

# Interactions of HIV Protease Inhibitors with ATP-Dependent Drug Export Proteins

HEIKE GUTMANN, GERT FRICKER, JÜRGEN DREWE, MICHAEL TOEROEK, and DAVID S. MILLER

*Divisions of Gastroenterology and Clinical Pharmacology, Departments of Internal Medicine and Research, University Clinic (Kantonsspital and Children's Hospital), Basel, Switzerland (H.G., J.D., M.T.); Institute for Pharmaceutical Technology and Biopharmacy, University of Heidelberg, Heidelberg, Germany (G.F.); Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina (D.S.M.); and Mount Desert Island Biological Laboratory, Salsbury Cove, Maine (H.G., G.F., D.S.M.)*

Received January 6, 1999; accepted May 11, 1999

This paper is available online at <http://www.molpharm.org>

## ABSTRACT

We used renal proximal tubules from a teleost fish (killifish; *Fundulus heteroclitus*), fluorescent substrates and confocal microscopy to study the interactions between human immunodeficiency virus protease inhibitors and drug-transporting ATPases. Both saquinavir and ritonavir inhibited luminal accumulation of a fluorescent cyclosporin A derivative (a substrate for P-glycoprotein) and of fluorescein methotrexate [a substrate for multidrug resistance-associated protein 2 (Mrp2)]. Of the two protease inhibitors, ritonavir was the more potent inhibitor of transport by a factor of at least 20. Ritonavir was at least as good an inhibitor of P-glycoprotein- and Mrp2-mediated transport as cyclosporin A and leukotriene C<sub>4</sub>, respectively. Inhibition of P-glycoprotein- and Mrp2-

mediated transport was not due to toxicity or impaired metabolism, because neither saquinavir nor ritonavir inhibited transport of fluorescein on the renal organic anion system. Experiments with a fluorescent saquinavir derivative showed strong secretion into the tubular lumen that was inhibited by verapamil, leukotriene C<sub>4</sub>, saquinavir, and ritonavir. Together, the data demonstrate that saquinavir, and especially ritonavir, are potent inhibitors of P-glycoprotein- and Mrp2-mediated transport. The experiments with the fluorescent saquinavir derivative suggest that these protease inhibitors may also be substrates for both P-glycoprotein and Mrp2.

A major strategy used to fight infection with HIV type 1 (HIV-1) is treatment with protease inhibitors, which are small polypeptide derivatives (Fig. 1). Several of these demonstrate high antiretroviral potency to HIV-1 *in vitro*. However, the low and variable oral bioavailability of protease inhibitors is a significant impediment to their use (e.g., saquinavir has the lowest oral bioavailability of the protease inhibitors, only about 4%). It is becoming clear that several types of mechanism contribute to this problem. Initially, saquinavir's low bioavailability was attributed to metabolism by the cytochrome P-450 3A4 isoform (CYP3A4; Eagling et al., 1997), but recent studies have implicated another process: active extrusion of saquinavir out of enterocytes back into the gut lumen, mediated by the ATP-driven, drug efflux pump, P-glycoprotein (Alsenz et al., 1998; Kim et al., 1998). P-glycoprotein-mediated transport at the blood-brain barrier has also been implicated in the near-exclusion of saquinavir and other protease inhibitors from the central nervous system, an important target of protease inhibitor therapy

(Drewe et al., 1999; Glynn and Yazdanian, 1998). The relevance of these mechanisms to the difficulties encountered during saquinavir treatment is further emphasized by recent studies in which the HIV protease inhibitor ritonavir increased saquinavir plasma concentrations in rats and humans by up to 50-fold (Kempf et al., 1997; Merry et al., 1997). Ritonavir has been shown to be a potent inhibitor of both CYP3A4 activity and P-glycoprotein-mediated transport (Eagling et al., 1997; Alsenz et al., 1998; Koudriakova et al., 1998; Lee et al., 1998).

