Involvement of P-Glycoprotein in the Transport of Saquinavir and Indinavir in Rat Brain Microvessel Endothelial and Microglia Cell Lines

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Purpose. Membrane-bound efflux transporters, such as P-glycoprotein (P-gp), may limit the brain entry and distribution of HIV-1 protease inhibitors and be in part responsible for HIV-1–associated dementia treatment failure. The purpose of this study was to characterize the transport properties of saquinavir and indinavir in a brain microvessel endothelial cell line and in microglia, the immune cells of the brain and primary HIV-1 cellular target.

Methods. Biochemical and transport studies were performed in an immortalized rat brain endothelial cell line (RBE4), a rat microglia cell line (MLS-9), and a P-gp overexpressing Chinese hamster ovary cell line (CH^RC5).

Results. Western blot analysis using the P-gp monoclonal antibody C219 detected a single band at approximately 170 to 180 kDa (a size previously reported for P-gp) in all cell lines. Cellular accumulation of [^{14}C]saquinavir and [^{3}H]indinavir by RBE4, MLS-9, and CH^RC5 monolayers was significantly enhanced in the presence of P-gp inhibitors, HIV-1 protease inhibitors, the ATPase inhibitor sodium azide, and the ATP depleting agent 2',4'-dinitrophenol respectively. [^{14}C]Saquinavir and [^{3}H]indinavir efflux from both cell systems was rapid and significantly reduced in the presence of PSC833.

Conclusions. These results provide evidence for P-gp mediated transport of saquinavir and indinavir in RBE4 and MLS-9 and suggest that this transporter can restrict, at least in part, the permeation of HIV-1 protease inhibitors at both the brain barrier site and in brain parenchyma.

KEY WORDS: central nervous system; drug transport; indinavir; P-glycoprotein; saquinavir.

INTRODUCTION

It is estimated that 15–20% of individuals with advanced acquired immunodeficiency syndrome (AIDS) will develop dementia, with an annual incidence of 5% (1). In the CNS, HIV-1 primarily infects microglia, the immune cells of the brain, and to a lesser extent astrocytes, leading to a productive and cytopathic infection (2). HIV-1 protease inhibitors (i.e., saquinavir, ritonavir, indinavir, nelfinavir) are often included in highly active antiretroviral therapy (HAART) regimens but permeate poorly into the CNS (3), thereby reducing the ability of pharmacotherapy to contribute to the long-term protection and/or complete reversal of HIV-1–associated dementia (HAD) (4). The suboptimal brain concentrations of HIV-1 protease inhibitors could permit the continued production of HIV-1 and the emergence of drug-resistant viral strains despite adequate plasma concentrations and acceptable systemic antiviral efficacy indicators (5).

One possible mechanism for the low brain permeation of HIV-1 protease inhibitors is the functional expression of ATP-dependent, membrane-bound efflux drug transporters [i.e., P-glycoprotein (P-gp) and multidrug resistance proteins (MRPs)] in various brain cellular compartments (6–8). P-gp, a 170-kDa (1280 amino acid) protein and member of the ATP-binding cassette (ABC) superfamily of drug transporters, is known to play a major role in cellular chemotherapy resistance (9,10). P-gp is a product of the multidrug resistance (MDR) genes MDR1 and MDR2 (also called MDR3) in humans and the mdr1a, mdr1b, and mdr2 genes in rodents. Overexpression of the proteins encoded by MDR1, mdr1a, and mdr1b confer the MDR phenotype (10), whereas the proteins encoded by MDR2 and mdr2 are involved in hepatic phospholipid transport into the bile (11). P-gp is involved in the efflux of many lipophilic and cationic xenobiotics, including anthracyclines, vinca alkaloids, epidophyllotoxins, taxanes, steroids, cardiac glycosides, immunosuppressive agents, and calcium channel blockers (10). Results from *in vitro* studies in intestinal and renal cell lines have shown that the HIV-1 protease inhibitors are substrates as well as inhibitors of P-gp (8,12). Choo *et al.* (8) reported IC_{50} values for the inhibition of P-gp mediated transport in Caco-2 cells of 6.5 μM for saquinavir and 44 μM respectively. *In vivo* studies have also shown that the brain concentrations of HIV-1 protease inhibitors are significantly enhanced (4- to 36-fold) in mice lacking the mdr1a gene (6,7). In addition, studies in wild-type mice have shown that pharmacological inhibition of P-gp by the specific and potent P-gp inhibitor zosuquidar trihydrochloride (LY-335979) enhances the uptake of HIV-1 protease inhibitors into the CNS (8). These data suggest that P-gp may be a significant contributing factor in the low drug accumulation of these antiretroviral drugs in the brain.

The molecular expression and cellular localization of P-gp in the CNS have been primarily investigated at the BBB and the blood-CSF barrier. To date, P-gp has been localized on the subapical side of the choroid plexus epithelia (13), on the luminal membrane of brain microvessel endothelial cells (14), and on the abluminal side of the BBB on adjacent astrocyte foot processes (15). Data from our laboratory has demonstrated the molecular expression, cellular/subcellular localization, and functional expression of P-gp in microglia (16) and in an immortalized rat brain endothelial cell line RBE4 (17).

Although the transport of HIV-1 protease inhibitors has been described in various *in vitro* cell culture systems (i.e., tumor cells, renal proximal tubular cells, intestinal epithelia), studies examining their direct transport properties across the endothelial lining of the brain microvasculature and in brain biological systems relevant to active HIV-1 infection (i.e., microglia) have not been undertaken. The goal of the current study was to characterize and compare, *in vitro,* the transport of saquinavir and indinavir, two commonly prescribed HIV-1

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ABBREVIATIONS: ABC, ATP-binding cassette; DNP, 2′,4′-dinitrophenol; EBSS, Earle's balanced saline solution; HAART, highly active antiretroviral therapy; HAD, HIV-1–associated dementia; MDR, multidrug resistance; MRP, multidrug resistance protein; P-gp, P-glycoprotein.

