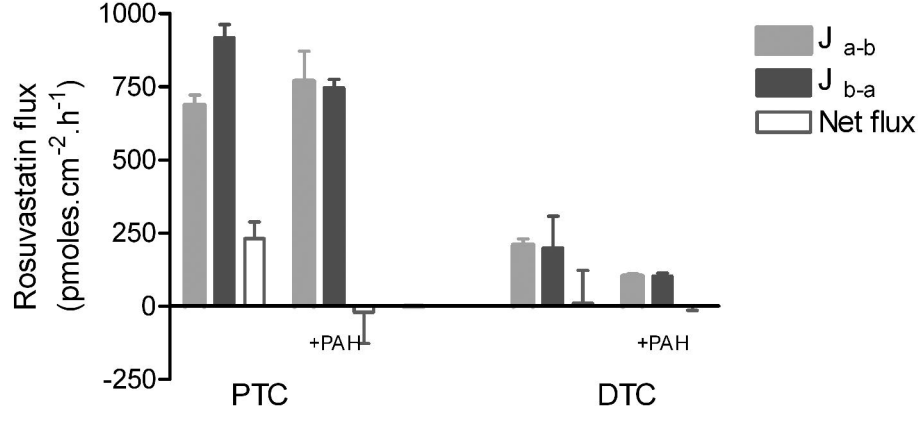

Figure 7b

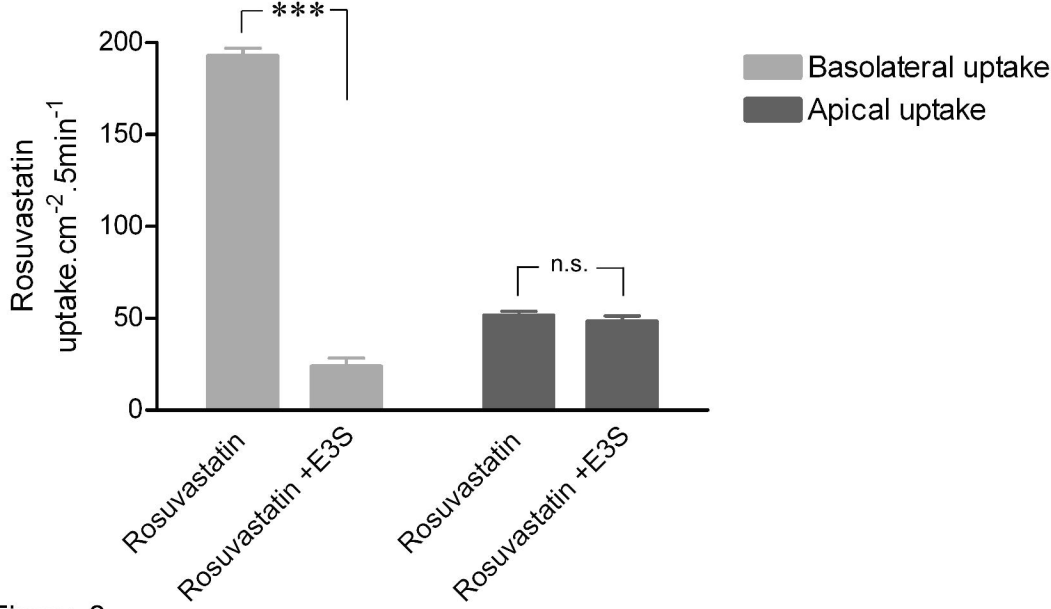
***
n.s.
200
150
100
50
0
Rosuvastatin uptake.cm-2.5min-1
Basolateral uptake
Apical uptake
Rosuvastatin
Rosuvastatin +E3S
Rosuvastatin
Rosuvastatin +E3S