The present report is concerned with the interactions between HIV-1 protease inhibitors and drug-transporting ATPases. These plasma membrane transport proteins play a major role in determining drug uptake, distribution, and excretion. P-glycoprotein and the recently characterized multidrug resistance-associated proteins (Mrps) were originally discovered as overexpressed proteins in tumor cells with a multidrug-resistant phenotype. Subsequently, P-glycoprotein and the Mrp2 isoform were also found to be at high levels in certain excretory or barrier tissues (e.g., gut, liver, and renal proximal tubule), where their polar distributions within the plasma membrane puts them in the correct orien-

This work was supported by National Institutes of Health Grant ES03828, North Atlantic Treaty Organization Grant CRG 960281, and Deutsche Forschungsgemeinschaft Grant FR1211.

tation to: 1) limit xenobiotic uptake into blood and 2) drive xenobiotic excretion into bile and urine (see, for example, Thiebault et al., 1987; Lieberman et al., 1989; Hsing et al., 1992; Muller and Jansen, 1997; Schaub et al., 1997; Makhey et al., 1998). Although P-glycoprotein and Mrp2 are both members of the ATP-binding cassette family of transporters and share a common distribution in epithelial tissues, they have different specificities. In general, P-glycoprotein transports uncharged and cationic drugs and Mrp2 transports primarily anionic compounds, although there is some overlap of specificities (Elbling et al., 1998; Kusuvara et al., 1998). Along with drug-metabolizing enzymes, these transporters are important determinants of drug effectiveness on the one hand and of drug toxicity on the other hand. In addition, because of their wide specificity limits, these transporters also provide a mechanism, namely competition for transport, by which drugs with very different structures may interact.

In the present study, we used isolated killifish renal prox-

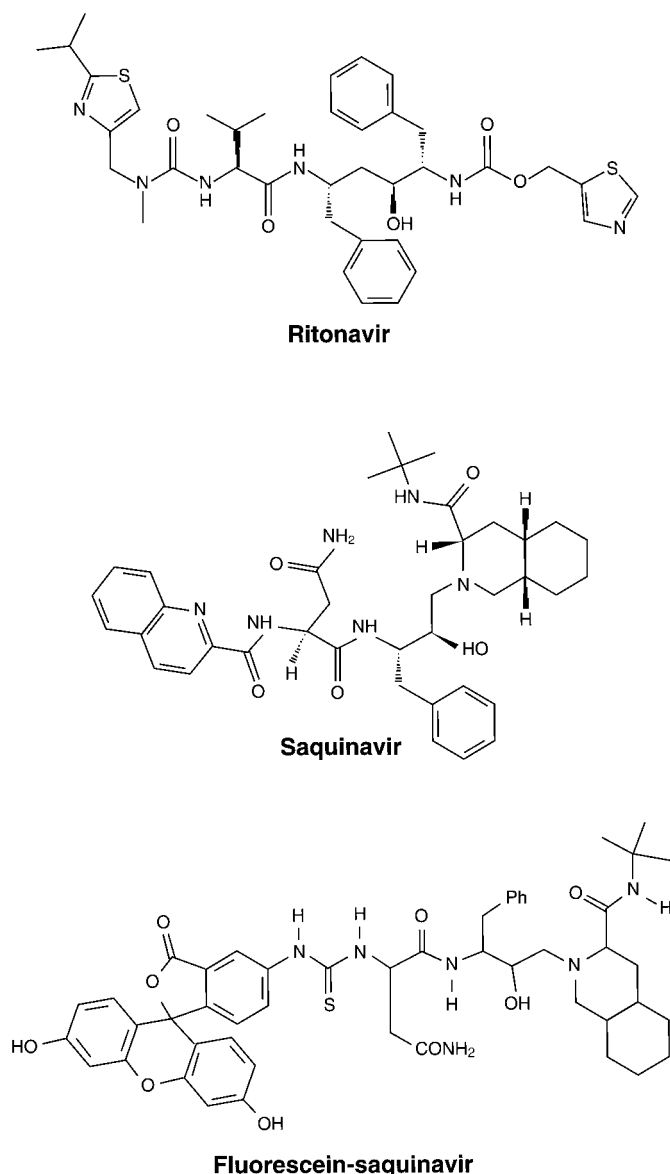
imal tubules, fluorescent substrates, confocal microscopy, and image analysis to assess the interactions between the HIV-1 protease inhibitors saquinavir and ritonavir and drug-transporting ATPases, P-glycoprotein, and Mrp2. Renal tissue from teleost fish offers several important advantages for the study of excretory transport mechanisms in the proximal tubule (Miller, 1987). Proximal tubules are easily isolated with broken ends resealed to form 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 transport. The use of imaging techniques allows us to probe mechanisms responsible for transport at both the basolateral and luminal membranes of the tubular epithelial cells. Moreover, the xenobiotic transport mechanisms found in teleost tubules appear to be identical to those found in mammalian renal proximal tubules. Finally, recent studies have identified fluorescent substrates and specific inhibitors of P-glycoprotein and Mrp2 (Miller, 1995; Schramm et al., 1995; Masereeuw et al., 1996; Miller and Pritchard, 1997; R. Masereeuw and D.S.M., unpublished data), so that the physiology and pharmacology of these transporters may be studied in an intact native epithelium.