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protease inhibitors, in a rat brain microvessel endothelial cell line and in microglia.

MATERIALS AND METHODS

Materials

[^{14}C]Saquinavir (3.2×10^{-4} μCi mmol^{-1}) and unlabeled saquinavir were a gift from Roche Products Ltd. (Hertfordshire, UK). [^{3}H]Indinavir (2.0×10^{6} μCi mmol^{-1}) and unlabeled indinavir were a gift from Merck Research Labs (West Point, PA, USA). Ritonavir was purchased from Moravek Biochemicals (Brea, CA, USA). Anti-actin (clone AC-40), 2′,4′-dinitrophenol (DNP), and sodium azide were purchased from Sigma-Aldrich (Oakville, ON, Canada). Cyclosporin A and PSC833 were a generous gift from Novartis Pharma (Basel, Switzerland). The P-gp monoclonal antibody C219 was purchased from ID Labs (London, ON, Canada).

Cell Culture

RBE4, an immortalized rat brain microvessel endothelial cell line kindly provided by Dr. F. Roux (Hôpital Fernand Widal, Paris, France), was prepared from second-passage rat brain endothelial cell cultures by transfection with the plasmid pE1A *neo* (18). This plasmid contains the entire E1A region of adenovirus 2, which confers immortalization without oncogenic transformation, and the *neo* gene for resistance to geneticin (aminoglycoside G418) (19,20). This cell line displays a phenotype that is consistent with brain endothelial cells *in vivo* (19). We have recently shown that, in culture, the cell line forms a confluent monolayer and displays typical morphology of brain endothelial cells (i.e., tight junctions) (17).

The MLS-9 cell line was kindly provided by Dr. L. C. Schlichter (Department of Physiology, University of Toronto, Toronto, Canada). This cell line was prepared from cultured microglia isolated from the neopallia of 2- or 3-day-old Wistar rats as previously described (21). Briefly, cultures that were greater than 98% pure microglia, as judged by labeling with isolectin B4, were induced to proliferate by the addition of a growth factor (i.e., colony-stimulating factor-1). After several weeks, the colonies were harvested and grown in the absence of growth factor. The MLS-9 cell line was established from one of these colonies. The MLS-9 cell line displays the typical morphology of rat microglia *in vivo* (i.e., spheroid cells with short surface villi) and expresses characteristic biochemical markers of microglia, including 100% staining with isolectin B4 and 98% staining with the OX-42 antibody (16,22).

Both brain cell lines (RBE4 passages 32–43; MLS-9 passages 9–22) were grown as a monolayer on 75 cm^2 untreated polystyrene cell culture flasks (Sarstedt, St. Leonard, PQ, Canada). Prior to plating, all the flasks for the RBE4 cell line were coated with type I rat tail collagen (Sigma-Aldrich). All cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. In both cell lines, culture medium was replaced every 2–3 days. The RBE4 cell line was fed with alpha-minimum essential medium/HAMS-F10 nutrient mixture (1:1 v/v) supplemented with L-glutamine (2.0 mM), geneticin (300 μg ml^{-1}), human fibroblast growth factor (1.0 ng ml^{-1}), gentamicin (50 μg ml^{-1}) and 10% (v/v) fetal bovine serum. Confluent cultures were subcultured with a 0.25% trypsin/EDTA solution. The MLS-9 cell line was grown in minimum essential medium, pH 7.2, containing L-glutamine (2.0 mM) and D-glucose (5.6 mM) and supplemented with 0.5% (v/v) penicillin-streptomycin suspension, 5% (v/v) horse serum, and 5% (v/v) fetal bovine serum. Confluent cultures were subcultured with a sodium citrate solution containing 130 mM NaCl, 15 mM sodium citrate, 10 mM D-glucose, and 10 mM HEPES, pH 7.4.

The P-gp overexpressing Chinese hamster ovary cell line selected for resistance to colchicine (CH^{R}C5) was kindly provided by Dr. V. Ling (University of British Columbia, Vancouver, Canada). This cell line (passages 4–13) was maintained at 37°C in the presence of 95% air and 5% CO_2. Culture medium consisted of α-minimum essential medium, pH 7.2, containing L-glutamine (2.0 mM), and supplemented with 0.5% (v/v) penicillin-streptomycin suspension and 10% (v/v) fetal bovine serum. Confluent cell monolayers were subcultured with a 0.1% (v/v) trypsin/EDTA solution.

Western Blotting

Crude membranes from cultured RBE4, MLS-9, and CH^{R}C5 cells were prepared by centrifuging the cell suspension at 400*g* at 4°C for 10 min. The supernatant was discarded, and the pellet was lysed for 30 min at 4°C in 250 mM sucrose buffer containing 1.0 mM EDTA and 0.1% (v/v) protease inhibitor cocktail (Sigma-Aldrich). The cell suspension was incubated for 15 min at 4°C and then homogenized in a Dounce homogenizer at 10,000 rpm for 3 cycles of 20 s each. Homogenates were collected and centrifuged at 3000*g* for 10 min to remove cellular debris. The supernatant was collected and centrifuged at 100,000*g* for 1 h at 4°C. The resultant pellet was resuspended in 10 mM Tris buffer, pH 8.8, and frozen at -20°C until further use. Protein concentrations of the crude membrane preparations were determined by the Bradford protein assay method.

