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P-glycoprotein- and mrp2-mediated octreotide transport in renal proximal tubule

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1 Transepithelial transport of a fluorescent derivative of octreotide (NBD-octreotide) was studied in freshly isolated, functionally intact renal proximal tubules from killifish (*Fundulus heteroclitus*).

2 Drug accumulation in the tubular lumen was visualized by means of confocal microscopy and was measured by image analysis. Secretion of NBD-octreotide into the tubular lumen was demonstrated and exhibited the all characteristics of specific and energy-dependent transport. Steady state luminal fluorescence averaged about five times cellular fluorescence and was reduced to cellular levels when metabolism was inhibited by NaCN.

3 NBD-octreotide secretion was inhibited in a concentration-dependent manner by unlabelled octreotide, verapamil and leukotriene C_4 (LTC_4). Conversely, unlabelled octreotide reduced in a concentration dependent manner the p-glycoprotein (Pgp)-mediated secretion of a fluorescent cyclosporin A derivative (NBDL-CS) and the mrp2-mediated secretion of fluorescein methotrexate (FL-MTX).

4 This inhibition was not due to impaired metabolism or toxicity since octreotide had no influence on the active transport of fluorescein (FL), a substrate for the classical renal organic anion transport system.

5 The data are consistent with octreotide being transported across the brush border membrane of proximal kidney tubules by both Pgp and mrp2.

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Abbreviations: FL, fluorescein; FL-MTX, fluorescein-methotrexate; LTC_4, leukotriene C_4; mdr, multidrug resistance; mrp2, multidrug resistance associated protein2; Pgp, p-glycoprotein

Introduction

Certain ATP-binding cassette (ABC) transporters like p-glycoprotein (p-gp, mdr1-gene product) and multidrug resistance associated protein2 (mrp2) function as ATP-driven drug efflux pumps. In the cells of tissues with excretory and barrier functions, e.g., renal proximal tubule, liver, intestine and brain capillary endothelium, these transporters have been shown to have a polar distribution, which places them in the correct location to drive drugs into urine and bile and prevent entry across the gut and into the central nervous system. As a result, these transporters play a major role by mediating drug uptake, distribution and excretion. They are important determinants of drug effectiveness on the one hand and of drug toxicity on the other hand. Although Pgp and mrp2 have similar distributions in epithelial tissues, their substrate specificities only partially overlap. In general, Pgp interacts with a wide range of uncharged and cationic drugs and mrp2 predominantly recognizes anionic compounds (Gottesman & Pastan, 1993; Oude Eleferink *et al*., 1995; Kusuhara, 1998; Abe *et al*., 1997; Seelig, 1998). Clearly, to be able to alter the uptake and distribution of drugs with difficult pharmacokinetic properties, it is important to know which specific transporter to target. Octreotide, a somatostatin analogue that is a cyclic octapeptide, is used to treat acromegaly and to normalize intestinal function in certain cancer patients. Octreotide pharmacodynamics show low oral bioavailability (Fuessl *et al*., 1987; Köhler *et al*., 1987; Fricker *et al*., 1992), a relatively high excretion (Fricker *et al*., 1994; Harris, 1994) and poor penetration into brain (Jaehde *et al*., 1994) as well as carrier-mediated excretion out of the central nervous system (Banks *et al*., 1990). In addition, recent experiments show that octreotide is actively secreted across the bile canalicular membrane, possibly (but not exclusively) by Pgp (Yamada *et al*., 1998).

In the present study, we used functionally and morphologically intact, isolated renal tubules from the teleost killifish (*Fundulus heteroclitus*) as a tool to identify mechanisms of transepithelial octreotide transport. In this regard, renal tissue from teleost fish offers several important advantages in an intact, functioning epithelium (Miller, 1987; Pritchard & Miller, 1991). Killifish renal tubules are easily isolated and remain viable for hours to days. The killifish nephron consists almost exclusively of proximal tubule, the segment in which the drug transport systems of interest are located. During the isolation, the broken ends of the tubules seal thereby forming a closed, fluid-filled luminal compartment that communicates with the medium only through the tubular epithelium. Thus, the preparation has the correct geometry to facilitate the study of excretory (bath to urinary space) transport. Several studies

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indicate that the xenobiotic transport mechanisms found in teleost tubules (including Pgp and mrp2) are functionally identical to those found in mammalian renal proximal tubules (Masereeuw *et al*., 1996; Miller, 1995; Miller *et al*., 1996; 1997; Miller & Pritchard, 1991; 1997; Pritchard & Miller, 1993; 1996; Schramm *et al*., 1995). Finally, the use of fluorescent substrates and confocal laser scanning microscopy allows the investigation of mechanisms of transport driving drugs across the luminal membrane in the intact tubules. Our recent studies have identified fluorescent substrates and specific inhibitors of Pgp, mrp2 and the classical renal organic anion transport system (Schramm *et al*., 1995; Miller *et al*., 1997; Fricker *et al*., 1999; Gutmann *et al*., 1999).

