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HIV Protease Inhibitor Ritonavir: A More Potent Inhibitor of P-Glycoprotein than the Cyclosporine Analog SDZ PSC 833

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ABSTRACT. The effect of P-glycoprotein inhibition on the uptake of the HIV type 1 protease inhibitor saquinavir into brain capillary endothelial cells was studied using porcine primary brain capillary endothelial cell monolayers as an *in vitro* test system. As confirmed by polymerase chain reaction and Western blot analysis, this system functionally expressed class I P-glycoprotein (pgp1A). P-Glycoprotein isoforms pgp1B or pgp1D could not be detected. The uptake of saquinavir into endothelial cells could be described as the result of a diffusional term of uptake and an oppositely directed saturable extrusion process. Net uptake of saquinavir into cultured brain endothelial cells could be increased significantly up to 2-fold by SDZ PSC 833 in a dose-dependent manner, with an IC_{50} of 1.13 μM. In addition, the HIV protease inhibitor ritonavir inhibited p-glycoprotein-mediated extrusion of saquinavir with an IC_{50} of 0.2 μM, indicating a high affinity of ritonavir for p-glycoprotein. In conclusion, we showed that the HIV protease inhibitor ritonavir is a more potent inhibitor of P-glycoprotein than the multidrug resistance (MDR)-reversing agent SDZ PSC 833. The inclusion of this drug in combination regimens may greatly facilitate brain uptake of HIV protease inhibitors, which is especially important in patients suffering from AIDS dementia complex. BIOCHEM PHARMACOL **57**;10:1147–1152, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. P-glycoprotein; HIV protease inhibitors; SDZ PSC 833; blood–brain barrier; ritonavir; saquinavir; indinavir; nelfinavir; AIDS; AIDS dementia complex

HIV protease inhibitors have been accepted as first-line treatment of infections with HIV-1 in order to slow down virus replication and disease progression. A relevant complication of infections with HIV-1 is the AIDS dementia complex [1], which occurs in one-third of adult and one-half of pediatric AIDS patients [2]. Therefore, an efficient antiretroviral therapeutic agent should be able to pass the blood–brain barrier. Experiments with *mdr1a* knockout mice have shown that the penetration of many lipophilic xenobiotics across the blood–brain barrier is limited by the action of the *MDR1*§ gene product, P-gp [3]. Recently, it was shown that HIV protease inhibitors are substrates of P-gp [4, 5]. In bovine BCEC, transendothelial permeability of saquinavir was very low and possibly also modulated by the action of P-gp [6]. These findings suggest that inhibition of P-gp may lead to increased cerebral uptake of HIV protease inhibitors, which could be beneficial in patients suffering from AIDS dementia complex. Therefore, in the present study the effect of P-gp inhibition on the uptake kinetics of saquinavir into BCEC was systematically characterized. Ritonavir co-administration significantly increases the enteral absorption of saquinavir [7–9]. *In vitro* studies using Caco-2 cell monolayers [5] suggest that this can be attributed at least partly to competition for the P-gp efflux mechanism during absorption. Thus, the dose-dependent effects of ritonavir on the uptake of saquinavir were investigated and compared to those of the well-known P-gp inhibitor SDZ PSC 833, a cyclosporine analog (Fig. 1).

MATERIALS AND METHODS

Chemicals

The HIV protease inhibitors saquinavir mesylate, [^{14}C]saquinavir mesylate, and ritonavir were a kind gift from Dr. H. Wiltshire, Roche Ltd. SDZ PSC 833 was obtained from Novartis LTD. All other chemicals were of analytical grade and obtained from commercial sources.

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§ *Abbreviations:* MDR, multidrug resistance; MEM, minimum essential medium; PCR, polymerase chain reaction; BCEC, brain capillary endothelial cells; P-gp, P-glycoprotein; CYP3A4, 3A4 isoform of the cytochrome P450 system; pGAPDH, porcine glyceraldehyde phosphate dehydrogenase; and TBS-T, Tris-buffered saline.