For immunoblotting, 50 mg aliquots of crude proteins were mixed in Laemmli buffer and resolved on an 8.5% sodium dodecyl sulfate-polyacrylamide gel. The gel was then electrotransferred onto a polyvinylidene difluoride membrane. Protein transfer was verified by Ponceau S staining. The membranes were blocked overnight at 4°C in Tris-buffered saline (15 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% (v/v) Tween-20 (TBS-T) and 5% (m/v) dry skim-milk powder. Following six washes (5 min each) with TBS-T, the membrane was incubated with the monoclonal P-gp antibody (C219; 1:1000 dilution in 5% milk) for 4 h at room temperature for P-gp detection, or the anti-actin antibody (AC-40, 1:500 dilution in 5% milk) for 4 h at room temperature for β-actin detection. Following a second wash, the membranes were incubated for 2 h in the presence of anti-mouse (1:5000) horseradish peroxidase–linked secondary antibodies (Serotec Inc., Raleigh, NC, USA) in 5% milk at room temperature. Protein bands were detected by enhanced chemiluminescence and exposed to X-ray film for 1 min. The CH^{R}C5 cell line was used as a positive control.

Functional Studies

Uptake and efflux studies were performed on confluent monolayers of RBE4 (passage 34–43), MLS-9 (passage 9–22), or CH^{R}C5 (passage 4–13) cells grown on 48-well polystyrene plates (Becton-Dickinson, Franklin Lakes, NJ, USA). The accumulation of [^{14}C]saquinavir and [^{3}H]indinavir was determined by a method previously developed in our laboratory (16,22). Briefly, the cells were washed and incubated at 37°C

for 30 min in Earle's balanced saline solution (EBSS) containing 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 138 mM NaCl, 1.0 mM Na_2HPO_4, 5.5 mM D-glucose, and 20 mM HEPES. The cells were then incubated for the desired time (i.e., 2 min, 5 min, 15 min, 30 min, 60 min, or 120 min) using [^{14}C]saquinavir (0.78 μM) or [^{3}H]indinavir (0.38 μM) in the presence or absence of standard P-gp inhibitors (50 μM cyclosporin A, 1.0 μM PSC833) or HIV-1 protease inhibitors (10 μM saquinavir, 50 μM indinavir, 10 μM ritonavir). At each time point, the incubation medium was aspirated, and the reaction was terminated with 750 μl of ice-cold 0.16 M NaCl solution. The cells were then solubilized with 375 μl of 1.0 N NaOH for 30 min and transferred to 7.0 ml scintillation vials containing 187.5 μl HCl (2.0 N) to neutralize the NaOH. [^{14}C]Saquinavir and [^{3}H]indinavir cellular accumulation were measured by a Beckman liquid scintillation counter with automated quench correction. All samples were corrected for "zero-time" and background radioactivity. Accumulation of [^{14}C]saquinavir and [^{3}H]indinavir were standardized to the protein concentration (mg ml^{-1}) determined by the Bradford colorimetric method using bovine serum albumin (Sigma-Aldrich) as the standard. Saquinavir cellular accumulation was expressed as micromoles per milligram of protein (μmol mg^{-1} protein). Indinavir cellular accumulation was expressed as picomoles per milligram of protein (pmol mg^{-1} protein).

Efflux by cultured RBE4 and MLS-9 cells was determined by incubating the cells for 30 min at 37°C with 0.78 μM [^{14}C]saquinavir or 0.38 μM [^{3}H]indinavir dissolved in standard EBSS incubation medium. The medium was then aspirated, and the cells were rapidly washed with 187.5 μl of ice-cold 0.16 M NaCl. The incubation medium alone or containing a P-gp inhibitor (i.e., 1.0 μM PSC833) was added for the desired times. After each interval, the incubation medium was removed, and the reaction was terminated by washing with 750 μl of ice-cold 0.16 N NaCl. The cells were solubilized with 1.0 N NaOH for 30 min. Efflux was calculated from the remaining cellular [^{14}C]saquinavir or [^{3}H]indinavir content.

The energy dependence of P-gp transport in cultured RBE4 and MLS-9 cells was investigated by measuring accumulation in the presence of 10 mM sodium azide, an ATPase inhibitor, or 5 mM DNP, an ATP depleting agent. The cells were incubated for 30 min with 187.5 μl of EBSS, pH 7.4, or with 187.5 μl of EBSS containing 5.5 mM mannitol (replacing D-glucose) and 10 mM sodium azide, pH 7.4, or 5 mM DNP, pH 7.4. Incubation medium containing [^{14}C]saquinavir or [^{3}H]indinavir, with or without sodium azide or DNP, was added for the desired times. After each time point, the incubation medium was aspirated, the reactions were stopped with ice cold 0.16 M NaCl, and the cells were solubilized with 1.0 N NaOH.

Data Analysis

Results are reported as a mean ± SD from a minimum of three separate experiments performed in monolayer cells pertaining to different passages. In an individual experiment, each data point represents quadruplicate trials. To determine the significance of transport inhibition, the Student's *t* test was used for unpaired experimental values. For multiple comparisons, ANOVA and/or the post hoc multiple-comparison Bonferroni *t* test was used. A value of at least $p < 0.05$ was considered to be statistically significant.

RESULTS

Protein Expression

Western blot analysis in RBE4 and MLS-9 cells using the monoclonal P-gp antibody, C219, detected a single band at approximately 170 to 180 kDa, a molecular weight previously reported for P-gp in CH^{R}C5 cells (9) (Fig. 1). The C219 antibody recognizes a highly specific intracellular epitope (amino acid sequence: VQEALD) on all isoforms of P-gp (23). The CH^{R}C5 cell line, an established P-gp overexpressing cell line selected for colchicine resistance (9), served as our positive control, and a single robust band was detected at the expected molecular weight. A single band of approximately 43 kDa corresponding to β-actin was detected in each lane suggesting that an appropriate amount of protein was loaded for each sample.