Here, we used killifish tubules to study the transport of a fluorescent labelled derivative of octreotide (NBD-octreotide). NBD-octreotide was previously used to study the intestinal absorption of octreotide (Fricker *et al*., 1991; Drewe *et al*., 1993) and has been shown to have similar kinetic properties as the unlabelled peptide. The present results show that NBD-octreotide transport from cell to tubular lumen was uphill, metabolism-dependent and specific.

Methods

Chemicals

NBD-Octreotide was synthesized by coupling the fluorescent residue 7-bromo-4-nitrobenzofurazan to octreotide. Fluorescein-methotrexate (FL-MTX) and fluorescein (FL) were obtained from Molecular Probes (Eugene, OR, U.S.A.). The fluorescent cyclosporin A derivative NBDL-CS was synthesized as described previously (Schramm *et al*., 1995). The Pgp blocker SDZ PSC-833 was obtained from Novartis AG, Basle, Switzerland. All other chemicals were obtained from commercial sources at the highest purity available.

Animals and tissue preparation

Killifish (*Fundulus heteroclitus*) were purchased from local fishermen in the vicinity of Mount Desert Island, Maine, and maintained at the Mount Desert Island Biological Laboratory in tanks with natural flowing, aerated sea water. Prior to kidney removal, the animals were decapitated.

Renal proximal tubules were prepared in marine teleost saline (Forster & Taggart, 1950), containing (in mM): 140 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$ and 20 Tris(hydroxymethyl)-aminomethane (TRIS), pH 8.0. Under a dissecting microscope the kidney tissue was teased with fine forceps in order to remove adherent hematopoietic tissue. Individual killifish proximal tubules were dissected and transferred to an aluminum foil-covered, teflon incubation chamber containing 1.5 ml of marine teleost saline with fluroescent compound and added effectors. The chamber floor was a 4×4 cm glass cover slip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted confocal laser scanning microscope. The fluorescent compounds were dissolved in dimethylsulphoxide (DMSO) and added to the incubation medium. Preliminary experiments showed that the concentrations of DMSO used ($<1\%$) had no significant effects on the uptake and distribution of the fluorescent labelled test compounds as measured by confocal and epifluorescence microscopy. HPTLC analysis of tubule extracts showed no degradation of NBD-octreotide, FL-methotrexate and NBDL-CS in the tissue after one hour incubations.

Fluorescence microscopy

The chamber containing the kidney tubules was mounted on the stage of a Olympus Fluo2 inverted confocal laser scanning microscope and viewed through a $40 \times$ water immersion objective (NA = 1.15). The 488-nm laser line, a 510-nm dichroic filter and a 515-nm long-pass emission filter were employed. Low laser intensity (6% of maximum) was used to avoid photobleaching of the dyes. With the photomultiplier gain set to give an average luminal fluorescence intensity of 1500 – 3000 (full scale, 4096), tissue autofluorescence was undetectable. Using image capture and analysis software (NIH Image 1.61) incoming images could be displayed at the video rate on a high resolution computer monitor, and frame averages stored on a Jaz Drive for later analysis. To make a measurement, tubules in the chamber were first viewed under reduced, transmitted light illumination. A suitable field with several tubules was selected and an epi-fluorescence image was acquired by averaging four frames. It has been shown with a similar video microscopy system and glass capillary tubes filled with solutions of known concentrations of fluorescein and other dyes that the relationship between image fluorescence intensity and concentration is approximately linear (Miller & Pritchard, 1991 and Miller, unpublished data). However, because there are uncertainties in relating cellular fluorescence to the actual concentration of an accumulated compound in cells with complex geometry (20; 21), data are reported here as an average measured pixel intensity rather than an estimated concentration of the fluorescent labelled compounds.

