HIV-1 Protease Inhibitors Are Substrates for the *MDR*1 Multidrug Transporter

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ABSTRACT: The FDA approved HIV-1 protease inhibitors, ritonavir, saquinavir, and indinavir, are very effective in inhibiting HIV-1 replication, but their long-term efficacy is unknown. Since in vivo efficacy depends on access of these drugs to intracellular sites where HIV-1 replicates, we determined whether these protease inhibitors are recognized by the *MDR*1 multidrug transporter (P-glycoprotein, or P-gp), thereby reducing their intracellular accumulation. In vitro studies in isolated membrane preparations from insect cells infected with *MDR*1-expressing recombinant baculovirus showed that these inhibitors significantly stimulated P-gp-specific ATPase activity and that this stimulation was inhibited by SDZ PSC 833, a potent inhibitor of P-gp. Furthermore, photoaffinity labeling of P-gp with the substrate analogue [^{125}I]iodoarylazidoprazosin (IAAP) was inhibited by all three inhibitors. Cell-based approaches to evaluate the ability of these protease inhibitors to compete for transport of known P-gp substrates showed that all three HIV-1 protease inhibitors were capable of inhibiting the transport of some of the known P-gp substrates but their effects were generally weaker than other documented P-gp modulators such as verapamil or cyclosporin A. Inhibition of HIV-1 replication by all three protease inhibitors was reduced but could be restored by *MDR*1 inhibitors in cells expressing *MDR*1. These results indicate that the HIV-1 protease inhibitors are substrates of the human multidrug transporter, suggesting that cells in patients that express the *MDR*1 transporter will be relatively resistant to the anti-viral effects of the HIV-1 protease inhibitors, and that absorption, excretion, and distribution of these inhibitors in the body may be affected by the multidrug transporter.

The HIV-1 protease, a product of the *pol* gene, is essential for the posttranslational processing of HIV-1 *gag-pol* polypeptide into its constituent proteins including itself (*1*, *2*). Numerous competitive inhibitors of the HIV-1 protease have been designed which mimic the protease substrates and prevent the formation of mature, infectious HIV-1 virions, effectively combating the HIV-1 virus especially in combination with other anti-retroviral drugs (*3*, *4*). Three of these inhibitors, ritonavir, saquinavir, and indinavir, have recently been approved for clinical use by the U.S. Food and Drug Administration (see ref *5*). However, major obstacles limit their efficacy. There are numerous reports of the development of HIV-1 strains resistant to these protease inhibitors (PIs) (*6*−*9*). Limited oral bioavailability requiring administration of very high doses of these drugs has mainly been attributed to inadequate absorption as well as first-pass metabolism in the liver where these drugs, especially saquinavir and indinavir, are rapidly metabolized by the hepatic cytochrome P450 3A system (see ref *10*). In addition, poor drug penetration into the brain may limit therapeutic efficacy in CNS infection.

Since the *MDR*1 multidrug transporter plays a role in limiting the efficacy of many drugs, we were interested in determining whether this transporter could have similar effects on the HIV-1 PIs. The *MDR*1 multidrug transporter (P-glycoprotein, or P-gp) is a 170 kDa cell-surface phosphoglycoprotein belonging to the ATP-binding cassette family of transporters. It is an energy-dependent efflux pump for a wide variety of structurally unrelated compounds (reviewed in ref *11*). High concentrations of P-gp can be found in the mucosa of the gastrointestinal tract and in brain capillary endothelial cells (*12*−*15*). Expression of *MDR*1 in CD4$^+$ T-lymphocytes, the major target for HIV-1 infection, has also been reported (*16*−*18*). Studies in mice with homozygous disruption of the *mdr*1 gene(s) demonstrate an important role of P-gp in excretion of some drugs and in limiting oral bioavailability and brain penetration of its substrate drugs (*19*). P-gp is believed to be responsible for the failure of some metastatic cancers to respond to long-term chemotherapy with anti-cancer drugs, which are substrates for the transporter (*20*, *21*). Hence, it is important to determine if the HIV-1 PIs are substrates of the *MDR*1

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transporter in order to devise ways to improve the bioavailability of these drugs in target cells.

In the present study, by a variety of assays, we found that the HIV-1 PIs are recognized by P-gp, and their effectiveness in inhibiting HIV-1 is reduced in *MDR*1-expressing cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Mammalian cell lines described in this report were cultured as monolayers at 37 °C, in 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. The previously described parental human carcinoma cell line, KB-3-1 (*22*), and its multidrug resistant derivative, KB-V1 (*23*), were maintained without selection or in 1 μg/mL vinblastine, respectively. The murine NIH3T3 cell line transfected with wild-type pHa*MDR*1/A, NIH-*MDR*-G185 (*24*), was grown in 60 ng/mL colchicine while its parental counterpart, NIH3T3, was cultured in nonselective medium. High Five insect cells were maintained in serum-free Excell 400 media at 27 °C (*25*). A recombinant baculovirus carrying the human *MDR*1 gene, with 6xHis-tag at the carboxy-terminal end, BV-*MDR*1(H6) [M. Ramachandra, S. V. Ambudkar, D. Chen, C. A. Hrycyna, S. Dey, M. M. Gottesman, and I. Pastan, *Biochemistry* (in press)] was used to infect confluent insect cells at a multiplicity of infection of 10. Cells were harvested 3 days post infection.