Here we report that the HIV-1 protease inhibitors saquinavir and especially ritonavir are potent inhibitors of drug secretion mediated by P-glycoprotein and Mrp2. These are the first data indicating that these compounds can interact with the Mrp family of major drug-transporting ATPases.

## Materials and Methods

**Chemicals.** The HIV protease inhibitors saquinavir, mesylate, and ritonavir, as well as fluorescein saquinavir (FL-Saq), were a kind gift from Dr. H. Wiltshire (Roche Ltd., Lewes, UK). The fluorescent labeled cyclosporin analog [*N*-ε(4-nitrobenzofurazan-7-yl)-D-Lys8]-cyclosporin (NBDL-CSA) was obtained from Novartis Ltd. (Basel, Switzerland), and the fluorescent methotrexate derivative fluorescein-methotrexate (FL-MTX) was obtained from Molecular Probes (Madison, WI). All other chemicals were of analytical grade and were obtained from commercial sources.

**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 aerated, natural flowing sea water. Renal proximal tubules were isolated in marine teleost saline based on the buffer system of Forster and Taggart (1950), containing 140 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, and 20 mM Tris, pH 8.0. Under a dissecting microscope, each kidney was teased with fine forceps 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 fluorescent compound and added effectors. The chamber floor was a 4 × 4-cm glass coverslip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted confocal laser microscope. Fluorescent compounds and inhibitors were added to the incubation medium as stock solutions in dimethyl sulfoxide. Previous experiments have shown that the concentrations of dimethyl sulfoxide used (≤1%) had no significant effects on the uptake and distribution of the fluorescent labeled test compounds as measured by confocal microscopy (Schramm et al., 1995; Masereeuw et al., 1996; D.S.M., unpublished data). Analysis of tubule extracts by HPLC showed no degradation of NBDL-CSA, FL-MTX, or FL-Saq during 60-min transport experiments (Schramm et al., 1995; Masereeuw et al., 1996; H.G., G.F., J.D. and D.S.M., unpublished data).



**Fig. 1.** Structures of HIV-1 protease inhibitors and the FL-Saq derivative used in the present study.

**Confocal Microscopy.** The chamber containing tubules was mounted onto the stage of an Olympus FluoView 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 used. 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 to 3000 (full scale, 4096), tissue autofluorescence was undetectable.

To make a measurement, tubules loaded with fluorescent compound in the chamber were viewed under reduced, transmitted light illumination. A tubule was selected, and an image was acquired by averaging four scans. In previous studies, it has been shown using video and confocal microscopy and glass capillary tubes filled with solutions of known concentrations of fluorescent dyes that the relationship between image fluorescence intensity and dye concentration is linear (Miller and Pritchard, 1991; D.S.M., unpublished data). However, because there are uncertainties in relating cellular fluorescence to the actual concentration of an accumulated compound in cells and tissues with complex geometry (Sullivan et al., 1990; Miller and Pritchard, 1991), data are reported here as average measured pixel intensity rather than as estimated dye concentration.

Fluorescence intensities were measured from stored images using National Institutes of Health Image 1.61 software as described previously (Miller, 1995). From each tubule under investigation, two or three adjacent cellular and luminal areas were selected. The background fluorescence intensity was subtracted, and the average pixel intensity for each area was calculated. The values used for that tubule were the means for all selected areas. Although some fish-to-fish variations in transport ability were noted, most of the differences in measured fluorescence intensities for control tubules reflect changes in photomultiplier settings.



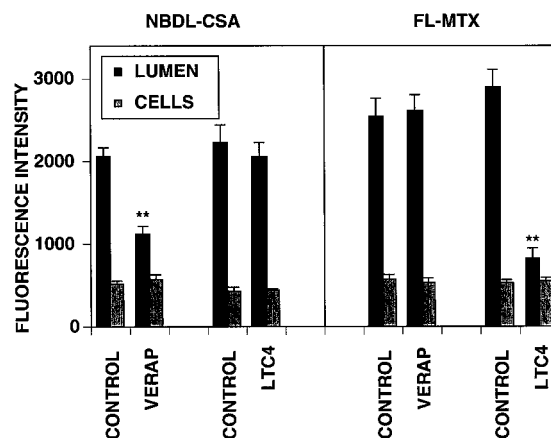
**Fig. 2.** Confocal micrograph showing steady-state distribution of NBDL-CSA fluorescence in a killifish renal proximal tubule. Tubules were incubated for 30 min in medium with 1  $\mu$ M NBDL-CSA, and confocal images were acquired as described in *Materials and Methods*. Notice the intensely fluorescent luminal space and the modest level of fluorescence within the cells. A similar distribution of fluorescence was found for tubules incubated in medium containing FL-MTX.