Fluorescence intensities were measured from stored images using the Image 1.61 software as described previously (Miller & Pritchard, 1991). From each tubule under investigation, several adjacent cellular and luminal areas (100 – 300 pixels each) were selected. The background fluorescence intensity was substracted and then, the average pixel intensity for each area was calculated. The values used for that tubule were the means for all selected areas.

Statistics

Data are given as means $\pm$ s.e.mean. Comparison tests were performed by ANOVA with Bonferroni adjustment for *post hoc* pairwise comparison probabilities.

Results

Figure 1 shows a representative confocal micrograph of a killifish proximal tubule after 30 min incubation in medium containing 1 μM NBD-octreotide. The fluorescence intensity of the lumen is substantially higher than the cells which, in turn, is higher than the fluorescence intensity of the medium. Figure 2 shows the time course of accumulation of 1 μM NBD-octreotide in tubules. Luminal and cellular fluorescence increased initially, but then reached a steady state value within 30 min. At all times, luminal fluorescence exceeded cellular fluorescence and at 30 – 60 min the lumen to cell fluorescence ratio averaged about six. In the steady state the lumen to medium ratio of NBD-octreotide ranged from 25 – 35. Figure 2 also shows that addition of 1 mM NaCN to the medium substantially decreased luminal fluorescence, but had little effect on cellular fluorescence. After 30 – 60 min luminal fluorescence in NaCN-treated tubules was about equal to cellular fluorescence. The control tubules exhibited the same fluorescence distribution seen previously with a variety of actively excreted fluorescent drugs and drug derivatives

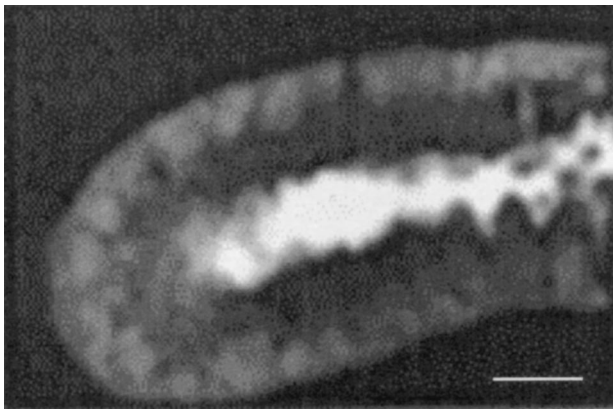

Figure 1 Confocal micrograph showing steady state distribution of NBD-octreotide fluorescence in a killifish renal proximal tubule. The bar represents 10 μM.

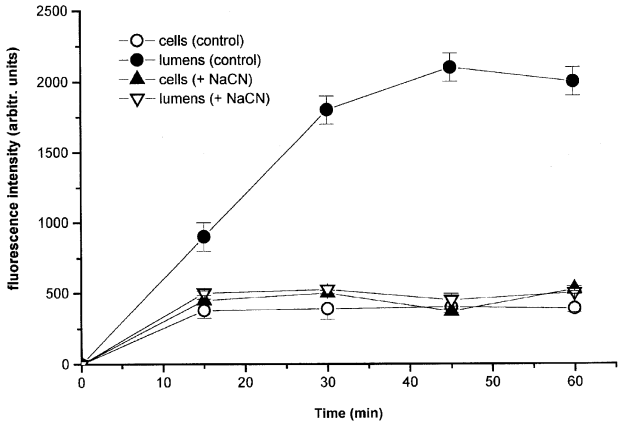

Figure 2 Time course of transport of NBD-octreotide in killifish proximal tubules. Tubular tissue was incubated with 1 μM NBD-octreotide in teleost Ringer solution (means $\pm$ s.e.mean of $n=12$).

(Schramm *et al*., 1995; Miller *et al*., 1997; Gutmann *et al*., 1999; Masereeuw *et al*., 1996). We take this to indicate accumulation of NBD-octreotide within the cells and tubular lumens. The profound effect of NaCN on luminal NBD-octreotide accumulation indicates energy-dependent transport of the peptide from cell to lumen. The absence of effect of NaCN on cellular NBD-octreotide accumulation indicates that uptake by cells was dependent on passive mechanisms, e.g, diffusion and compartmentation.