P-gp ATPase Activity. P-gp-mediated ATPase activity was measured in isolated membranes from High Five insect cells infected with a recombinant baculovirus encoding the human *MDR*1 gene prepared as previously reported (*26*). Vanadate-sensitive release of inorganic phosphate from MgATP in the presence and absence of drugs was measured using a colorimetric assay as described previously (*27*) with minor modifications (*26*). To evaluate the ability of the PIs to stimulate P-gp ATPase activity, membranes were preincubated with either verapamil, a known reversing agent of P-gp, or the various PIs for 3−5 min at 37 °C prior to the addition of 5 mM ATP and determination of ATP hydrolysis. The ability of SDZ PSC 833, a potent inhibitor of the *MDR*1 efflux pump, to modulate drug-stimulated ATPase activity was assessed by preincubating the membranes for 3 min with varying concentrations of SDZ PSC 833 before the addition of verapamil or PIs.

Photoaffinity Labeling with [^{125}I]IAAP. Photoaffinity labeling of isolated membranes from infected insect cells was performed as previously described (*26*). Briefly, membranes were preincubated with the indicated concentrations of competing drugs for 10 min prior to incubation with 5 nM [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) at room temperature (23 °C) followed by illumination with a UV lamp (wavelength 365 nm), analysis by SDS−PAGE, and autoradiography. Radioactivity associated with P-gp in the gel was quantified in a STORM 860 phosphoimaging system (Molecular Dynamics).

Drug Transport Analyses. Drug accumulation analyses were performed using Bodipy FL-vinblastine (0.5 μM) (Molecular Probes), Bodipy FL-prazosin (0.5 μM) (Molecular Probes), and Bodipy FL-verapamil (0.5 μM) (Molecular Probes), daunomycin (3 μM) (Calbiochem), and rhodamine-123 (0.263 μM) (Sigma). For studies with Bodipy analogues, approximately 1×10^5 of either NIH3T3 or NIH-*MDR*-G185 cells were incubated with the various drugs in the presence or absence of either cyclosporin A (Calbiochem), another known *MDR*1 modulator, or the HIV-1 PIs in prewarmed Iscove's Modified Dulbecco's Media (IMDM) supplemented with 5% calf serum at 37 °C for 1 h. Cells were then pelleted, resuspended in PBS and analyzed. For daunomycin and rhodamine-123, cells were initially loaded with the drugs in the presence or absence of cyclosporin A or the HIV-1 PIs at 37 °C for 1 h. The cells were then centrifuged at 200*g* for 5 min, resuspended in IMDM + 5% calf serum containing only cyclosporin A or the PIs, and incubated for another hour. Thereafter, the cells were pelleted, resuspended in PBS and analyzed. Analyses were performed with a FACSort flow cytometer equipped with CellQuest software.

Colony-Forming Assay. Vinblastine sensitivity of parental KB-3-1 and multidrug-resistant KB-V1 were evaluated by a colony formation assay as previously described (*28*). The colonies were counted automatically using the AlphaImager IS-1000 Digital Imaging System and its associated software. The vinblastine LD_{10} value in the presence or absence of verapamil or the HIV-1 PIs was expressed as the drug concentration which reduced the cloning efficiency of the cell lines to 10% of the control without drug. Verapamil was used as a positive control for reversing activity.

Anti-HIV-1 Activity Analyses. The role of P-gp in influencing the ability of the HIV-1 PIs to inhibit HIV-1 was determined. KB-3-1 and KB-V1 cells were preincubated for 30 min with the indicated concentrations of HIV-1 PIs and/or *MDR*1 inhibitors before the HIV-1 molecular clone, pNL4-3, was introduced into these cells by calcium phosphate precipitation (*29*). As a negative control, an unrelated plamid was transfected in place of pNL4-3. Four hours later, the transfection solution was removed and fresh medium containing the PIs and/or *MDR*1 inhibitors was added. Two days after transfection, the media were collected and centrifuged to get rid of floating cells/cell debris, before HIV-1 reverse transcriptase (RT) activity was determined (*30*). Quantitation of RT activity was performed using the STORM 860 phosphorimaging system. Transfection efficiency differences were normalized by cotransfection of pCMV-βgal plasmid. β-Galactosidase activity in these transfected cells was determined using chlorophenol-red-β-D-galactopyranoside (CPRG) as substrate (*31*). The rate of CPRG hydrolysis was monitored at 570 nm at room temperature using SpectraMAX 250 microplate reader (Molecular Devices). The results are reported as percent RT (% RT), which is the normalized reverse transcriptase activity, expressed as a percentage of cells that were treated with the various concentrations of the HIV-1 PIs as compared to the untreated cells.