Functional Studies

The time course of saquinavir and indinavir accumulation at 37°C by RBE4, MLS-9, and CH^{R}C5 cells (Figs. 2A, 2B, 2C, 3A, 3B, 3C) shows increasing uptake until a steady state is reached by approximately 1 h. Accumulation was significantly enhanced in the presence of 1.0 μM PSC833, a non-immunosuppressive cyclosporin A analog and potent P-gp inhibitor, in all three cell lines.

Saquinavir efflux (expressed as the remaining cellular content of [^{14}C]saquinavir) by the RBE4 and MLS-9 monolayer cells was rapid and the remaining intracellular concentration of saquinavir was significantly enhanced in the presence of 1.0 μM PSC833 (Figs. 4A, 4B). Indinavir efflux was also rapid in both the RBE4 monolayer cells and in MLS-9. Efflux was significantly reduced ($p < 0.05$) by 1.0 μM PSC833 at 5 min and 15 min in the RBE4 monolayer cells (Fig. 5A). In MLS-9, the remaining intracellular content of indinavir was slightly enhanced by 1.0 μM PSC833 but the results did not reach statistical significance (Fig. 5B).

Because it is well established that P-gp–mediated transport is dependent on the cellular production of ATP, we measured the accumulation of saquinavir and indinavir in the

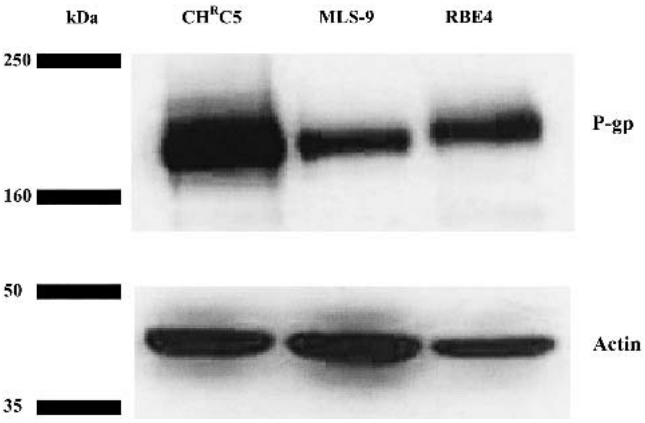

Fig. 1. Western blot analysis of P-gp in cultured RBE4, MLS-9, and CH^{R}C5 cells. Crude membrane preparations (50 μg) from CH^{R}C5 cells (lane 1), MLS-9 cells (lane 2), and RBE4 cells (lane 3) were resolved on an 8.5% sodium-dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blot was incubated with the monoclonal P-gp antibody C219 (1:1000 dilution) and the anti-actin antibody AC-40 (1:500).

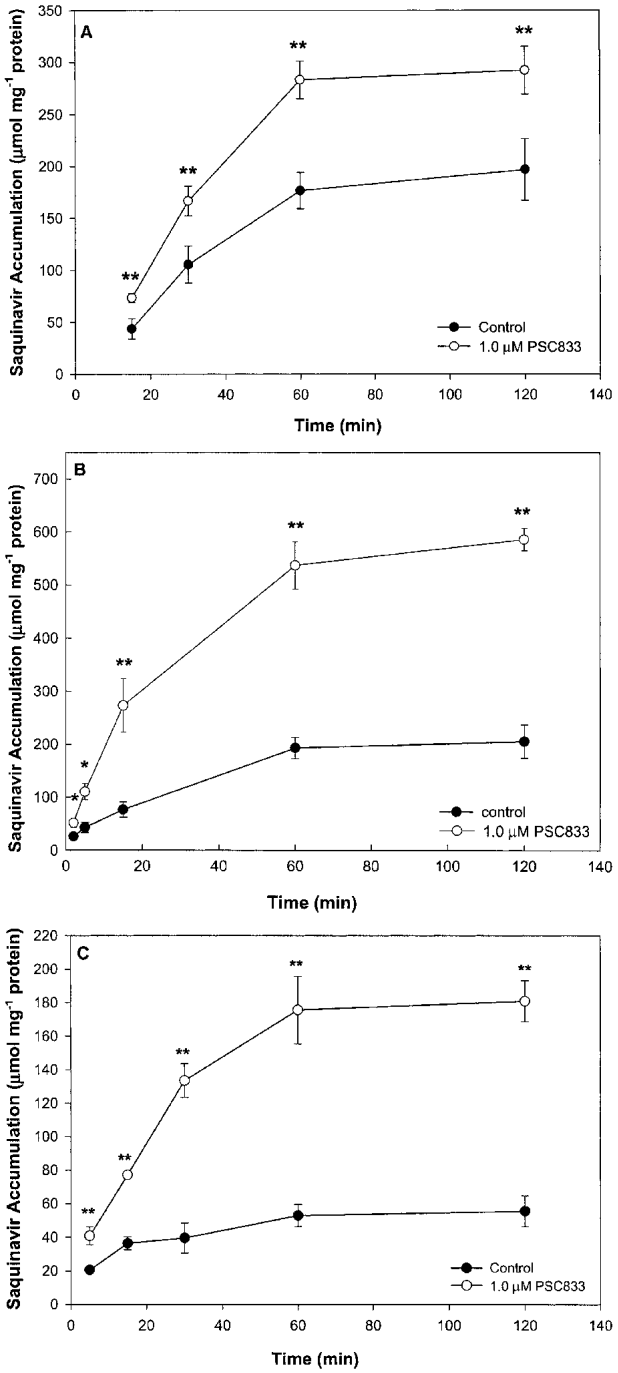

Fig. 2. Effect of PSC833 on the accumulation of saquinavir by (A) RBE4, (B) MLS-9, and (C) CH^RC5 monolayer cells. Saquinavir accumulation (0.78 μM) was measured at 37°C in the presence of 1.0 μM PSC833. Results are expressed as mean ± SD of 3 separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control (*$p < 0.05$; **$p < 0.001$).