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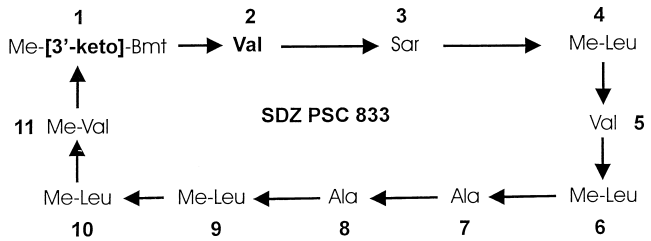

FIG. 1. Structure of SDZ PSC 833. Me-Bmt, *N*-methyl-4-butenyl-4-methyl threonine; Sar, sarcosine; Me-Val, *N*-methyl-L-valine; Me-Leu, *N*-methyl-L-leucine.

Cell Cultures

The cell culture model was validated as described in detail [10]. Primary cultures of porcine BCEC were prepared according to Audus and Borchardt [11] with the following modifications: Cortical gray matter from six freshly obtained porcine brains was minced and incubated in MEM (Sigma) containing 0.5% dispase (Boehringer Mannheim) for 2 hr. Cerebral microvessels were obtained after centrifugation in MEM containing 13% dextran (Sigma). The microvessels were subsequently incubated in MEM containing 1 mg/mL collagenase–dispase (Boehringer Mannheim) for 4.25 hr. The resulting cell suspension was supplemented with 10% horse serum and filtered through a 150 μm nylon mesh. BCEC were isolated on a continuous 50% Percoll gradient (Pharmacia) (centrifugation: 100 *g* for 10 min). For uptake experiments, isolated endothelial cells were filtered through a 35 μm nylon mesh before seeding with a density of 100,000 cells per cm^2 onto collagen/fibronectin (Boehringer)-coated 24-well cell culture plates. Cells were cultured under standard cell culture [11] (cell culture medium: 45% MEM, 45% F12-HAM nutrient mixture, 100 μg/mL streptomycin, 100 μg/mL penicillin G, 100 μg/mL heparin, 13 mM $NaHCO_3$, and 20 mM HEPES containing 10% heat-inactivated horse serum (GIBCO BRL).

Uptake Assays

Uptake assays were performed at room temperature using confluent monolayers of porcine BCEC at day 10 of culture. Cells were grown in 24-well cell culture plates. The surface area was 2 cm^2 per well. Cells were washed with uptake buffer consisting of 142 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 4 mM D-glucose, 10 mM HEPES, and 1.5 mM K_2HPO_4, pH 7.4. The incubation was initiated by addition of 250 μL uptake buffer containing 0.3 μCi of [^{14}C] isotope-labeled tracer of the respective substrate, sufficient unlabeled substrate to bring the medium to the desired final concentration, and 0.3 μCi of the extracellular marker [^{3}H]sucrose.

For [^{14}C]saquinavir and [^{14}C]sucrose, partition coefficients were determined in octanol: 100 mM phosphate buffer (pH 7.0) following equilibration over 24 hr at room temperature. Binding of the compounds to the plastic of cell culture plates was assessed by their incubation in uptake buffer in the presence and absence of 1% BSA.

Stock solutions of inhibitors which were poorly soluble in buffer were prepared using DMSO or ethanol. The final concentration of DMSO or ethanol in the assay did not exceed 1% (v/v) or 0.5% (v/v), respectively. Control experiments were performed in the absence and the presence of the respective solvent. At the concentrations used, the solvents had no detectable effect on the measured cell parameters. Lactate dehydrogenase release was negligible, and uptake of the extracellular marker sucrose did not change. Viability of the cells was determined by trypan blue exclusion before the experiment and was always greater than 95%. Total cell numbers were measured using a hemocytometer.

Incubations were terminated after 5 min by removing the incubation medium. Cells were washed with ice-cold uptake buffer, removed from the wells by incubation for 10 min in trypsin (0.25%)/EDTA, and subsequently transferred to scintillation vials. Cells were solubilized overnight in scintillation cocktail, and the amount of radiolabeled substrate taken up was determined by scintillation counting.