RESULTS

HIV-1 Protease Inibitors Stimulate P-gp-Specific ATPase Activity. P-gp is an ATP-dependent efflux pump which prevents accumulation of drugs within cells. Known substrates of P-gp, e.g., vinblastine, daunorubicin, and doxorubicin, as well as agents such as verapamil which modulate transport of other substrates have been shown to stimulate P-gp-specific ATPase activity (*32*, *33*) (Figure 1A). As

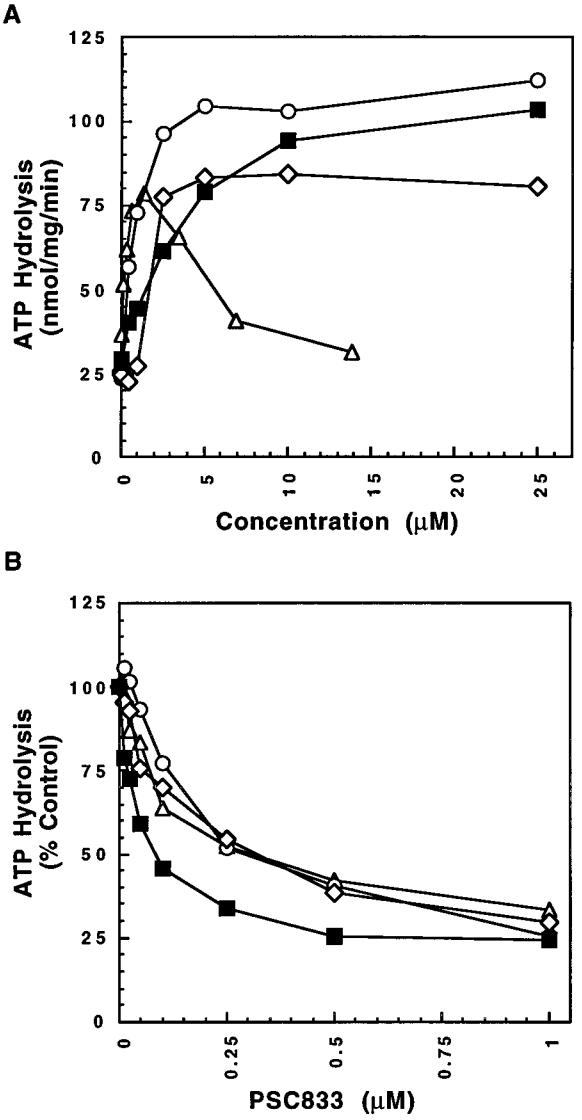


FIGURE 1: (A) Stimulation of Pgp-mediated ATP hydrolysis by HIV-1 protease inhibitors. The vanadate sensitive Pgp-ATPase activity was measured in isolated membranes (10 μg of protein) from insect cells infected with recombinant baculovirus virus, BV-*MDR*1(H6), in the presence of 5 mM ATP and 10 mM $MgCl_2$. Assays were done in the presence of the indicated concentration of either of the four drugs, verapamil (■), ritonavir (△), saquinavir (◇), or indinavir (○), following the modified protocol of Dey et al. (*26*). (B) Effect of PSC833 on HIV-1 protease inhibitor-stimulated Pgp-ATPase activity. Drug-stimulated ATP hydrolysis by Pgp was carried out following preincubation of the membranes for 3 min at 37 °C with the indicated concentrations of SDZ PSC 833. Following preincubation, either of the four stimulatory drugs were added to final concentrations of 3 μM for ritonavir (△), 25 μM for verapamil (■), 25 μM for saquinavir (◇), and 25 μM for indinavir (○). The rate of ATP hydrolysis was measured by a colorimetric assay as described in Materials and Methods.

shown in Figure 1A, the HIV-1 PIs, ritonavir, saquinavir, and indinavir, stimulated P-gp-dependent ATPase activity in a concentration-dependent manner. The K_m and V_{max} for stimulation of each of the three drugs (ritonavir, $K_m = 0.06$ μM, $V_{max} = 77.3$ nmol/mg/min; saquinavir, $K_m = 1.186$ μM, $V_{max} = 93.6$ nmol/mg/min; indinavir, $K_m = 0.47$ μM, $V_{max} = 112.2$ nmol/mg/min) were comparable to that of verapamil ($K_m = 1.1$ μM, $V_{max} = 101.7$ nmol/mg/min). Furthermore, SDZ PSC 833, a nonimmunosuppressive analogue of cyclosporin A and a potent inhibitor of the multidrug transporter, inhibited the ATPase activity that was stimulated by the HIV-1 PIs (Figure 1B). The observed K_is for inhibition by SDZ PSC 833 of the stimulated activity were of the same order of magnitude for all the drugs (ritonavir $K_i = 0.005$ μM; saquinavir $K_i = 0.008$ μM; indinavir $K_i = 0.009$ μM) including verapamil ($K_i = 0.002$ μM). This suggests a mechanism of stimulation of *MDR*1-associated ATPase activity by the HIV-1 PIs similar to verapamil.

HIV-1 Protease Inhibitors Compete with [^{125}*I*] *Iodoarylazidoprazosin* (*IAAP*) *for Binding to the Multidrug Transporter*. IAAP, a photoaffinity analogue of the P-gp substrate prazosin, has been extensively used for studying the drug-binding properties of the transporter (*34*). When photoaffinity labeling of P-gp was carried out in membranes from insect cells infected with an *MDR*1-encoding recombinant baculovirus, the labeling of P-gp was inhibited in a dose-dependent manner by all three PIs (Figure 2). This result suggests a mutually exclusive binding between the HIV-1 PIs and IAAP with the *MDR*1 transporter.