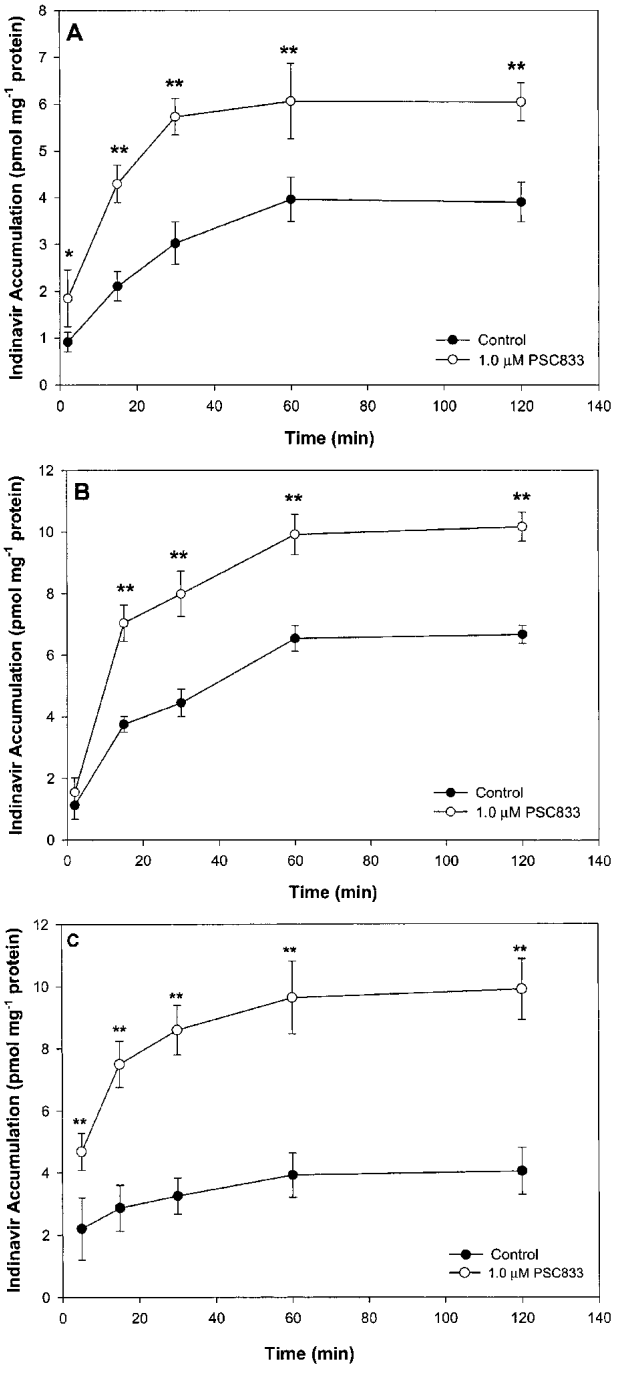

Fig. 3. Effect of PSC833 on the accumulation of indinavir by (A) RBE4, (B) MLS-9, and (C) CH^RC5 monolayer cells. Indinavir accumulation (0.38 μM) was measured at 37°C in the presence of 1.0 μM PSC833. Results are expressed as mean ± SD of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control (*$p < 0.05$; **$p < 0.001$).

presence of sodium azide, an ATPase inhibitor, and DNP, an ATP depleting agent, respectively. The accumulation of saquinavir by both cell lines was greatly enhanced by 2.3- to 2.9-fold ($p < 0.001$) at 1 h in the presence of 10 mM sodium azide (Table I). Similarly, the 1 h accumulation of indinavir by both cell lines was enhanced by 2.2- to 2.5-fold ($p < 0.001$) in the presence of 5.0 mM DNP (Table I). This suggests that a fully functional ATPase is required to effectively transport saquinavir and indinavir from RBE4 and MLS-9 cell monolayers. Taken together, these data suggest that a P-gp mediated process may be involved in the transport of saquinavir and indinavir in the two brain cell lines.

Previous studies have demonstrated that HIV-1 protease inhibitors are P-gp substrates and may modulate the transport