Assessment of P-gp Effect in P388 Cells

In control experiments, the effect of P-gp on the uptake of 20 μM [^{14}C]saquinavir was assessed in murine monocytic leukemia P388 cells. Parental (Par-P388) and multidrug-resistant (MDR-P388) sublines of P388 were cultured as described by Boesch *et al.* [12]. The generation time of Par-P388 and MDR-P388 cells was 9 to 10 hr.

Kinetic Experiments

Uptake of [^{14}C]saquinavir into cultured BCEC was measured as a function of its concentration in the incubation mixture. Incubations were performed either at room temperature or at 4°. Higher temperatures were not chosen because uptake into the cell monolayers was too rapid, thereby diminishing the reproducibility of the kinetic data. The range of concentrations used was from 1 to 40 μM, the latter being the limit for solubility.

Immunodetection of P-gp and CYP3A4

P-gp was detected by western blot analysis using the monoclonal antibody C219. SDS-PAGE was performed with a Mini-Protean II apparatus (Bio-Rad). Endothelial cell homogenates were resuspended in PBS at a final protein concentration of 1 mg/mL. Then, one-fifth of the volume sample buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 5% SDS, 40 mM dithiothreitol, and 0.00625% bromophenol blue) was added. Samples were loaded on 7.5% acrylamide/bisacrylamide gels. Electrophoresis was carried out at 80 V in the stacking gel and at a constant voltage of 120 V in the separating gel. Proteins were transferred electrophoretically to a nitrocellulose membrane (0.45 μm pore size) using a Mini Trans-Blot cell

(Bio-Rad). The transfer buffer contained 192 mM glycine, 25 mM Tris, and 20% methanol. The transfer was carried out for 2 hr at a constant amperage of 2 mA/cm^2. Hydrophobic or non-specific sites were blocked overnight at 4° with 5% powdered skimmed milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.3% Tween 20 (TBS-T). Membranes were washed three times for 15 min in TBS-T. The nitrocellulose membranes were incubated with monoclonal antibody C219 (200 ng/mL; Centocor Inc.) in TBS-T, 1% BSA, and 0.05% NaN_3 for 2 hr at 37°. Membranes were washed four times for 15 min and incubated for 1 hr at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (1:1000) in TBS-T containing 2% milk powder. Membranes were washed four times for 10 min in TBS-T, and P-gp was detected using enhanced chemiluminescence reagent (ECL) (Amersham). Molecular weights were determined in comparison to commercially available standards: myosin (206 kDa), β-galactosidase (117 kDa), BSA (89 kDa), and ovalbumin (47 kDa). For detection of CYP3A4, a polyclonal goat anti-rat antibody against rat CYP3A2 was used as primary antibody, one which recognizes human CYP3A4 (Daiichi Pure Chemical Co.). Peroxidase-conjugated rabbit anti-goat immunoglobulin G (DAKO) was utilized as secondary antibody.

RNA and cDNA

Total RNA was isolated from confluent monolayers of porcine BCEC using the RNeasy Mini Kit (Qiagen). RNA was quantified after DNase digestion with a GeneQuant photometer (Pharmacia). Its integrity was checked by ethidium bromide agarose gel electrophoresis. The purity of the RNA preparations was high, as demonstrated by the 260 nm/280 nm ratio (range, 1.8–2.0). One μg of total RNA was reverse-transcribed by Superscript II (GIBCO BRL) according to the manufacturer's protocol using random hexamers as a primer.