Transport of Various P-gp Substrates Is Affected by HIV-1 Protease Inhibitors. Transport of various fluorescent P-gp substrates was examined by FACS analysis, and the results are shown in Figure 3. NIH3T3 cells with low expression of P-gp exhibit high fluorescence due to increased accumulation and reduced efflux of fluorescent substrates. The high fluorescence intensity is largely unaffected by the addition of either cyclosporin A or any of the HIV-1 PIs. On the other hand, the NIH-*MDR*-G185 cells, which express high levels of P-gp, appear less fluorescent. In the presence of cyclosporin A, a known inhibitor of P-gp, P-gp expressing cells fluoresce more intensely. Increased fluorescence of NIH-*MDR*-G185 cells is also evident for all the substrates tested (Bodipy FL-vinblastine, Bodipy FL-verapamil, Bodipy FL-prazosin, daunomycin, and rhodamine) in the presence of 7 or 21 μM of ritonavir. However, for saquinavir and indinavir, the fluorescence intensity of the NIH-*MDR*-G185 cells was slightly increased only when Bodipy FL-vinblastine was used as the substrate and in the presence of high concentrations (37 and 89 μM saquinavir; 35 and 84 μM indinavir) of the HIV-1 PIs. Similarly, ritonavir (at 1.4 and 3.5 μM) but not saquinavir (at 10 and 25 μM) or indinavir (at 10 and 25 μM) reversed the reduced accumulation of [^{3}H]vinblastine in drug resistant NIH-*MDR*-G185 cells (data not shown).

Vinblastine Resistance in Multidrug Resistant Cells Can be Reversed by HIV-1 Protease Inhibitors. By themselves, the HIV-1 PIs are not very toxic to cells. The drug-sensitive KB-3-1 cells which exhibit an LD_{50} value of 0.572 nM with vinblastine required 14 μM of ritonavir, >45 μM of saquinavir, and 232 μM of indinavir to reduce colony formation ability by 50% (data not shown). By comparison, the P-gp-expressing drug resistant cells, KB-V1, which showed an LD_{50} of 473 nM for vinblastine, are also cross-resistant to the PIs, ritonavir or indinavir, requiring about twice as much of these drugs for 50% cell killing (data not shown). To further establish the interaction of HIV-1 PIs with P-gp in these drug resistant KB-V1 cells, the vinblastine sensitivity of KB-V1 cells was examined in the presence of either verapamil, a known P-gp reversing agent, or the HIV-1 PIs. Verapamil as well as the HIV-1 PIs only marginally enhanced the cytotoxic activity of vinblastine against the

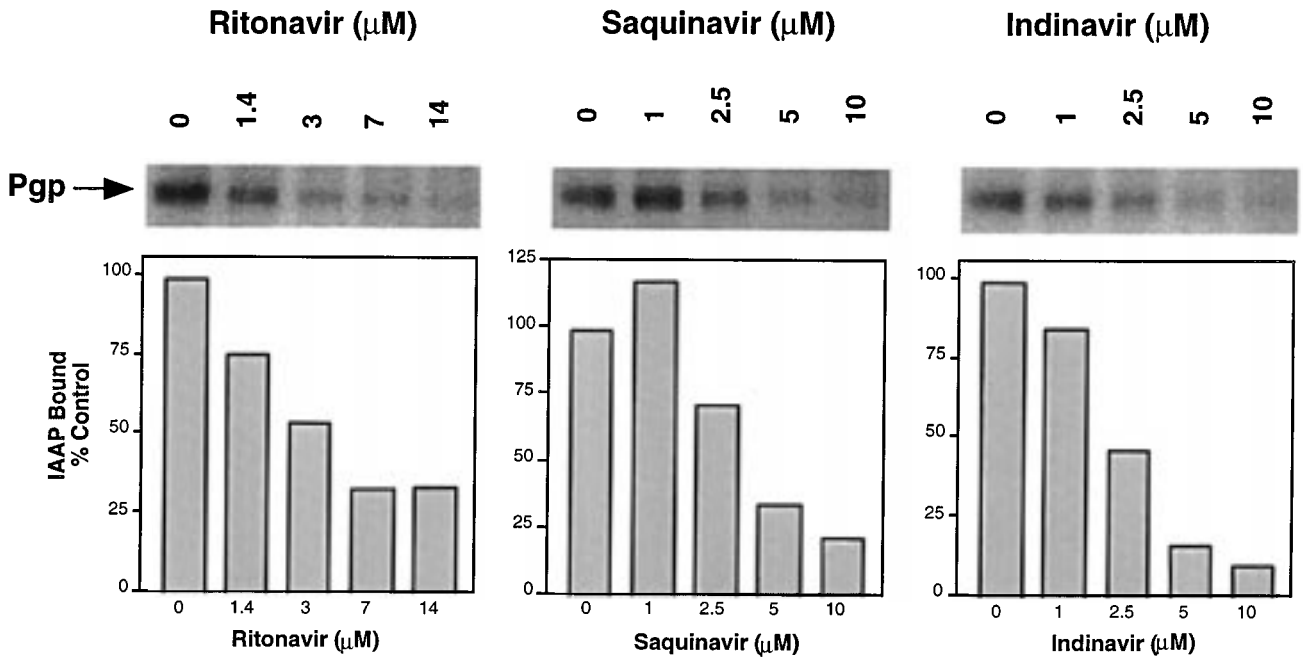
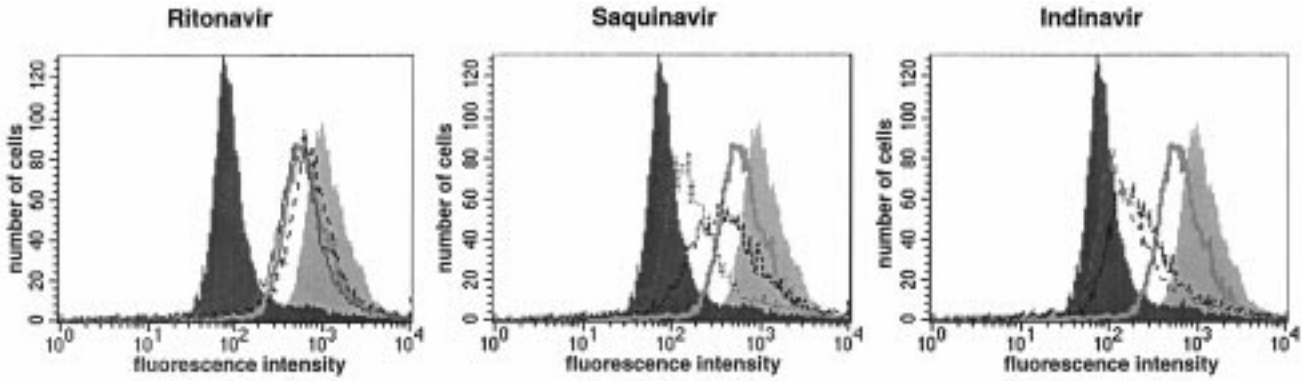