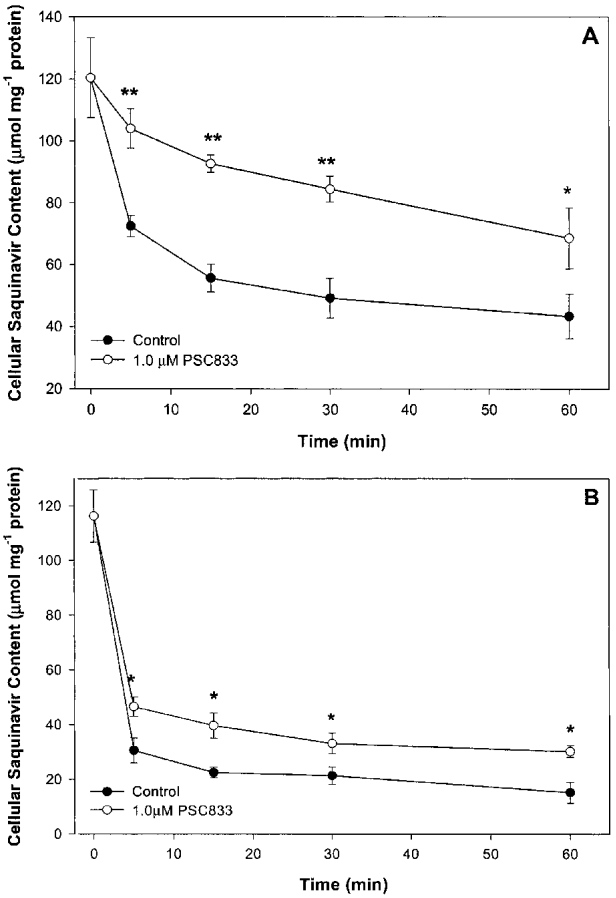

Fig. 4. Effect of PSC833 on saquinavir efflux by (A) RBE4 and (B) MLS-9 monolayer cells. Saquinavir efflux was measured at 37°C in the presence or absence of 1.0 μM PSC833 in RBE4 and MLS-9 cells. The time-course of efflux was expressed as the remaining intracellular drug content following a 30-min cellular incubation with radiolabeled saquinavir (0.78 μM). Results are expressed as mean ± SD of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control (*$p < 0.05$; **$p < 0.001$).

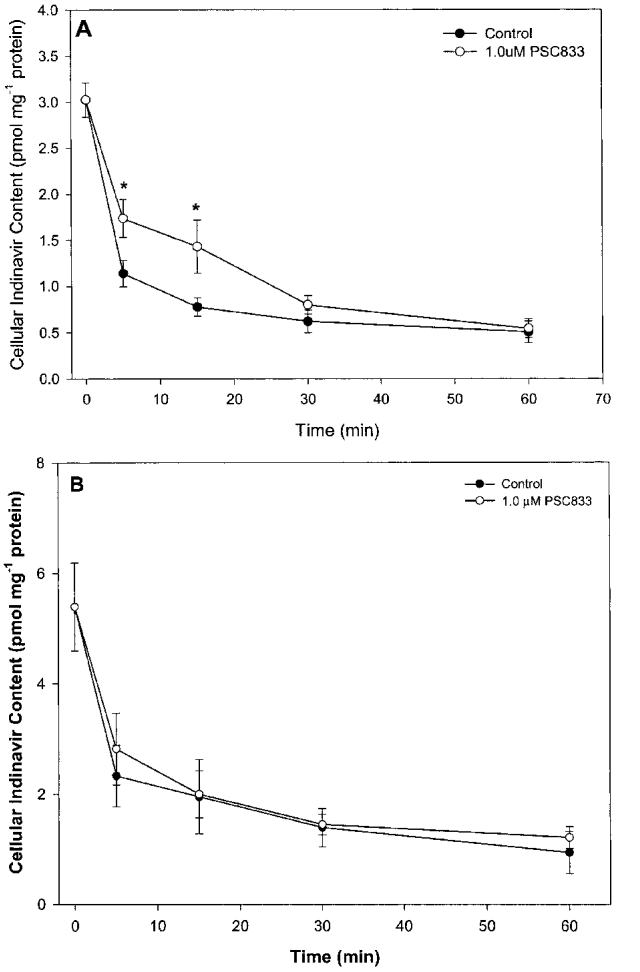

Fig. 5. Effect of PSC833 on indinavir efflux by (A) RBE4 and (B) MLS-9 monolayer cells. Indinavir efflux was measured at 37°C in the presence or absence of 1.0 μM PSC833 in RBE4 and MLS-9 cells. The time-course of efflux was expressed as the remaining intracellular drug content following a 30-min cellular incubation with radiolabeled indinavir (0.38 μM). Results are expressed as mean ± SD of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control (*$p < 0.05$).

of other P-gp substrates in normal and tumor cell lines (7,24,25). To investigate the P-gp–mediated transport characteristics of saquinavir and indinavir in our cell culture systems, we measured their cellular accumulation in the presence of standard P-gp inhibitors and HIV-1 protease inhibitors (Table II). The accumulation of 0.78 μM saquinavir at 1 h was significantly enhanced ($p < 0.001$) by 1.7- to 2.3-fold in RBE4 cells, 2.2- to 2.7-fold in MLS-9, and 1.8- to 6.2-fold in CH^RC5 in the presence of 50 μM cyclosporin A, 1.0 μM PSC833, 10 μM saquinavir, 10 μM ritonavir, and 50 μM indinavir. The accumulation of 0.38 μM indinavir at 1 h was significantly enhanced by 1.6- to 2.4-fold in RBE4, 1.9- to 2.6-fold in MLS-9, and 1.5- to 4.0-fold in CH^RC5 in the presence of 50 μM cyclosporin A, 1.0 μM PSC833, 10 μM saquinavir, or 10 μM ritonavir (Table III). Unlabeled indinavir (50 μM) enhanced the accumulation of [^{3}H]indinavir up to 5.2-fold ($p < 0.001$) in RBE4 and up to 4.8-fold in MLS-9 but only 2.4-fold in the CH^RC5 cells, suggesting the possible involvement of additional membrane transport systems in the efflux of indinavir by the brain cell culture systems.