Addition of unlabelled octreotide to the medium caused a concentration dependent decrease in luminal NBD-octreotide accumulation (Figure 3). The concentration of octreotide causing a 50% reduction in luminal accumulation was between 5 and 10 μM. Cellular accumulation of the labelled drug was not affected, except at the highest concentration of octreotide tested (20 μM caused a 32% decrease, $P<0.05$). In addition, several inhibitors of transport mediated by Pgp and Mrp2 were potent inhibitors of luminal NBD-octreotide accumulation (Figure 4). These included CSA and SDZ-PSC 833 (IC_{50} between 5 and 10 μM), verapamil (IC_{50} about 10 μM) and LTC_4 (IC_{50} between 0.3 and 0.5 μM). In renal proximal tubule, the latter two inhibitors have been shown previously to be specific for p-glycoprotein- and Mrp2-mediated transport, respectively (Masereeuw *et al*., 1996; Gutmann *et al*., 1999). None of these compounds affected cellular NBD-octreotide accumulation (Figure 4).

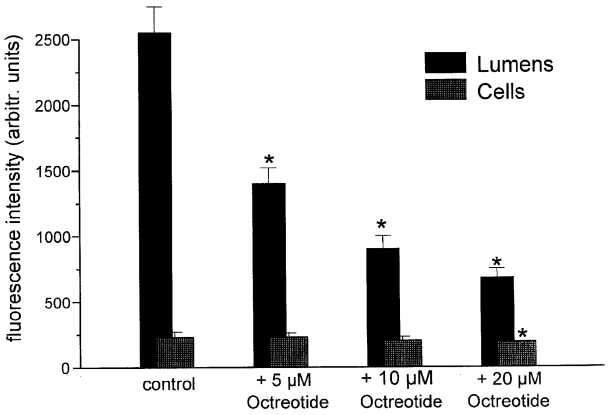

Figure 3 Effects of octreotide on the transport of NBD-octreotide. Tubules were incubated in medium with 1 μM NBD-octreotide without or with the indicated concentration of unlabelled octreotide. Data are given as mean $\pm$ s.e.mean for 10 tubules. Incubation time was 30 min.

Based on substrate and inhibitor specificity studies and immunostaining experiments with mammalian antibodies specific to Pgp and Mrp2, we have found in killifish proximal tubules that cell to lumen transport mediated by Pgp and Mrp2 can be monitored using NBDL-CS and FL-MTX, respectively (Schramm *et al*., 1995; Masereeuw *et al*., 1996, Gutmann *et al*., 1999). Consistent with this, Figure 5 shows that the Pgp inhibitor, verapamil, reduced cell to lumen transport of NBDL-CS, but had no effects on the transport of FL-MTX and that the Mrp2 inhibitor, LTC_4, reduced cell to lumen transport of FL-MTX, but had no effects on transport of NBDL-CS. Neither verapamil nor LTC_4 affected cellular accumulation of NBDL-CS or FL-MTX.

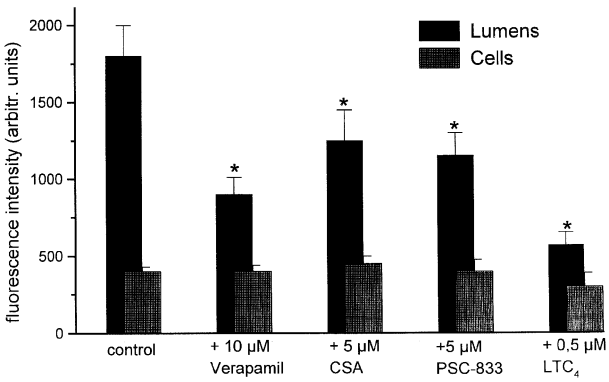

Figure 4 Effects of inhibitors of Pgp and Mrp2 on NBD-octreotide transport. Tubules were incubated in medium with 1 μM NBD-octreotide without (control) or with 10 μM verapamil, 5 μM CSA, 5 μM SDZ PSC-833 or 0.5 μM LTC_4. Data are given as mean $\pm$ s.e.mean for 12 tubules (*significantly lower than controls, $P<0.05$). Incubation time was 30 min.