**Statistics.** Data are given as mean  $\pm$  S.E.. Mean values were considered to be statistically different at a value of  $P < .05$  by use of the appropriate paired or unpaired  $t$  test or by one-way ANOVA.

## Results

**NBDL-CSA and FL-MTX Transport.** The confocal micrograph shown in Fig. 2 demonstrates the basic characteristics of NBDL-CSA and FL-MTX transport in killifish renal proximal tubules. In control tubules, the steady-state distributions of both fluorescent compounds were similar: the lumens were much brighter than the cells, which in turn were brighter than the medium (Fig. 2). Although both compounds exhibited similar steady-state distribution patterns in control tubules, they were differentially affected by compounds that interact specifically with each of the ATP-driven transporters (Fig. 3). For example, verapamil and leukotriene (LT) $C_4$  are potent competitive inhibitors of P-glycoprotein and Mrp2, respectively (Leier et al., 1996; Ling, 1997; Kusu-hara et al., 1998). In agreement with previous experiments using killifish tubules (Schramm et al., 1995; Masereeuw et al., 1996), NBDL-CSA transport from cell to tubular lumen was reduced by 10  $\mu$ M verapamil but not by 500 nM LTC $_4$ ; conversely, LTC $_4$  inhibited cell-to-lumen transport of FL-MTX, but 10  $\mu$ M verapamil was without effect (Fig. 3). Neither inhibitor altered the cellular accumulation of NBDL-CSA or FL-MTX, a result consistent with previous studies (Schramm et al., 1995; Masereeuw et al., 1996) and taken to indicate that events at the luminal membrane do not significantly affect the steady-state cellular accumulation of these fluorescent substrates.

The data in Fig. 3 demonstrate that NBDL-CSA and FL-MTX are handled by different transporters at the luminal membrane of the renal tubule cells. For the fluorescent cyclosporin derivative, these and previous data (Schramm et al., 1995) implicate P-glycoprotein, which has been localized to the luminal membrane of mammalian (Lieberman et al., 1989) and teleost (Sussman-Turner and Renfro, 1994) renal proximal tubule cells. For the fluorescent MTX derivative, the present and previous inhibition studies (Masereeuw et al., 1996; Miller and Pritchard, 1997) are consistent with



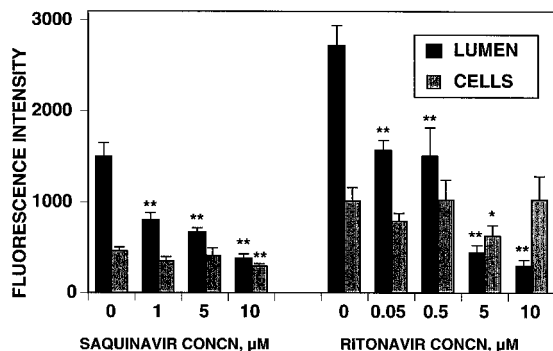
**Fig. 3.** Effects of 10  $\mu$ M verapamil (VERAP) and 0.5  $\mu$ M LTC $_4$  on the transport of NBDL-CSA and FL-MTX. Killifish tubules were incubated for 30 min in medium containing 1  $\mu$ M NBDL-CSA or FL-MTX and the indicated additions. Confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for 15 to 22 tubules. \*\* $P < .01$ , significantly lower than control.

Mrp2-mediated transport. In support of this, recent experiments with killifish renal proximal tubules (R. Masereeuw, F.G. Russel and D.S.M., unpublished data) have shown 1) specific immunolocalization of a mammalian anti-Mrp2 antibody to the luminal membrane of the epithelial cells and 2) strong inhibition of FL-MTX secretion by vanadate, a potent inhibitor of P-type ATPases. Based on these findings, we consider FL-MTX transport from cell to lumen to be mediated by Mrp2.