PCR for P-gp and CYP3A4

A total of 50 ng cDNA was used as a template for PCR. Primers were synthesized (GIBCO BRL) for class I pig P-gp (5′AAGCGCTCATCAACTGTG3′ and 5′GGCACTTTATGCAAACATTC3′) according to Childs and Ling [13]. pGAPDH primers (5′ACCACAGTCCATGCCATCAC3′ and 5′TCCCACCACCCTGTTGCTGTA3′) were used as an internal control. PCR was performed with a thermocycler (Biometra). Each sample was amplified for 35 cycles of denaturation (95° for 30 sec, 60° for 50 sec, 72° for 50 sec). The reaction mixture contained 5 μL of the cDNA template, 1.25 U AmpliTaq Gold DNA polymerase (Perkin Elmer), 5 μL 10× PCR buffer (Mg^{2+}-free, Perkin Elmer), $MgCl_2$ at a final concentration of 3.5 mM, 4 μL of dNTP reaction mixture (2.5 mM each, Perkin Elmer), 15 pmol of each primer and water to a final volume of 50 μL. Each PCR reaction included a 50 μL aliquot of the reaction mixture without cDNA as a negative control. The PCR products were separated by electrophoresis in 1.5% agarose and visualized by UV in the presence of ethidium bromide (Bio-Rad GelDetection System, BioRad). CYP3A4 was assessed with primers according to Kivistö *et al.* [14].

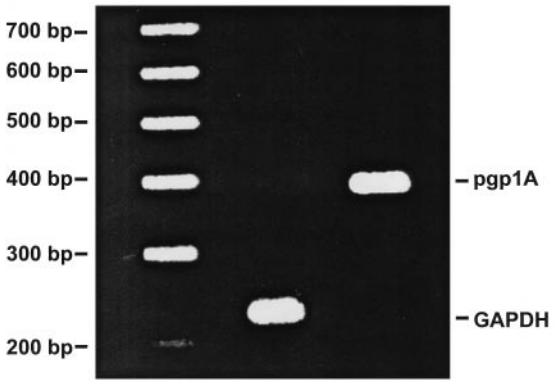

FIG. 2. Polymerase chain reaction reveals transcription of Class I p-pg (p-pg1A) in capillary brain endothelial cells (lane 3). P-gp1A product (389 bp) was compared with the constitutive expression (lane 2) of porcine glyceraldehyde phosphate dehydrogenase (226 bp). None of the other Class I P-gp isoforms (658 bp product of p-gp1B or p-gp1D) were detected. Lane 1 represents size markers.

Statistical Analysis

Data were compared by analysis of variance using the SPSS for Windows software [15]. The level of significance was $P = 0.05$. In the case of more than two groups, pairwise comparisons between groups were performed by the Tukey HSD multicomparison test. Kinetic analysis of saquinavir uptake was performed with non-linear regression using Origin Software (Version 5.0, Microcal). All data were given the same weight.

RESULTS

Expression of P-gp

The expression of P-gp in BCEC was demonstrated by several experiments: PCR analysis (Fig. 2) indicated that class I P-gp (p-gp1A) was present in BCEC at 389 bp. No 658 bp product of p-gp1B or p-gp1D was detected. The expression was of the same magnitude as the constitutive expression of pGAPDH. Western blot analysis (Fig. 3) demonstrated that P-gp-overexpressing MDR-P388 exhibited immunoreactivity in the molecular weight range of 150 kDa that was not present in Par-P388 cells devoid of P-gp expression. A stained band was observed in BCEC at the same molecular weight.

Characterization of Saquinavir Uptake into BCEC

Saquinavir demonstrated high lipophilicity compared to the reference compounds: The partition coefficients were (log P) 2.03 for [^{14}C]saquinavir and −2.96 for [^{14}C]sucrose.

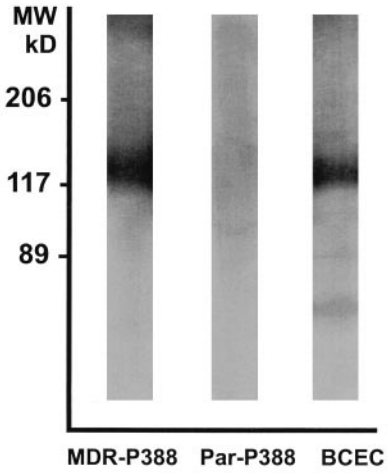

FIG. 3. Western blot analysis of P-glycoprotein in BCEC. Par-P388 and MDR-P388 cells were used as negative and positive controls, respectively. P-gp was detected by monoclonal antibody C219. Immunoreaction was shown in the molecular weight range of 150 kD in P-gp overexpressing MDR-P388 cells and was not present in Par-P388 cells, which are devoid of Pgp.