FIGURE 2: Effect of HIV-protease inhibitors on [^{125}I]IAAP photoaffinity labeling of Pgp. Membranes (10 μg of protein) isolated from High Five insect cells, expressing Pgp, were preincubated with either of the three HIV-1 protease inhibitors, ritonavir, saquinavir, and indinavir, prior to addition of 2 nM [^{125}I]IAAP. Photoaffinity labeling was carried out as mentioned in the Materials and Methods. A total of 2 μg of membrane protein/lane was separated in an 8% gel by SDS−PAGE. The upper panels are autoradiograms of the gels showing the area corresponding to Pgp (140 kDa). The lower panels show quantification of radioactivity associated with Pgp expressed as % bound with binding in the absence of protease inhibitors taken as 100%.



	NIH3T3	NIH-MDR-G185						
		no drug	CyA	Ritonavir		Saquinavir		Indinavir	
			5 μM	7 μM	21 μM	37 μM	89 μM	35 μM	84 μM
Daunomycin	382	71	655	325	403	74	104	70	80
rhodamine 123	799	141	599	184	615	131	155	144	151
bodipy FL-vinblastine	982	79	557	500	673	158	372	132	189
bodipy FL-verapamil	764	64	231	244	757	100	84	124	100
bodipy FL-prazosine	733	58	346	461	770	135	133	139	165

FIGURE 3: *MDR*1-mediated transport of Bodipy FL-vinblastine in NIH3T3 or NIH-*MDR*-G185 cells. Cells were incubated with bodipy-vinblastine for 1 h at 37 °C, pelleted, and fluorescence intensity was analyzed by FACS. Fluorescence histograms are shown in the upper panel. (shaded area) NIH3T3 cells; (dark area) NIH-*MDR*-G185; solid line: NIH-*MDR*-G185 + 5 μM cyclosporin A (CyA); (light dotted line) NIH-*MD*R−G185 + 7 μM ritonavir or 37 μM saquinavir or 35 μM indinavir; (dark dotted line) NIH-*MDR*-G185 + 21 μM ritonavir or 89 μM saquinavir or 84 μM indinavir. The lower panel shows a table representing the median fluorescence intensity of NIH3T3 or NIH-*MDR*-G185 cells that were incubated with the indicated *MDR*1 fluorescence substrates in the presence/absence of CyA or HIV-1 protease inhibitors.

drug-sensitive KB-3-1 cells (data not shown). However, in KB-V1 cells, the addition of 6 μM verapamil, 4 μM ritonavir, 15 μM saquinavir, or 14 μM indinavir sensitizes these resistant cells to vinblastine probably by competing with vinblastine for the multidrug transporter (Figure 4). Verapamil displays the most potent effect, shifting the LD_{10} value of vinblastine in the drug-resistant KB-V1 cells from 880 to 2.2 nM, hence making these cells as sensitive as the parental