The addition of protease inhibitors to the medium bathing the tubules caused a profound reduction in the transport of both NBDL-CSA and FL-MTX. Figure 4 shows that saquinavir and ritonavir substantially reduced luminal accumulation of NBDL-CSA. With both protease inhibitors, reductions in NBDL-CSA transport were concentration dependent. Of the two compounds, ritonavir appeared to be the most potent inhibitor, with 0.05  $\mu$ M reducing luminal fluorescence by about 50% ( $I_{50}$  value). Ritonavir did not affect cellular fluorescence, and saquinavir only significantly reduced cellular fluorescence at the highest concentration tested (36% reduction with 10  $\mu$ M, Fig. 4). A similar inhibition pattern was found with FL-MTX as substrate (Fig. 5). Again, both saquinavir and ritonavir reduced luminal accumulation in a concentration-dependent manner. Of the two, ritonavir was by the far more potent inhibitor, with an  $I_{50}$  value of about 0.05  $\mu$ M. As with NBDL-CSA, only the highest concentration of saquinavir significantly reduced cellular fluorescence.

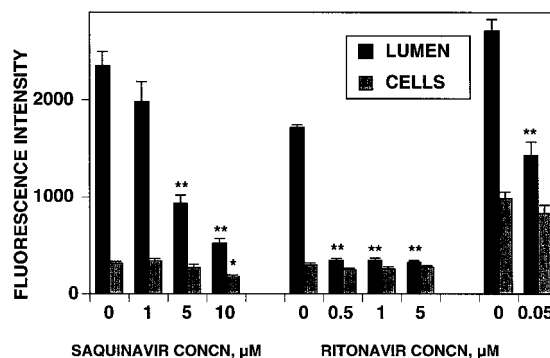
To determine whether the observed reductions in NBDL-CSA and FL-MTX transport by the protease inhibitors were specific, we measured the effects of saquinavir and ritonavir on the uptake and distribution of another fluorescent compound, FL. Previous studies have demonstrated that: 1) FL is actively transported in mammalian and teleost proximal tubule, 2) FL is handled by the classic renal organic anion transport system, and 3) transport through that system is particularly sensitive to treatments that impair metabolism or reduce cellular ion gradients (Miller, 1981; Sullivan and Grantham, 1990; Miller and Pritchard, 1991; Pritchard and Miller, 1993). As shown in Fig. 6, neither saquinavir nor ritonavir, at the same concentrations that substantially inhibited NBDL-CSA and FL-MTX secretion, reduced FL transport.

**FL-Saq Transport.** To determine whether protease inhibitors are substrates for renal P-glycoprotein and Mrp2, we

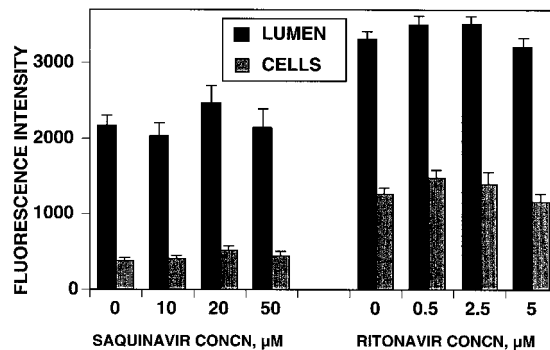


**Fig. 4.** Inhibition of NBDL-CSA transport by saquinavir and ritonavir. Tubules were incubated for 30 min in medium containing 1  $\mu$ M NBDL-CSA and the indicated concentration of protease inhibitor. Confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for 6 to 21 tubules. \* $P$  < .05, \*\* $P$  < .01, significantly lower than control.

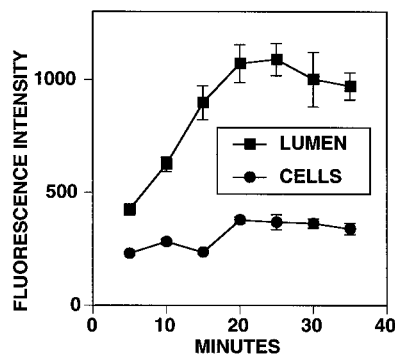
measured the transport of a FL-Saq derivative. Figure 7 shows the time course of FL-Saq accumulation in killifish tubules. FL-Saq was rapidly taken up by the tissue, with a steady-state distribution of the drug attained within 20 min of incubation. At steady state, the lumen-to-cell fluorescence ratio averaged 3 to 5, which is similar to that found for NBDL-CSA and FL-MTX (above). When verapamil or LTC<sub>4</sub> was added to the medium bathing the tubules, steady-state luminal fluorescence was partially reduced (Fig. 8). The effect of verapamil and LTC<sub>4</sub> in combination on luminal fluo-



**Fig. 5.** Inhibition of FL-MTX transport by saquinavir and ritonavir. Tubules were incubated for 30 min in medium containing 1  $\mu$ M FL-MTX with the indicated concentration of protease inhibitor. Confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for 8 to 18 tubules. \* $P$  < .05, \*\* $P$  < .01, significantly lower than control.