However, control experiments showed that binding to cell culture plastic material was negligible for saquinavir. After 24-hr incubation, recovery with and without addition of 1% BSA to the uptake buffer was 95.5% and 95.9% for [^{14}C]saquinavir, 99.5% and 98.6% for [^{14}C]sucrose, and 65.8% and 61.6% for [^{3}H]imipramine, respectively.

Uptake of saquinavir into BCEC increased in a curvilinear manner with increasing dose (Fig. 4). Using non-linear regression analysis, this relationship could be described as the result of a linear diffusion term of uptake (K_d of 13.6 cm·min^{-1}) and an oppositely directed saturable (Michaelis–Menten-type) extrusion process (V_{max} of 497.6 pmol/min/mg and K_m of 36.5 μM).

Recent reports have demonstrated that saquinavir is a substrate of P-gp [4, 5, 16]. In our experiments, this was confirmed by a significantly ($P < 0.0001$) decreased uptake of [^{14}C]saquinavir into P-gp-overexpressing MDR-P388 cells compared to Par-P388 cells, which showed no P-gp expression (Fig. 5). When saquinavir was given to MDR-P388 cells together with 100 μM verapamil or 2 μM PSC833, the uptake of saquinavir was significantly enhanced ($P < 0.0001$), resulting in an accumulation that was about 2.5-fold higher than in untreated MDR-P388 cells.

Effect of SDZ PSC 833

Uptake of saquinavir into BCEC was enhanced by SDZ PSC 833 in a dose-dependent manner (Fig. 6), maximum inhibition resulting in an approximately 2-fold increase in uptake. The IC_{50} for this inhibition was estimated to be 1.13 μM. All investigated protease inhibitors (ritonavir, indinavir, and nelfinavir) increased the net uptake of saquinavir into BCEC. However, ritonavir was more than 50 times more potent than nelfinavir and indinavir (data not shown). Therefore, only ritonavir was further investigated. Maximum inhibition of P-gp appeared to occur at a significantly lower concentration of ritonavir compared to SDZ PSC 833 (Fig. 6). The IC_{50} was estimated to be 0.20 μM. Assuming that the IC_{50} in inhibition experiments reflects the K_m of an inhibitor, these results are indicative of a very high affinity of ritonavir for P-gp. Alternatively, these effects may be explained by an inhibitory effect of ritonavir on CYP3A4-mediated metabolism of saquinavir. To investigate the impact of endothelial metabolism by CYP3A4 on the net uptake of saquinavir, Western blot and PCR analyses were performed, but showed no signs of CYP3A4 expression in BCEC. In addition, the effect of imipramine, a known substrate of CYP3A4 but not of P-gp, on saquinavir uptake was investigated. Saquinavir uptake in BCEC was identical with and without the addition of 10 μM imipramine.

DISCUSSION

Therapeutic strategies in AIDS have been hampered by a variety of problems. These include strict and rigid drug regimens (and potential non-compliance) but also pharmacokinetic barriers, which limit access of the different drugs