Figure 8

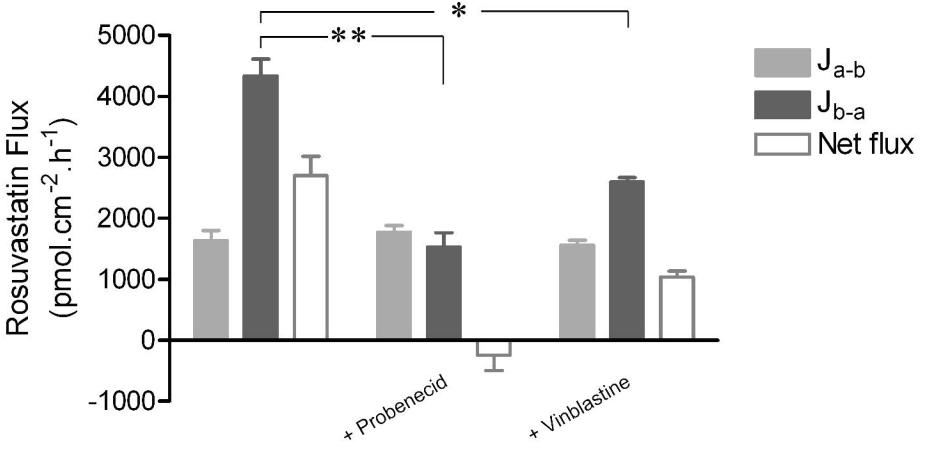

Figure 9a

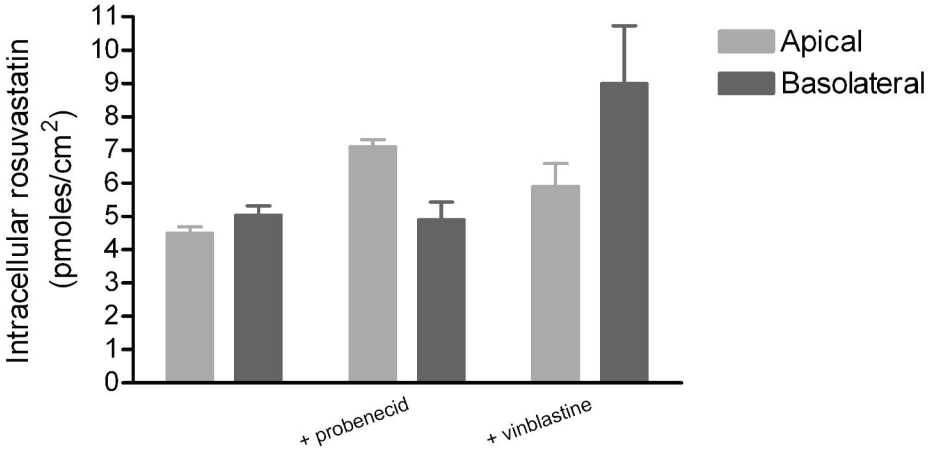

Figure 9b

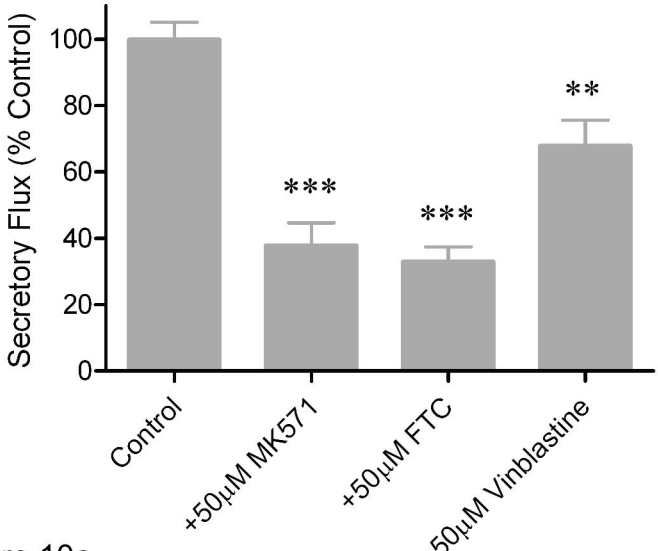

Figure 10a

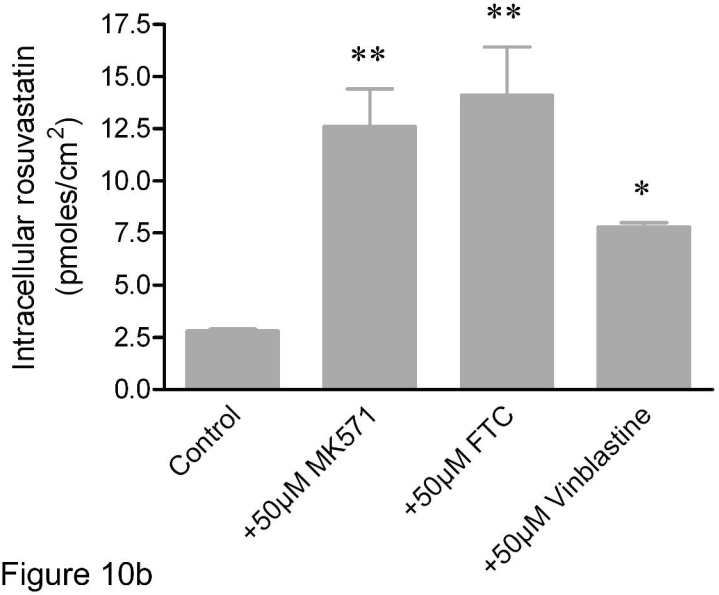

Figure 10b