**Fig. 6.** Lack of effects of saquinavir and ritonavir on fluorescein (FL) transport in killifish tubules. Tubules were incubated for 30 min in medium containing 1  $\mu$ M FL and the indicated concentration of protease inhibitor. Confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for 9 to 23 tubules.



**Fig. 7.** Time course of NBD-Saq accumulation in killifish renal tubules. Tubules were incubated for the time indicated in medium containing 1  $\mu$ M NBD-Saq, and confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for five tubules.

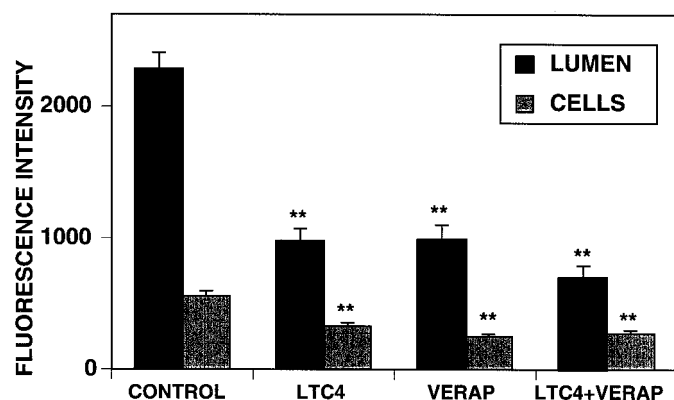


rescence was significantly greater than that of LTC<sub>4</sub> alone (one-way ANOVA; Fig. 8). Both verapamil and LTC<sub>4</sub> also caused a significant reduction in cellular fluorescence. No additional reduction in cellular fluorescence was found when the two inhibitors were used in combination. These data indicate that the fluorescent saquinavir derivative is secreted into the tubular lumen by a process that is uphill and specific and that may involve both P-glycoprotein and Mrp2.

Transport of FL-Saq was also inhibited by both saquinavir and ritonavir. Figure 9 shows significant inhibition of FL-Saq transport into the tubular lumen by 10 to 50  $\mu$ M saquinavir. Only the highest concentration of saquinavir tested significantly reduced cellular FL-Saq accumulation. Ritonavir was a much more potent inhibitor of FL-Saq transport, although the inhibition pattern was more complicated than that for saquinavir (Fig. 9). At 0.05 to 10  $\mu$ M, ritonavir reduced luminal accumulation of FL-Saq in a concentration-dependent manner; we estimate an I<sub>50</sub> value of 0.1  $\mu$ M for this inhibitor. Although 0.05  $\mu$ M ritonavir had no effects on cellular fluorescence, higher concentrations significantly reduced the cellular accumulation of NBD-Saq.

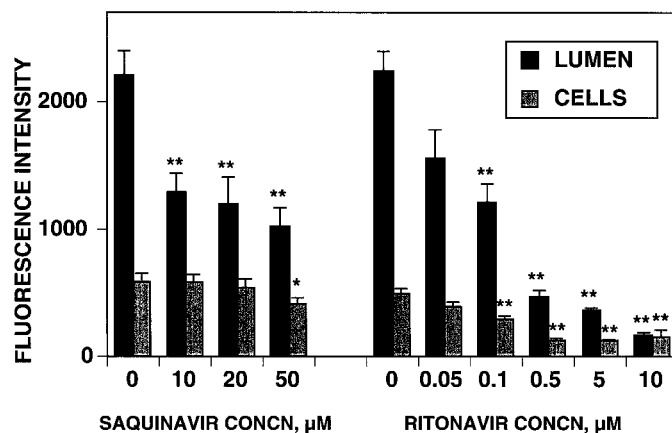
## Discussion

The success of protease inhibitor-based therapies against acquired immunodeficiency syndrome have been limited by a variety of problems; these include not only strict and rigid drug regimens with the potential for noncompliance but also the presence of pharmacodynamic barriers, which limit entry of orally administered drugs from the gastrointestinal tract and access of absorbed drugs to sites of HIV-1 infection in the central nervous system. Although first-pass metabolism by the 3A4 isoform (CYP3A4) of the cytochrome P-450 system may partially account for low bioavailability after oral administration, another important factor affecting drug bioavailability, specific transport, is just beginning to receive attention. Indeed, recent studies implicate P-glycoprotein in the excretory transport of saquinavir and ritonavir in an intestinal cell line in vitro and in the blood-brain barrier in vitro and in vivo (Alsenz et al., 1998; Drewe et al., 1999; Glynn and Yazdanian, 1998; Kim et al., 1998).