Figure 6 shows that unlabelled octreotide caused concentration-dependent reductions in the luminal accumulation of NBDL-CS and FL-MTX. For both substrates, the concentration of octreotide causing 50% reduction in luminal accumulation was about 10 μM. Octreotide did not significantly affect the cellular accumulation of FL-MTX or NBD-CSA (Figure 6A,B). In contrast to the results of experiments with NBD-octreotide, NBDL-CS and FL-MTX as substrates, octreotide had no effects at all on the luminal or cellular accumulation of FL. FL is a substrate for the Na-dependent renal organic anion transport system, which is particularly

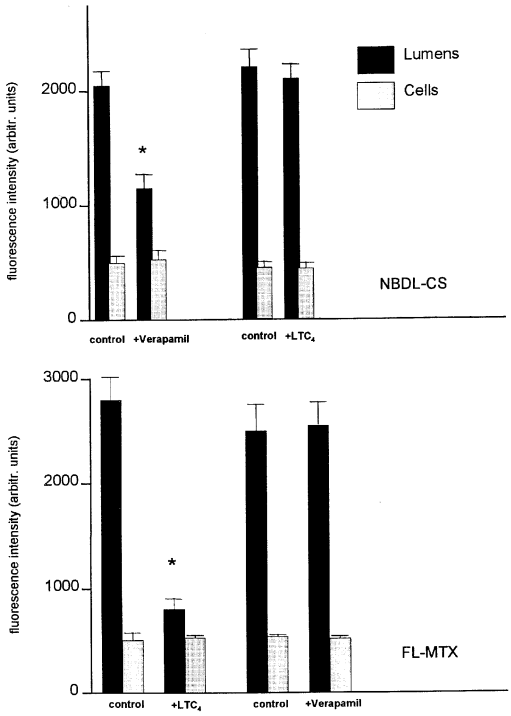

Figure 5 Effects of 10 μM verapamil and 0.3 μM LTC_4 on the transport of NBDL-CS and FL-MTX. Killifish tubules were incubated in medium containing 1 μM NBDL-CS or FL-MTX and LTC_4 or verapamil as additives. Data are given as mean $\pm$ s.e.mean for 15–22 tubules. (*significantly lower than control, $P<0.05$). Incubation time was 30 min.

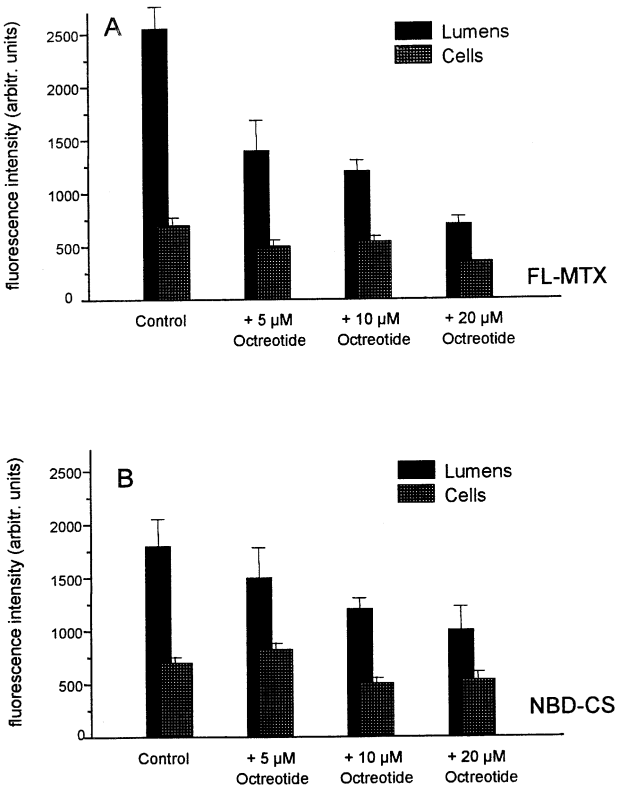

Figure 6 Effects of octreotide on transport of FL-MTX (A) and NBDL-CS (B). Tubules were incubated in medium with 1 μM fluorescent compound without (control) or with the indicated concentrations of octreotide. Data are given as mean $\pm$ s.e.mean for 12 tubules (*significantly lower than controls, $P<0.05$). Incubation time was 30 min.

sensitive to treatments that reduce cell metabolism or viability (Pritchard & Miller, 1993).

