HIV Protease Inhibitors Are Inhibitors but Not Substrates of the Human Breast Cancer Resistance Protein (BCRP/ABCG2)

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d) **Abbreviations**:  
BCRP, breast cancer resistance protein; MRP2, multidrug-resistance protein 2; P-gp, P-glycoprotein; HIV, human immunodeficiency virus; HPI, HIV protease inhibitor; ABC, ATP-binding cassette; MX, mitoxantrone; HEK cells, human embryonic kidney cells; mAb, monoclonal antibody; FTC, fumitremorgin C.

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Abstract

BCRP is a recently discovered ABC drug transporter. Hence, the full spectrum of therapeutic agents that interact with BCRP remains to be elucidated. Since HIV protease inhibitors (HPIs) are well known P-gp substrates and there is an overlap in substrate specificity between P-gp and BCRP, this study was performed to investigate whether HPIs are substrates and/or inhibitors of BCRP. First, the effect of HPIs on BCRP efflux activity in HEK cells stably expressing wild-type BCRP (482R) and its two mutants (482T and 482G) was studied by measuring intracellular mitoxantrone fluorescence using flow cytometry. We found that ritonavir, saquinavir and nelfinavir were effective inhibitors of wild-type BCRP (482R) with IC₅₀ values of 19.5 ± 0.8 µM, 19.5 ± 7.6 µM and 12.5 ± 4.1 µM, respectively. Ritonavir, saquinavir and nelfinavir inhibited 482T and 482G with IC₅₀ values that were approximately 2 times greater then that for 482R. Indinavir and amprenavir had no significant inhibition on BCRP activity. Direct efflux of radiolabeled HPIs in HEK cells was measured to determine whether the HPIs are substrates of BCRP. None of the HPIs were found to be transported by BCRP. Taken together, ritonavir, saquinavir, nelfinavir, indinavir and amprenavir are not substrates for BCRP. However, ritonavir, saquinavir and nelfinavir are effective inhibitors of the transporter. These results suggest that BCRP may play an important role in drug-drug interactions involving co-administration of the HPIs with drugs that are substrates of the transporter.
The human breast cancer resistance protein (BCRP/MXR/ABCG2) is a relatively new ABC transporter originally cloned from drug-selected human cancer cell lines and human placenta (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). Like P-glycoprotein (P-gp), BCRP confers high levels of resistance to anthracyclines, mitoxantrone and the camptothecins by enhancing drug efflux from the cell (Litman et al., 2000; Bates et al., 2001; Ejendal and Hrycyna, 2002). Indeed, in acute leukemia, BCRP may play an important role in resistance to flavopiridol (Robey et al., 2001b; Nakanishi et al., 2003b). In addition to its role in resistance to chemotherapeutic agents, BCRP actively transports structurally diverse organic drugs, conjugated or unconjugated, such as estrone-3-sulfate, 17β-estradiol 17-(β-D-glucuronide) and methotrexate (Volk et al., 2002; Chen et al., 2003; Imai et al., 2003; Suzuki et al., 2003; Volk and Schneider, 2003). Sequence analysis of BCRP cDNA revealed mutations at position 482 in several drug-selected cell lines (Honjo et al., 2001; Allen et al., 2002). Subsequent studies showed that position 482 is important in determining substrate specificity of BCRP (Honjo et al., 2001; Allen et al., 2002; Volk et al., 2002; Chen et al., 2003; Robey et al., 2003). For instance, wild-type BCRP (482R) does not efflux rhodamine 123 but the mutants 482T and 482G can readily transport rhodamine (Honjo et al., 2001). BCRP is prominently expressed in placental syncytiotrophoblasts, in the epithelium of the small intestine, and in the liver canalicular membrane (Maliepaard et al., 2001; Doyle and Ross, 2003). In fact, BCRP transcript is expressed in the intestine in greater amounts than P-gp (Taipalensuu et al., 2001). This strategic and substantial tissue localization implies that BCRP also functions as a protective drug efflux pump in the placenta and the intestine. Indeed, Jonker et al. (Jonker et al., 2000) have shown that treatment with the BCRP inhibitor, GF120918 (also a P-gp inhibitor), decreases plasma clearance and hepatobiliary excretion of the anticancer agent, topotecan, and increases
absorption of this drug from the small intestine in P-gp knockout mice. In pregnant GF120918-
treated P-gp-deficient mice, the relative fetal concentration of topotecan was 2-fold higher than
that in pregnant vehicle-treated mice. These observations suggest that Bcrp1 (the murine
ortholog of BCRP) mediates apically directed drug transport, reduces drug bioavailability, and
protects fetuses against therapeutic agents. A recent clinical study showed that inhibition of
BCRP significantly increases the oral bioavailability of topotecan in cancer patients from 40 %
to 97 % (Kruijtzer et al., 2002). Thus, BCRP is important in determining absorption, distribution,
and elimination of drugs that are substrates for this transporter.