**Fig. 8.** Effects of 10  $\mu$ M verapamil (VERAP) and 0.3  $\mu$ M LTC<sub>4</sub> on the transport of NBD-Saq in killifish tubules. Tubules were incubated for 30 min in medium containing 1  $\mu$ M NBD-Saq and the indicated additions. Confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for 19 to 31 tubules. \*\* $P$  < .01, significantly lower than control.

**Interactions with P-Glycoprotein and Mrp2.** Previous studies have established killifish renal proximal tubules as a model system in which to study excretory drug transport mediated by P-glycoprotein and Mrp2 in an intact, native epithelium (Schramm et al., 1995; Masereeuw et al., 1996). This system provides information of two types about interactions of drugs with these transporters: 1) a quantitative measure of the drug's ability to specifically inhibit the transport of model substrates and, thus, interact at the transport level with other drugs handled by that carrier, and 2) an indication of whether the drug itself or a fluorescent analog is transported by P-glycoprotein or Mrp2. The results of the present study show that two protease inhibitors extensively used in anti-HIV-1 therapy were potent inhibitors of the luminal accumulation of fluorescent substrates for both P-glycoprotein (NBDL-CSA) and Mrp2 (FL-MTX). Except for the highest concentration of saquinavir used, this decrease in luminal substrate accumulation was generally seen in the absence of reduced cellular accumulation, indicating that uptake at the basolateral membrane and intracellular sequestration of the two substrates were not affected but that cell-to-lumen transport was reduced. Inhibition of transport was not due to toxicity or inhibition of cellular metabolism because neither saquinavir nor ritonavir, at concentrations that substantially reduced NBDL-CSA and FL-MTX transport, had any effect on the transport of FL, a substrate for the classic renal organic anion system. The organic anion transport system is particularly sensitive to disruption of metab-



**Fig. 9.** Inhibition of NBD-Saq transport by saquinavir and ritonavir. Tubules were incubated for 30 min in medium containing 1  $\mu$ M NBD-Saq and the indicated concentration of protease inhibitor. Confocal images were acquired as described in *Materials and Methods*. Data given as mean  $\pm$  S.E. for 14 to 28 tubules. \* $P$  < .05, \*\* $P$  < .01, significantly lower than control.

**TABLE 1**

Effectiveness of several compounds as inhibitors of P-glycoprotein- and Mrp2-mediated transport in killifish renal proximal tubules

Values were estimated from dose-response curves in Figs. 3 and 4 and from data of Schramm et al., 1995; Masereeuw et al., 1996; and Miller et al., unpublished observations.

	Concentration Causing 50% Inhibition				
	CSA	Verapamil	LTC <sub>4</sub>	Saquinavir	Ritonavir
	$\mu$ M				
P-glycoprotein (NBDL-CSA)	0.2	5–10	>1	1	0.05
Mrp2 (FL-MTX)	2	50–100	0.3	2	0.05

olism or ion gradients (Miller, 1981; Sullivan and Grantham, 1990; Miller and Pritchard, 1991; Pritchard and Miller, 1993). Together, these inhibition data indicate that in renal proximal tubule, both saquinavir and ritonavir inhibit drug transport mediated by both P-glycoprotein and Mrp2. Although it can be inferred from recent transport studies with Caco-2 cell monolayers that saquinavir and ritonavir can be transported by P-glycoprotein and can inhibit P-glycoprotein-mediated transport (Alsenz et al., 1998; Kim et al., 1998), the present data are the first published evidence that these protease inhibitors interact with the second major epithelial drug transporter, Mrp2.

Data from the present and previous experiments with killifish renal proximal tubules provide the first direct comparison of the inhibitory effectiveness of saquinavir and ritonavir with other compounds that interact with P-glycoprotein and Mrp2. They are summarized in Table 1, which lists estimated  $I_{50}$  values for inhibition of NBDL-CSA and FL-MTX transport by saquinavir, ritonavir, CSA, verapamil, and LTC<sub>4</sub>. Note that unlike CSA, verapamil, and LTC<sub>4</sub>, neither saquinavir nor ritonavir was an appreciably better inhibitor of one drug-transporting ATPase than the other. Note also that although the  $I_{50}$  values for saquinavir fell in the middle of the range of values for the compounds tested, ritonavir was clearly one of the best inhibitors of P-glycoprotein- and Mrp2-mediated transport (Table 1). This result is consistent with flux studies using monolayers of Caco-2 cells, which show that unlabeled ritonavir is a better inhibitor of the basal-to-apical transport of <sup>14</sup>C-saquinavir and <sup>14</sup>C-ritonavir than unlabeled saquinavir and that ritonavir is roughly as potent an inhibitor as CSA (Alsenz et al., 1998).