Discussion

It was the aim of the present work to study the mechanisms underlying the epithelial transport of the cyclic somatostatin analogue peptide octreotide. Transport of this peptide across epithelial tissues is of special interest for several reasons: First, octreotide is pharmacologically active after oral administration, however its bioavailability is normally below 1% with a perferential absorption site in the upper GI-tract (Fricker *et al*., 1992). One reason for differential absorption in distinct regions of the gut may be an interaction with ATP-dependent excretory systems located in the apical membrane of enterocytes as has been observed for another cyclic peptide, cyclosporin A (Augustjins *et al*., 1993; Fricker *et al*. 1996). Second, octreotide has a rather short plasma half life and a relatively high hepatic and renal elimination (Chanson *et al*., 1993; Harris, 1994; Fricker *et al*., 1994). There is evidence that the peptide is secreted by one or more of the hepatic excretory carrier systems in the bile canalicular plasma membrane (Yamada *et al*., 1996; 1998). Third, small somatostatin analogs permeate the blood–brain barrier only to a neglegible extent (Jaehde *et al*., 1994; Banks *et al*., 1990), most probably due to an interaction with excretory proteins (Kitazawa *et al*., 1998). Finally, 111In-DTPA-conjugated octreotide analogues showed accumulation in the kidney with about 60% injected radioactivity being excreted in the urine by 24 h post injection. Thereby, over 85% of the radioactivity in the urine represented intact peptide (Akizawa *et al*., 1998). The fate of such radiolabelled octreotide derivatives in the kidney is of interest, because they are used for radiodiagnostic purposes in oncology and exhibit high renal accumulation (Bernard *et al*., 1997; de Jong *et al*., 1996; Akizawa *et al*., 1998; Breman *et al*., 1998).

In the present study, the transport of an NBD derivative of octreotide from bath to urinary space was studied in killifish renal proximal tubules, a well-established comparative model for the investigation of carrier-mediated excretory drug transport. NBD octreotide was accumulated by the cells and excreted into the lumen by a concentrative process. Previous studies from our laboratories have demonstrated that, in addition to the classical renal organic anion and organic cation transport systems, these tubules possess both Pgp and Mrp2 (Schramm *et al*., 1995; Miller, 1995; Masereuw *et al*., 1996, Miller *et al*., 1997; Gutmann *et al*., 1999; Fricker *et al*., 1999). Moreover, we have shown, that Pgp, the mdr1 gene product, is able to transport lipophilic cyclic oligopeptides like cyclosporin A in killifish proximal tubules (Schramm *et al*., 1995) and that Mrp2, the multidrug resistance associated protein, recognizes substrates with peptide like structures, like HIV-protease inhibitors (Gutmann *et al*., 1999).

The transport of NBD-octreotide into the luminal space of proximal tubules had all the hallmarks of an active, carrier-mediated process. First, luminal accumulation exceeded cellular accumulation by a factor of 4–6; second, luminal accumulation was reduced to cellular levels when metabolism was inhibited by NaCN; third, luminal accumulation was reduced in a concentration-dependent manner by octreotide itself and by compounds that competitively inhibit transport mediated by Pgp and Mrp2, i.e. CSA, PSC-833, verapamil and LTC_4. In killifish tubules, inhibition by the latter two compounds has been taken as evidence for the involvement of Pgp and Mrp2, respectively (Gutmann *et al*., 1999; Fricker *et al*., 1999).

In contrast, neither NaCN nor any of the other inhibitors of transport had any consistent effects on cellular accumulation of NBD-octreotide. This suggests that cellular accumulation of this fluorescent derivative is passive and non-mediated. As with other lipophillic drugs, e.g., CSA and rapamycin (Schramm *et al*., 1995; Miller, *et al*., 1997), the increase in fluorescence in cells over that in the medium probably reflects accumulation of the drug in cellular membranes and other compartments. The lack of effect of inhibitors on steady state cellular fluorescence indicates that drug efflux to the lumen is not a major determinant of cell levels, as has been found previously for several secreted compounds (Schramm *et al*., 1995; Miller, *et al*., 1997; Gutmann *et al*., 1999).

Consistent with octreotide interacting with both Pgp and Mrp2, we found that unlabelled octreotide was a potent inhibitor of cell to lumen transport of NBDL-CS and FL-MTX. This inhibition was not due to toxicity or non-specific effects, since octeotide had no effects on the transport of the small organic anion, FL. This compound is handled by the classical renal organic anion transport system, which is particularly sensitive to disrupted metabolism or ion gradients (Pritchard & Miller, 1993). Taken together, these data provide evidence that octreotide is a substrate for both Pgp and mrp2. Knowledge of the excretory mechanisms underlying epithelial transport of octreotide may be of relevance to influence the pharmacokinetic properties of this therapeutic peptide. In summary, these date demonstrate that octreotide inhibits drug secretion mediated by p-glycoprotein and Mrp2 and may therefore contribute to clinical relevant drug–drug interactions. The experiments with the fluorescent octreotide derivative suggest that efflux of octreotide in renal proximal tubules may be mediated by p-glycoprotein and Mrp2.

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