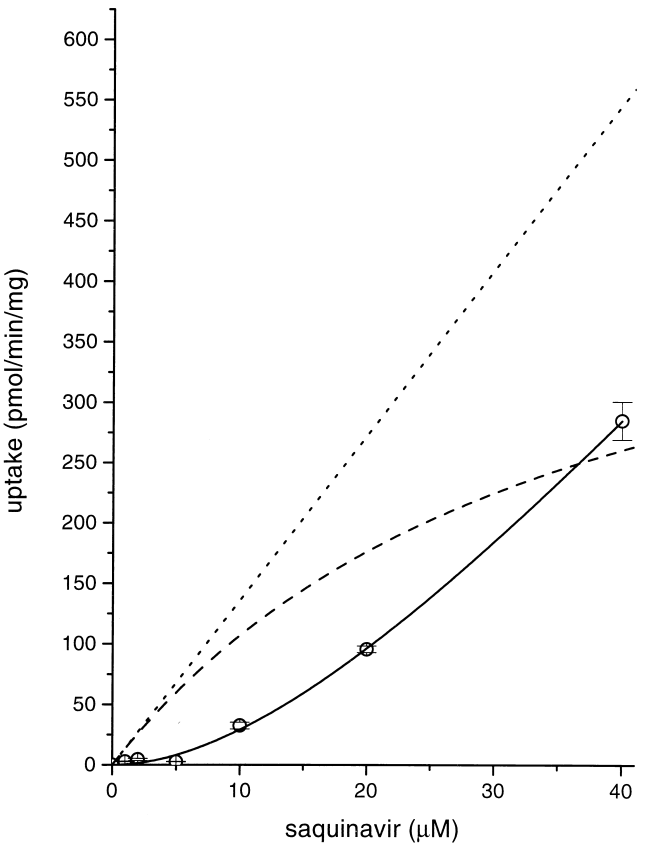

FIG. 4. Dose-dependent uptake of [^{14}C]saquinavir into BCEC. Net uptake (solid line) could be described as the result of a linear diffusion term of uptake (dotted line) and an oppositely directed saturable extrusion process (dashed line) (V_{max} of 0.011 nmol/min/cm^2 and K_m of 36 μM). Values represent means ± SEM of 5 experiments.

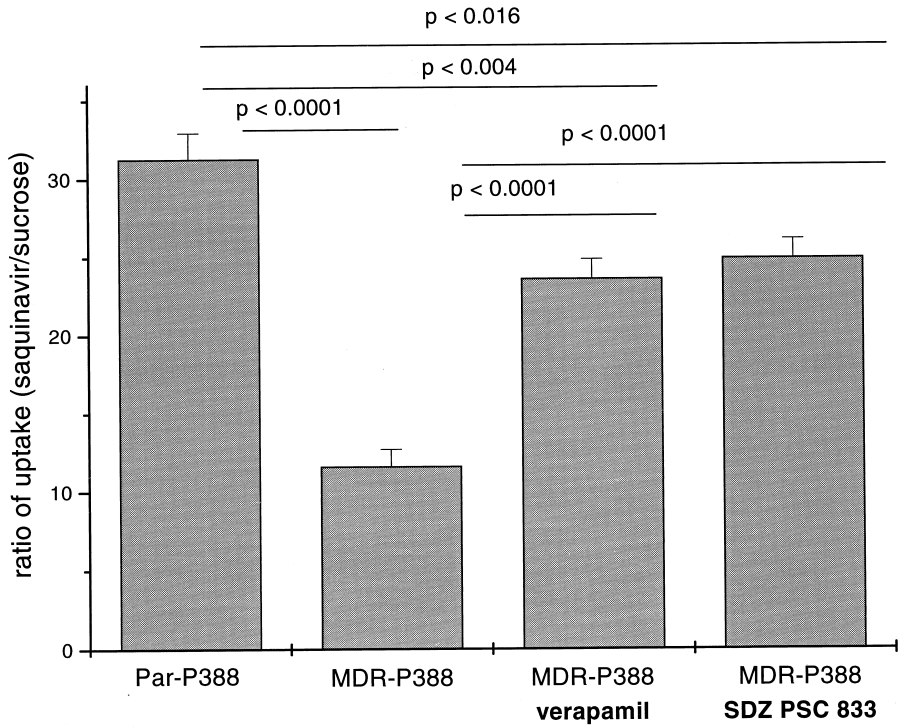

FIG. 5. Uptake of [^{14}C]saquinavir into murine leukaemia P388 cells. Par-P388 cells are devoid of P-gp activity and MDR-P388 cells overexpress P-gp. Uptake was decreased significantly ($P < 0.0001$) in MDR-P388 cells, this decrease being significantly ($P < 0.0001$) reduced by addition of 100 μM verapamil or 2 μM SDZ PSC 833. Values represent means ± SEM of 5 experiments.