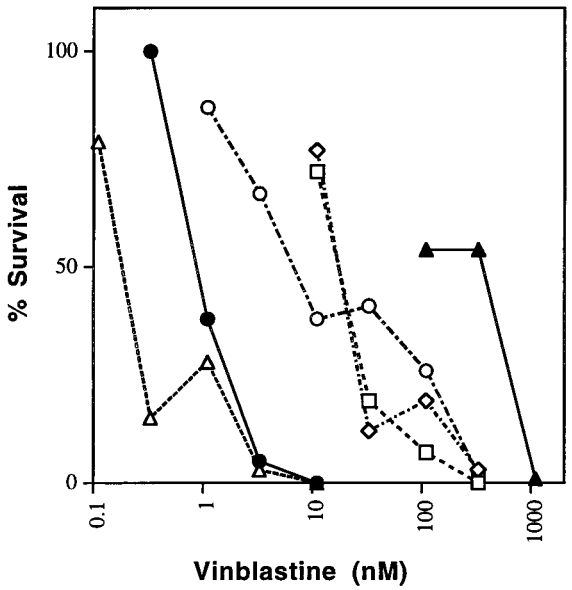


FIGURE 4: Response of KB-3-1 or KB-V1 cells to vinblastine in the presence of verapamil or the HIV-1 protease inhibitors. Cell survival in the presence of increasing concentrations of vinblastine was determined by colony formation assays as described in Materials and Methods. ● denotes KB-3-1; KB-V1 is indicated by ▲; KB-V1 + 6 μM verapamil is indicated by △; KB-V1 + 4 μM ritonavir is indicated by □; KB-V1 + 15 μM saquinavir is indicated by ○ and KB-V1 + 14 μM indinavir is indicated by ◇.

KB-3-1 cells which have an LD_{10} of 2.75 nM. Of the HIV-1 PIs, ritonavir displays the greatest ability to reverse the vinblastine resistance of KB-V1 attenuating the vinblastine LD_{10} value to 77 nM. In the presence of saquinavir or indinavir, the vinblastine LD_{10} is reduced to 225 and 209 nM, respectively.

HIV-1 Protease Inhibitors Are Less Effective and Can be Made More Effective Using MDR1-Specific Inhibitors in P-gp-Expressing Cells. We next determined the biological significance of the observation that the HIV-1 PIs are substrates for the multidrug transporter. To ascertain if this observation translates into less effective inhibition of HIV-1 by the HIV-1 PIs in cells containing *MDR*1, the antiviral activity of these compounds in drug sensitive KB-3-1 and drug resistant KB-V1 cells was determined. The antiviral activity was assessed by measuring the amount of virus produced, as reflected by the reverse transcriptase activity, in the media of cells that were transfected with the HIV-1 molecular clone, pNL4-3. Figure 5 shows that in the parental drug-sensitive KB-3-1 cells, all three PIs demonstrated a dose-dependent reduction in the reverse transcriptase production to less than 25% of the untreated cells at 1 μM drug. However, in the drug-resistant KB-V1 cells, while there is an observable dose-dependent reduction, high concentrations of the PIs were needed to achieve this inhibition and the same extent of inhibition is not achieved in KB-V1 cells at any protease inhibitor concentration tested. This relative resistance to PIs is reversed by agents which inhibit P-gp (Table 1). These results provide evidence for a role of P-gp in subverting the antiviral efficacy of HIV-1 PIs.

DISCUSSION

Significant advances in the treatment of HIV-1 infection have been achieved with the development of HIV-1 PIs.