DISCUSSION

Although current HIV-1 drug therapy with antiretrovirals, including HIV-1 protease inhibitors, has proven to significantly reduce systemic viral loads in patients, treatment failure and the emergence of resistant strains of HIV-1 in the brain have posed a major problem (4,26). Recent *in vitro* studies from our laboratory suggest that HIV-1 protease inhibitors, including saquinavir and indinavir, may interact with P-gp in brain microvessel endothelial cells and in microglia (16,17). However, the direct investigation of P-gp mediated transport of HIV-1 protease inhibitors at the BBB and in brain parenchyma, two brain compartments that are clinically relevant to HIV-1 infection, has not been extensively investigated. The objective of this study was to characterize the *in vitro* transport properties of saquinavir and indinavir in two CNS cellular compartments using rat brain cell culture models (i.e., RBE4 and MLS-9). These systems have been shown

Table I. Effect of Sodium Azide or 2′, 4′-Dinitrophenol (DNP) on Radiolabeled Saquinavir or Indinavir Accumulation by RBE4 and MLS-9 Monolayer Cells

	Increase in accumulation (% control)			
	[^{14}C]Saquinavir*		[^{3}]Indinavir†	
Cell line	30 min	1 h	15 min	1 h
RBE4	205%‡	234%‡	188%‡	220%‡
MLS-9	269%‡	289%‡	283%‡	247%‡

n = 3 separate experiments.
* Sodium azide, 10 mM.
† DNP, 5 mM.
‡ $p < 0.001$.

to retain morphological and biochemical characteristics of brain microvessel endothelial cells and microglia respectively (17,19,27).

Results from Western blotting analysis show that P-gp encoding genes are actively translated in cultured rat brain microvessel endothelia and in rat microglia. We observe the presence of a single band at approximately 170–180 kDa, a size previously reported for P-gp (9), in both the RBE4 and the MLS-9 cell lines. This band was detected using the monoclonal C219 antibody, which recognizes a highly conserved cytoplasmic epitope in the ATP-binding domain of mdr1a, mdr1b, and mdr2 (23). A robust band was also detected in the P-gp overexpressing cell line, CH^{R}C5, further confirming the antibody specificity. These data are consistent with other published results and our recent studies that report the protein expression of P-gp in brain microvessel endothelial cells and in microglia (14,16–18,20).

Results from our functional studies show that saquinavir uptake by RBE4 and MLS-9 cell monolayers reached a steady state by approximately 1 h and was significantly enhanced ($p < 0.001$) in the presence of PSC833, a nonimmunosuppressive cyclosporin A analog. This compound has been identified as one of the most potent P-gp inhibitors in *in vitro* cell culture systems (IC_{50} = 0.11 μM) (8), and has been shown to reduce the basolateral-to-apical transport of saquinavir in Caco-2 cells by 70% (7). In our study, PSC833 reduced saquinavir efflux in both cell culture systems by up to 67% in RBE4 and 72% in MLS-9, respectively. Cyclosporin A, another standard P-gp inhibitor, and the three HIV-1 protease inhibitors used (saquinavir, indinavir, ritonavir) also significantly enhanced intracellular saquinavir accumulation up to 129% in RBE4 cells and up to 168% in MLS-9 cells.

As expected, a larger effect was observed in CH^{R}C5, the P-gp overexpressing cell line, where these same inhibitors increased saquinavir accumulation up to 523%. Previous studies have reported that PSC833 (2 μM) enhanced the accumulation of saquinavir up to 108% in MDR-P388 cells, a drug resistant murine monocytic leukemia cell line (28) and that cyclosporin A (4 μM) significantly reduced the transepithelial flux of saquinavir in HCT-8 cells, a human intestinal adenocarcinoma cell line (3).

Incubation of RBE4 and MLS-9 monolayer cells with sodium azide, an ATPase inhibitor, led to a significant enhancement in the intracellular accumulation of saquinavir suggesting that the transport requires coupling to an energy-dependent mechanism. The ATP-dependent transport of saquinavir across renal and intestinal epithelia (7,12) and the ability of saquinavir to stimulate P-gp specific ATPase activity (24) were previously demonstrated.

Results from our functional studies also suggest that, at least in part, indinavir is transported by P-gp in brain microvessel endothelial cells and in microglia. In the two brain cell lines, indinavir accumulation reached a steady state by 1 h and was significantly enhanced in the presence of PSC833, cyclosporin A, and all three HIV-1 protease inhibitors. Results from our efflux studies show that radiolabeled indinavir was rapidly extruded from both rat brain cell lines. Indinavir accumulation was also significantly enhanced in the presence of DNP, an ATP depleting agent, suggesting the involvement of an energy-dependent mechanism. In the CH^{R}C5 cell line, unlabeled indinavir increased the intracellular accumulation of radiolabeled indinavir 2.4-fold. This enhancement was 2.8-fold lower than in the RBE4 cell line and 4.1-fold lower than in the MLS-9 cells, suggesting that other membrane efflux transport systems [i.e., MRP1, MRP2, breast cancer resistance protein (BCRP)] may also be involved in regulating indinavir cellular permeation.

Similar to P-gp, MRP isoforms are also members of the ABC superfamily of membrane transporters and play a major role in chemotherapy cell resistance (29) but, in contrast,

Table II. Effect of Various P-gp Inhibitors and HIV-1 Protease Inhibitors on Saquinavir 1-Hour Accumulation by RBE4, MLS-9, and CH^{R}C5 Monolayer Cells

	Intracellular [^{14}C]saquinavir accumulation (μmol mg^{-1} protein)					
Treatment	RBE4	Fold increase	MLS-9	Fold increase	CH^{R}C5	Fold increase
Control	210.3 ± 24.5		151.4 ± 15.6		60.9 ± 3.8	
P-gp inhibitors						
Cyclosporin A (50 μM)	466.6 ± 39.2**	2.2	332.8 ± 27.4**	2.2	379.6 ± 7.0**	6.2
PSC833 (1.0 μM)	419.5 ± 25.1**	2.0	337.7 ± 57.6**	2.2	226.4 ± 12.8**	3.7
Protease inhibitors						
Ritonavir (10 μM)	481.2 ± 33.9**	2.3	405.6 ± 58.7**	2.7	169.3 ± 8.7**	2.8
Saquinavir (10 μM)	398.9 ± 52.3**	1.9	369.0 ± 44.9**	2.4	109.0 ± 1.4*	1.8
Indinavir (50 μM)	353.0 ± 35.3**	1.7	330.8 ± 51.2**	2.2	160.1 ± 25.7**	2.6

n = 3–6 separate experiments.
* $p < 0.05$.
** $p < 0.001$.