Based on these data, saquinavir and especially ritonavir have the potential to affect the transport of the large number of drugs and metabolites handled by the drug efflux pumps P-glycoprotein and Mrp2. For a large number of therapeutic drugs, both of these transporters are important determinants of uptake from the gut, transport into the central nervous system, and excretion into bile and urine. It follows that inhibition of P-glycoprotein and Mrp2 in these barrier and excretory tissues would increase drug levels in plasma and the central nervous system. Such an effect has been documented for ritonavir, which increases plasma concentrations of coadministered drugs, such as saquinavir (Hsu et al., 1998). Because HIV-1-infected patients may be taking several other drugs during protease inhibitor therapy (e.g., antiviral agents and antibiotics), these interactions should be considered when dose levels are calculated. Moreover, because of its potent inhibition of both drug-transporting ATPases, ritonavir could provide a useful tool to modify the barrier and excretory functions of tissues during therapy with other classes of drugs handled by P-glycoprotein or Mrp2.

**FL-Saq Transport.** Although the NBDL-CSA and FL-MTX inhibition studies provided clear evidence of interactions between the HIV-1 protease inhibitors and P-glycoprotein and Mrp2, the experiments designed to characterize the mechanisms driving transport of the fluorescent saquinavir derivative were more difficult to interpret. Confocal micrographs showed both cellular accumulation and uphill transport of FL-Saq from cell to tubular lumen. Little is known about the renal handling of protease inhibitors. Renal excretion can account for as little as a few percent of administered

dose to as much as 20% (Hilgeroth, 1998). Hsu et al. (1998) reported that the renal clearance of saquinavir in patients is 0.5 liter/h, a value well below the glomerular filtration rate. However, interpretation of this low value is confounded by high (98%) binding of saquinavir to plasma proteins (Hilgeroth, 1998) and the drug's nonpolar nature. Extensive binding in plasma and reabsorption in more distal nephron segments could mask active secretion in proximal tubule.

Luminal accumulation of FL-Saq in killifish tubules was inhibited by both verapamil and LTC<sub>4</sub>, and there was evidence for the additive effects of these two compounds, suggesting cell-to-lumen transport was mediated by both P-glycoprotein and Mrp2. Luminal accumulation of FL-Saq was also inhibited by saquinavir and ritonavir, indicating that the fluorescent saquinavir derivative and the two protease inhibitors share one or more common transport pathways. Of the two protease inhibitors, ritonavir was by far the more potent inhibitor of FL-Saq transport; at 0.1  $\mu$ M, ritonavir inhibited luminal accumulation of FL-Saq by 50%, and at the highest concentration tested (10  $\mu$ M), ritonavir nearly abolished luminal accumulation. However, interpretation of the data was complicated by the observations that in addition to reducing luminal accumulation of FL-Saq, verapamil, LTC<sub>4</sub>, and higher concentrations of saquinavir and ritonavir also reduced cellular accumulation. This could mean that transport of FL-Saq across the basolateral membrane involved more than simple diffusion or that FL-Saq was sequestered intracellularly and the inhibitors altered sequestration patterns. Because there is emerging evidence of renal toxicity associated with protease inhibitor therapy (Rutstein et al., 1997; Witzke et al., 1997), it is of importance to understand the mechanisms that contribute to accumulation in renal tubular cells. Additional studies will be needed to better define these mechanisms.

In summary, the present results for intact renal proximal tubules demonstrate that the HIV-1 protease inhibitors saquinavir and ritonavir are potent inhibitors of drug secretion mediated by P-glycoprotein and Mrp2. The data suggest that saquinavir and ritonavir may be substrates for both P-glycoprotein and Mrp2, a circumstance that could further complicate the search for strategies to improve protease inhibitor bioavailability. Finally, the remarkable ability of ritonavir to block transport on both P-glycoprotein and Mrp2 may imply clinical uses in indications other from HIV treatment, such as reversal of multidrug resistance.

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**Send reprint requests to:** Dr. David S. Miller, Laboratory of Pharmacology and Chemistry, National Institutes of Health/National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709. E-mail: miller@niehs.nih.gov

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