to the body and to different target organs. These pharmacokinetic barriers include the gastrointestinal tract with partly low bioavailability of orally administered protease inhibitors, but also a limited effectiveness of these compounds in the treatment of HIV-1 infection with central nervous system involvement. While first pass metabolism by the 3A4 isoform of the CYP system may partially account for low bioavailability after oral administration, another important factor in drug bioavailability and brain disposition, P-gp, has thus far received little attention.

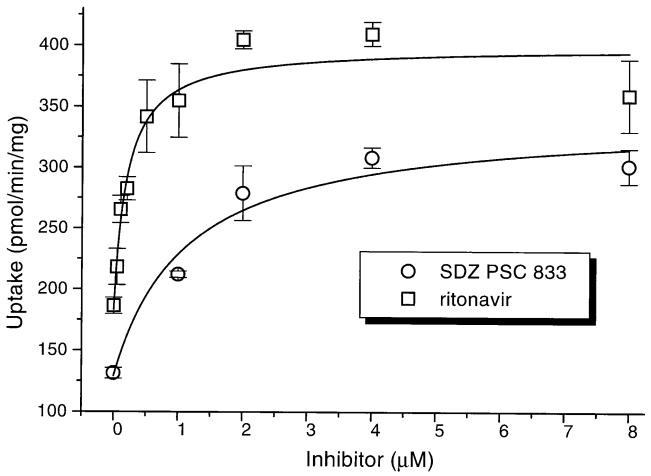

FIG. 6. Dose-dependent effect of SDZ PSC 833 on the net uptake of 20 μM saquinavir into BCEC (open circle). The inhibition constant K_i was estimated to be 1.13 μM. Dose-dependent effect of ritonavir on the net uptake of [^{14}C]saquinavir into BCEC (open square). The inhibition constant K_i was estimated to be 0.20 μM.

The role of P-gp in the CNS absorption and distribution of protease inhibitors has recently been documented [4, 6]. The finding that saquinavir is a substrate of P-gp was confirmed by our experiments in P388 cells; in P-gp-overexpressing cells (MDR-P388), the uptake of saquinavir was significantly decreased. This decrease was abolished in the presence of the P-gp inhibitors SDZ PSC 833 and verapamil [17]. The importance of these findings for blood–brain barrier penetration and CNS uptake of drugs and HIV protease inhibitors in particular was indicated by further experiments: ritonavir enhanced the uptake of saquinavir in BCEC; and, in contrast to earlier reports, we have shown for the first time a dose-dependent inhibitory effect of ritonavir on P-gp function. This was compared to the effect of the cyclosporine analog SDZ PSC 833, which is assumed to be one of the most potent inhibitors of P-gp [17]. Ritonavir was demonstrated to be an approximately 6-fold more potent inhibitor of P-gp than SDZ PSC 833.

What are the implications of the present findings? One strategy to improve the brain bioavailability of saquinavir or other protease inhibitors could be to transiently block P-gp function by co-administration of a P-gp inhibitor. Combination therapy based on administration of saquinavir with other protease inhibitors may result in significantly higher cerebral availability of the drugs. Based on our results, ritonavir seems to be an excellent candidate for such an effect. This assumption is supported by casual

evidence [18]: a therapeutic regimen based on zidovudine, lamivudine, and indinavir in a patient with AIDS dementia complex was able to reduce peripheral plasma viral RNA by 2.85 $\log_{10}$, but neurologic symptoms of AIDS dementia worsened. Two months after the addition of ritonavir to the treatment, neurological symptoms improved, with a concomitant decrease in viral RNA in CSF by 2.65 $\log_{10}$ in the cerebrospinal fluid.