Many of the drugs transported by BCRP (listed above) are also substrates of P-gp. Some
have suggested that the substrate selectivity of BCRP substantially overlaps with that of P-gp
(Litman et al., 2000; Doyle and Ross, 2003). Therefore, to comprehensively evaluate the
importance of BCRP in the \textit{in vivo} disposition of drugs, it is important to determine the substrate
profile of BCRP. Hence, we have asked if the HIV protease inhibitors (HPIs), excellent
substrates of P-gp, are substrates of BCRP. In this paper, we present data that addresses this
question.
Materials and Methods

Materials. Ritonavir was a gift from NIH AIDS Research and Reference Reagent Program (NIH, Bethesda, MD). Saquinavir and [14C]-saquinavir (27.71 mCi/mmol) were from Roche Laboratories (Nutley, NJ) and amprenavir was from GlaxoSmithKline (Research Triangle Park, NC). Nelfinavir and [3H]-nelfinavir (60 mCi/mmol) were from Pfizer Global Research and Development (San Diego, CA). [3H]-Ritonavir (1.2 Ci/mmol), [3H]-amprenavir (2.0 Ci/mmol) and [3H]-mitoxantrone (1.5 Ci/mmol) were purchased from Moravek (Brea, CA). Indinavir was a gift from Merck (West Point, Pennsylvania). Mitoxantrone (MX) hydrochloride was from Sigma (St. Louis, MO). Fumitremorgin C (FTC) was a generous gift from Dr. Susan E. Bates (NCI, Bethesda, MD). HPLC grade DMSO was obtained from Fisher Scientific (Pittsburgh, PA) and used as the solvent for making stock solutions of all the drugs and FTC. Eagle’s Minimum essential medium (MEM), penicillin-streptomycin-glutamine solution and trypsin-EDTA were purchased from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s phenol-free low-glucose medium (DMEM), Phosphate buffered saline (PBS), trypsin-EDTA solution and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA).

Cell Culture and Whole Cell Lysate Preparation. HEK293 cells stably transfected with pcDNA empty vector and cDNAs coding for wild-type BCRP (482R) and its two mutants (482T and 482G) were obtained from Dr. Susan E. Bates (NCI, Bethesda, MD) (Robey et al., 2003). All the cell lines were grown and maintained in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 mg/ml G418 (Mediatech Inc., Herndon, VA) at 37°C in a 5% CO2 incubator. Cells were grown to 80-90% confluence and
treated with trypsin-EDTA prior to harvesting for subculturing or efflux assays. Only cells within six passages were used in subsequent transport experiments.

Whole cell lysates were prepared as follows: Briefly, cell pellet from 5 x 10⁶ cells was suspended in approximately 100 µl of lysis buffer containing 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 50 µg/ml PMSF, 0.25% (w/v) SDS, 250 µg/ml DNase I (Invitrogen), and protease inhibitors (Complete™, Roche Molecular Biochemicals). The mixture was then incubated on ice for 1 h with gentle vortexing occasionally and centrifuged at 2,400 x g for 10 min at 4°C. The supernatant was subjected to immunoblotting as described below. Protein concentrations were determined using a modified Lowry assay (Peterson, 1979) and bovine serum albumin as standard.

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting.** Gel electrophoresis of whole cell lysates from various cell lines was carried out using 10% SDS-polyacrylamide mini gels in a Bio-Rad Mini-protein II electrophoresis cell. Standard western blot procedure was followed. Briefly, proteins were transferred on Immuno-P nitrocellulose membranes (Millipore, Billerica, MA) using 25 mM Tris, 192 mM glycine and 20% methanol buffer. The blot was blocked with 5% skim milk in TBS-T buffer (10 mM Tris/HCl, pH 7.5, 0.15 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. The blot was incubated with mAb BXP-21 (which recognizes an internal epitope in the nucletotide-binding domain (aa 271-396) of BCRP (Maliepaard et al., 2001)) (Kamiya Biomedical Co., Seattle, WA) at 1:500 dilution as the primary antibody for 2 h at room temperature followed by washing the blot 3 times with TBS-T. The blot was then incubated with the secondary antibody, goat anti-mouse (GAM)-HRP conjugate (dilution 1:10,000) (BioRad, Hercules, CA) for 1 h at room temperature. The blot was then washed 3
times with TBS-T buffer and developed using a Supersignal West Pico chemiluminescence detection kit (Pierce chemical Co., Rockford, IL). Relative levels of BCRP expression were quantitated by densitometric analysis of the immunoblot with Chemi Doc system (BioRad, Hercules, CA) and Quantity One software version 4.3.0. For immunoblotting of P-gp, MRP1 and MRP2, the same procedure as described above was used except that the primary antibody used was mAb F4 (Kamiya) for P-gp (dilution 1:1,000), mAb QCRL-1 (Signet, Dedham, MA) for MRP1 (dilution 1:500) and mAb M2III-6 (Alexis Biochemicals) for MRP2 (dilution 1:100).