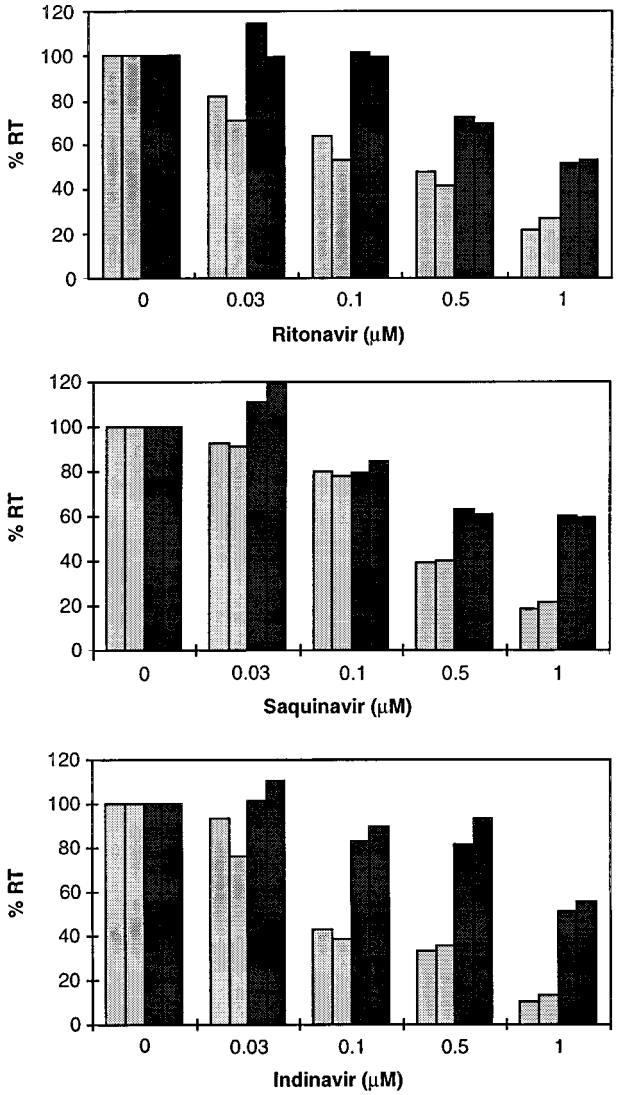


FIGURE 5: Effectiveness of HIV-1 protease inhibitors against HIV-1 in KB-3-1 or KB-V1 cells. Antiviral activity, as measured by reverse transcriptase activity, was determined in the media of cells that had been transfected with pNL4-3, a molecular cDNA clone of HIV-1. The results are expressed as the % RT, which is the reverse transcriptase activity of the cells treated with the various concentrations of the HIV-1 protease inhibitors in relation to the untreated cells expressed as a percentage. The % RT activity of KB-3-1 cells is represented by light-colored bars while the % RT activity of KB-V1 cells is shown as dark-colored bars. The two light-/dark-colored bars for each concentration point represent data from duplicate transfections. The RT activity in medium from transfected KB-3-1 cells was 29 000 RT units and in medium from transfected KB-V1 cells, it was 11 000 RT units.

These inhibitors, when used in combination with other antiretroviral drugs, are highly efficient at reducing viral replication and correcting $CD4^+$ T-cell defects (*35*). However, in addition to development of HIV-1 protease mutants, problems with absorption, metabolism, and distribution of these drugs in the body may limit their effectiveness. The studies reported in this paper suggest that the HIV-1 PIs are substrates for the *MDR*1 encoded multidrug transporter (P-gp), and therefore, this transporter may contribute to these problems.

HIV-1 Protease Inhibitors Are Substrates of the MDR1 Transporter. Several different analyses suggest that the three HIV-1 PIs, ritonavir, saquinavir and indinavir are recognized

Table 1: *MDR*1 Inhibitors Increase the Ability of the HIV-1 Protease Inhibitors (PIs) to Inhibit HIV-1 in *MDR*1 Expressing Cells[a]

	KB-V1 cells				
		MDR1 inhibitor			
HIV-1 protease inhibitor	none	quinidine (20 μM)	rapamycin (5 μM)	PSC 833 (1 μM)	KB-3-1 cells none
none	100	100	100	100	100
ritonavir (0.1 μM)	94	38	59	69	51
saquinavir (1 μM)	51	19	19	25	15
indinavir (1 μM)	52	16	14	23	16

[a] Results are expressed as % reverse transcriptase (RT) activity (mean of at least two determinations) in *MDR*1-expressing cells (KB-V1) in the absence (row 1) or presence (rows 2−4) of the various HIV-1 Pls as well as in the absence (col 2) or presence (col 3−5) of three different *MDR*1 inhibitors. The last column shows the inhibitory effect of the HIV-1 Pls on non-*MDR*1 parental KB-3-1 cells for comparison. The % RT activity obtained in the presence of both HIV-1 Pls and *MDR*1 inhibitors were normalized against the RT obtained in the presence of *MDR*1 inhibitors alone which were expressed as 100%. For each column, 100% in relative normalized RT units (mean of at least two determinations) was ~3700 (col 2), 9400 (col 3), 4100 (col 4), 6400 (col 5), and 25 000 (col 6).

by the multidrug transporter. In vitro studies, utilizing crude membranes isolated from insect cells infected with *MDR*1-expressing recombinant baculovirus, showed that these PIs stimulate P-gp-associated ATPase activity as well as verapamil, a known P-gp substrate (Figure 1A). Furthermore, this stimulation of ATPase activity was inhibited by SDZ PSC 833, a nonimmunosuppressive analogue of cyclosporin A (Figure 1B), which is a potent inhibitor of P-glycoprotein in current use in clinical trials to sensitize drug-resistant cancer cells to anti-cancer drugs. In addition, these PIs significantly inhibited the binding of [^{125}I]IAAP to the multidrug transporter in a concentration-dependent manner in these membrane preparations (Figure 2), suggesting that all three PIs are interacting with the major substrate domain of P-glycoprotein.

Cell-based approaches including analyses of transport of fluorescent substrates and reversal of drug resistance in colony forming assays were also undertaken to examine the effects of the HIV-1 PIs on the transport of known *MDR*1 substrates. These studies show that the HIV-1 PIs can reduce drug resistance as would be expected for P-gp substrates, but they are less potent than other documented *MDR*1 reversing agents such as verapamil and cyclosporin A. Of the PIs, ritonavir is the most effective in modulating the effects of the other P-gp substrates, since a lower concentration of ritonavir (4 μM) was needed to sensitize P-gp-expressing cells to vinblastine (Figure 4) and it competed effectively with all the fluorescent P-gp substrates tested (Figure 3, lower panel). Additional evidence that the multidrug transporter reduces accumulation of the HIV-1 PIs was obtained in KB-V1 cells overexpressing P-gp. Production of the HIV-1 virus, as detected by the appearance of reverse transcriptase in medium, was reduced by all three HIV-1 PIs in the parental KB-3-1 cells. The *MDR*1-expressing KB-V1 cells, despite reduced production of HIV-1, showed less sensitivity to these HIV-1-PIs, consistent with lower intracellular concentrations of these agents (Figure 5).