In conclusion, we have shown that the HIV protease inhibitor ritonavir is a more potent inhibitor of P-glycoprotein than the MDR-reversing agent SDZ PSC 833. The inclusion of this drug in combination regimens may greatly facilitate brain uptake of HIV protease inhibitors, which is especially important in patients with AIDS dementia complex. The study of ritonavir in the modulation of multidrug-resistant phenotypes in other therapeutic areas is also warranted.

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References

1. Price RW, Brew B, Sidtis J, Rosenblum M, Scheck AC and Cleary P, The brain and AIDS: Central nervous system HIV-1 infection and AIDS dementia complex. *Science* **239:** 586–592, 1988.
2. Lipton SA and Gendelman HE, Dementia associated with the acquired immunodeficiency syndrome. *N Engl J Med* **332:** 934–940, 1995.
3. Schinkel AH, Smit JJM, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CAAM, van der Valk MA, Robanus–Maandag EC, te Riele HPJ, Berns AJM and Borst P, Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. *Cell* **77:** 491–502, 1994.
4. Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM and Wilkinson GR, The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J Clin Invest* **101:** 289–294, 1998.
5. Alsenz J, Steffen H and Alex R, Active apical secretory efflux of the HIV protease inhibitors saquinavir and ritonavir in Caco-2 cell monolayers. *Pharmacol Res* **15:** 423–428, 1998.
6. Glynn SL and Yazdanian M, *In vitro* blood–brain barrier permeability of nevirapine compared to other HIV antiretroviral agents. *J Pharm Sci* **87:** 306–310, 1998.
7. Kempf DJ, Marsh KC, Kumar G, Rodrigues AD, Denissen JF, McDonald E, Kukulka MJ, Hsu A, Granneman GR, Baroldi PA, Sun E, Pizzuti D, Plattner JJ, Norbeck DW and Leonard JM, Pharmacokinetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. *Antimicrob Agents Chemother* **41:** 654–660, 1997.
8. Merry C, Barry MG, Mulcahy F, Ryan M, Heavey J, Tjia JF, Gibbons SE, Breckenridge AM and Back DJ, Saquinavir pharmacokinetics alone and in combination with ritonavir in HIV-infected patients. *AIDS* **11:** F29–33, 1997.
9. Hsu A, Granneman GR, Cao G, Carothers L, el-Shourbagy T, Baroldi P, Erdman K, Brown F, Sun E and Leonard JM, Pharmacokinetic interactions between two human immunodeficiency virus protease inhibitors, ritonavir and saquinavir. *Clin Pharmacol Ther* **63:** 453–464, 1998.
10. Huwyler J, Drewe J, Klusemann C and Fricker G, Evidence for P-glycoprotein-modulated penetration of morphine-6-glucuronide into brain capillary endothelium. *Br J Pharmacol* **118:** 1879–1885, 1996.
11. Audus KL and Borchardt RT, Bovine brain microvessel endothelial cell monolayers as a model system for the blood–brain barrier. *Ann NY Acad Sci* **507:** 9–18, 1987.
12. Boesch D, Gaveriaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P and Loor F, *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res* **51:** 4226–4233, 1991.
13. Childs S and Ling V, Duplication and evolution of the P-glycoprotein genes in pig. *Biochim Biophys Acta* **1307:** 205–212, 1996.
14. Kivistö KT, Griese E-U, Fritz P, Linder A, Hakkola J, Raunio H, Beaune P and Kroemer HK, Expression of cytochrome P 450 3A enzymes in human lung: A combined RT-PCR and immunohistochemical analysis of normal tissue and lung tumours. *Naunyn Schmiedeberg's Arch Pharmacol* **353:** 207–212, 1996.
15. SPSS for Windows. SPSS Inc., Chicago IL, 1997.
16. Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, Pastan I and Dey S, HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* **37:** 3594–3601, 1998.
17. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
18. Pialoux G, Fournier S, Moulignier A, Poveda JD, Clavel F and Dupont B, Central nervous system as a sanctuary for HIV-1 infection despite treatment with zidovudine, lamivudine and indinavir. *AIDS* **11:** 1302–1303, 1997.