Table III. Effect of Various P-gp Inhibitors and HIV-1 Protease Inhibitors on Indinavir 1-Hour Accumulation by RBE4, MLS-9, and CH^RC5 Monolayer Cells

	Intracellular [^{3}H]indinavir accumulation (pmol mg^{-1} protein)					
Treatment	RBE4	Fold increase	MLS-9	Fold increase	CH^RC5	Fold increase
Control	3.7 ± 0.4		5.9 ± 0.9		2.2 ± 0.6	
P-gp inhibitors						
Cyclosporin A (50 μM)	9.0 ± 0.8**	2.4	13.0 ± 2.0**	2.2	8.8 ± 0.8**	4.0
PSC833 (1.0 μM)	7.3 ± 1.3**	2.0	11.3 ± 2.0**	1.9	6.7 ± 0.4**	3.0
Protease inhibitors						
Ritonavir (10 μM)	6.3 ± 0.5**	1.7	15.2 ± 2.1**	2.6	4.8 ± 0.4**	2.2
Saquinavir (10 μM)	6.0 ± 0.4**	1.6	12.4 ± 1.5**	2.1	3.3 ± 0.3*	1.5
Indinavir (50 μM)	19.3 ± 4.7**	5.2	28.3 ± 12.4**	4.8	5.2 ± 0.7**	2.4

n = 3–6 separate experiments.
* $p < 0.05$.
** $p < 0.001$.

MRPs appear to be more selective for organic anions as well as glutathione and glucuronide conjugates. However, the determination of the functional significance of MRP is more complex due to the existence of several different isoforms (30). The expression of MRP1 has been demonstrated in the RBE4 cell line (20) and in the MLS-9 cell line (22). In addition, results from RT-PCR analysis detected the presence of various MRP isoforms in bovine brain endothelial cells and bovine brain homogenates (i.e., MRP1, MRP4, MRP5, MRP6) (31) and in primary cultures of rat microglia (i.e., MRP1, MRP3, MRP4, MRP5) (32).

Saquinavir and indinavir have previously been shown to cross-react with MRP1 (33,34). Jones *et al.* (33) observed that the intracellular accumulation of saquinavir (1 μM) and indinavir (1 μM) was reduced in CEM-MRP1 cells, a human lymphocyte cell line that overexpresses MRP1, as compared to the parent, drug-sensitive CEM cell line. In addition, Williams *et al.* (34) reported that MK-571, a well-characterized MRP inhibitor, could significantly inhibit the efflux of radiolabeled saquinavir (3 μM) in MDCKII cells (a continuous canine renal epithelial cell line) transfected with human MRP1 or MRP2. Recent data from our laboratory show that the intracellular accumulation of vincristine (30 nM), an MRP1 substrate, can be significantly enhanced in a human MRP1 overexpressing cell line (WT-MRP1) in the presence of saquinavir (10 μM) and indinavir (50 μM) (22). Therefore, MRP-mediated transport may also contribute to the pattern of HIV-1 protease inhibitor accumulation observed in RBE4 and MLS-9 monolayers. Recently, another member of the ABC superfamily of membrane transporters, BCRP, has been identified in cultured human brain microvessel endothelial cells (35). The expression of a homologue of BCRP, named brain multidrug resistance protein (BMRP), was also reported in porcine brain capillary endothelial cells (36). Both BCRP and BMRP possess one half of the MDR1 P-gp structure with only six transmembrane domains and one ATP-binding domain (37). In addition to this structural similarity, most known substrates for BCRP/BMRP are similar to those for P-gp (i.e., hydrophobic, amphiphilic xenobiotics), suggesting that saquinavir and indinavir may also interact with BCRP/BMRP (38,39). In fact, results from a recent study suggest that saquinavir, ritonavir, and nelfinavir are inhibitors but not substrates of BCRP (40). Currently, the expression of BCRP/BMRP in RBE4 cells and MLS-9 cells is unknown. Once evidence for the molecular expression of BCRP/BMRP is clearly demonstrated in the brain microvessel endothelial microglia cell lines, the relative contribution of these transporters to the brain permeation of saquinavir and indinavir could be examined.

In summary, our results provide direct evidence for P-gp–mediated transport of saquinavir and indinavir in cultured rat brain endothelial cells (RBE4) and in cultured rat brain microglia (MLS-9). The possible involvement of other membrane transporters (i.e., MRPs) known to be functionally expressed in the two brain cell systems cannot be excluded and deserves further investigation. Combined with our previous work showing the localization and functional expression of P-gp in both cell lines (16,17), our current data show that the low CNS accumulation of HIV-1 protease inhibitors may be, in part, due to the expression of P-gp in brain microvessel endothelial cells and brain parenchyma (i.e., microglia) and astrocytes (41). Functional expression of P-gp in brain cellular compartments relevant to HIV-1 infection may play a significant role in antiretroviral drug response and drug resistance.

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