Implications of the Findings That the HIV-1 Protease Inhibitors Are Substrates of the MDR1 Transporter. One of the obvious consequences of the PIs being recognized by the multidrug transporter is that the same concentration of these drugs would be less effective against HIV-1 in P-gp-expressing cells. The major targets for HIV-1 infection are the CD4$^+$ T-lymphocytes, monocytes/macrophages (*36*), and the brain (*37*). These targets were also found to express P-gp to varying degrees. Subsets of CD4$^+$ T-lymphocytes were reported to express P-gp (*17*). Two reports (*38*, *39*) suggest that HIV-1 infection induced the surface expression of P-gp but that the increased expression was abnormal in HIV-1 patients since these *MDR*1-expressing cells are still rhodamine bright and cannot be reversed by cyclosporin A. On the other hand, Lucia et al. (*16*) found lower expression of the *MDR*1 transporter in the CD4$^+$ T-cells from HIV-1 infected patients, and these cells are rhodamine bright when compared with noninfected controls (*16*). P-gp was also found to be expressed in monocytes/macrophages (*40*, *41*) as well as on the lumenal surfaces of capillary endothelial cells in the brain, where it serves as part of the blood-brain barrier to restrict entry of xenobiotics into the brain (*13*, *14*, *42*−*44*). On the basis of our results, depending on the level of expression of *MDR*1 in these target cells, the effectiveness of these PIs on CD4$^+$ T-cells and brain cells may be significantly reduced. The expression of P-gp in capillary endothelial cells of the brain may explain the poor ability of indinavir (20%) or the inability of ritonavir and saquinavir to penetrate the blood-brain barrier in animal studies (*45*).

The presence of the *MDR*1 transporter in the epithelial cells of the gastrointestinal tract may play a role in the relatively poor absorption of these drugs which are administered orally. The current recommended doses for ritonavir, saquinavir and indinavir are 600 mg, twice daily, 600 mg, three times daily, and 800 mg, three times daily, respectively (*45*). Besides the multidrug transporter, poor absorption due to other factors as well as first pass metabolism by the cytochrome P450 system, especially for saquinavir and indinavir, limit the plasma bioavailability of these drugs to ~11.2 mg/L for ritonavir, 0.04−0.1 mg/L for saquinavir and 5−11 mg/L for indinavir (*46*, *47*). The concentration of the drugs in the plasma, if fully available to the target cells, would be sufficient to effectively inhibit HIV-1 replication, as can be deduced from Figure 5 and the reported literature in which the EC_{50} (drug concentration that inhibits 50% of viral replication) was found to be 4−153 nM (*48*) for ritonavir, 1−30 nM for saquinavir (*49*), and 25−100 nM for indinavir (*50*). However, the presence of the *MDR*1 transporter in the target cells and in the blood-brain barrier may further reduce the intracellular availability of these drugs and compromise their effectiveness.

The improved delivery of these HIV-1 PIs to sites where HIV-1 is replicating may be possible by inhibiting P-gp, thus increasing absorption from the gastrointestinal tract, and accumulation in target cells. Noncytotoxic chemomodulators or reversing agents, like verapamil, cyclosporin A, SDZ PSC 833, FK 506, rapamycin, or quinidine, can be used to inhibit the *MDR*1 efflux pump and make the PIs more available to target cells (*20*, *51*, *52*). For example, we showed that SDZ PSC 833, a nonimmunosuppressive analogue of cyclosporin A and a potent inhibitor of P-gp, was able to inhibit the stimulation of P-gp-specific ATPase activity by the three HIV-1 PIs (Figure 1B). We further demonstrated that PSC 833 and other P-gp inhibitors, while increasing HIV-1

production in *MDR*1-expressing cells, also sensitized these cells to the HIV-1 PIs (Table 1). PSC 833 has also been reported to improve the penetration of two different P-gp substrates, ivermectin (*53*) and colchicine (*54*), into the brain of mice and rats, respectively. Nonetheless, because of the direct and indirect effects of P-gp function and reversing agents on HIV-1 replication (Lee, C. G. L., Ramachandra, M., Jeang, K. T., Martin, M. A., Pastan, I. and Gottesman, M. M., unpublished observations; Figure 5, Table 1), the choice of a P-gp inhibitor to be used together with the HIV-1 PIs would have to be carefully determined. This is especially true in HIV-1 therapy since PIs have been found to be the most effective when used in combination with other antiviral drugs. The ultimate reversing agent should not potentiate HIV-1 replication nor have other undesirable effects on the already immunocompromised patients nor interact negatively with the other antiviral drugs.

In summary, the HIV-1 PIs, ritonavir, saquinavir, and indinavir, were found to be recognized by the multidrug transporter. The effectiveness of these drugs was diminished in P-gp-expressing cells. Further studies are needed to determine if P-gp expression in target cells is related to the failure of some patients to respond to these protease inhibitor drugs and to identify appropriate reversing agents to improve the delivery of these drugs to target cells.

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