**Flow Cytometric Efflux Assays.** The efflux assays were essentially the same as previously described (Wang et al., 2000; Robey et al., 2001a) with minor modifications. Briefly, BCRP-expressing or vector control HEK cells were harvested and suspended in incubation buffer (DMEM supplemented with 5% FBS and 5 mM HEPES buffer) at cell concentration of approximately 10^6 cells per reaction in 1 ml of volume. In the accumulation phase, cell suspensions were co-incubated with 10 µM MX and variable concentrations (0-150 µM) of HPIs or 10 µM FTC as a positive control for 30 min at 37°C to allow accumulation of drugs. Cells were then immediately transferred on ice, washed once with ice-cold PBS and resuspended in 1 ml of incubation buffer containing only the respective HPIs or FTC at their respective concentrations as in the accumulation phase and incubation was continued for 1 h at 37°C to allow maximum efflux of MX (efflux phase). Cells were washed once and finally resuspended in 1 ml of ice-cold PBS. Intracellular MX fluorescence was measured within 1h with a 488 nm argon laser and a 650 nm bandpass filter in a BD FACSCAN flow cytometer. Cells were kept on ice until intracellular MX fluorescence was read. Ten thousand (10^4) events were collected for all the samples. Cell debris was eliminated by gating on forward versus side scatter. Cells in
medium containing vehicle (4% (v/v) DMSO) yielded the blank histogram, a measure of cellular autofluorescence. Cells in medium containing MX alone or medium containing MX and FTC or HPI generated the control and FTC or HPI histograms, respectively. The difference ($\Delta F$) between the median fluorescence of the control histogram and the median fluorescence of the FTC or HPI histogram was used to express inhibition of MX efflux by FTC or HPIs, respectively. The maximum concentration of DMSO used as vehicle for HPIs was 4% (v/v) in all the efflux assays. No effects of the vehicle on MX efflux were observed at this concentration.

**Inhibition of BCRP-mediated efflux of $[^3H]$-MX by HPIs.** Direct efflux of $[^3H]$-MX in BCRP-expressing or vector-control HEK cells in the presence and absence of cold HPIs was also measured to examine whether HPIs inhibit BCRP-mediated efflux of $[^3H]$-MX. Briefly, 0.5 x 10^6 cells were incubated with $[^3H]$-MX (20 nM, 0.03 $\mu$Ci) and various concentrations of HPIs (25 or 100 $\mu$M) in 0.5 ml of incubation buffer (DMEM supplemented with 5 mM HEPES buffer) for 30 min at 37°C. To avoid possible protein binding of $[^3H]$-MX, FBS was omitted from the incubation buffer. The cells were then transferred on ice, washed once with ice-cold PBS and resuspended in 0.5 ml of incubation buffer in the presence of respective HPIs for 1 h at 37°C. Efflux from the cells was terminated by washing once in ice-cold PBS. The cell pellet was lysed with 1 ml of 1% SDS and 900 $\mu$l of the lysate was subjected to counting in a scintillation counter. Counts were normalized to protein concentration that was measured using the remaining lysate by the modified Lowry assay. The intracellular $[^3H]$-MX was calculated based on radioactivity associated with the cell pellet and expressed as pmols of MX/µg protein. The concentration of DMSO used as vehicle for HPIs was 0.5 % (v/v). No effects of the vehicle on $[^3H]$-MX efflux were observed at this concentration.
**Efflux of Radiolabeled HPIs.** Direct efflux assays using radiolabeled HPIs were carried out to confirm whether the HPIs are substrates of BCRP as follows. Briefly, the BCRP-expressing or vector-control HEK cells (0.5 x 10^6 cells) were incubated with a radiolabeled HPI at a desired concentration (with or without FTC) in 0.5 ml of incubation buffer (DMEM supplemented with 5 mM HEPES buffer) for 30 min at 37°C to allow maximum accumulation of HPIs in cells. The concentrations (specific activities) of [3H]-ritonavir, [14C]-saquinavir, [3H]-amprenavir and [3H]-nelfinavir used in the accumulation phase were 0.5 µM (0.6 µCi/ml), 1 µM (0.028 µCi/ml), 0.25 µM (0.5 µCi/ml) and 1 µM (0.06 µCi/ml), respectively. The cells were then transferred on ice, washed once with ice-cold PBS and re-suspended in 0.5 ml of incubation buffer (with or without FTC) and incubation was continued for 1 h at 37°C to allow maximum efflux of the drug. The efflux reactions were stopped by washing the cells once with ice-cold PBS. Cell pellet was dissolved in one ml of 1% SDS and 900 µl of the lysate was subjected to counting in a scintillation counter. Counts were normalized to protein concentration that was measured by the modified Lowry assay using the remaining lysate. The intracellular levels of HPIs were calculated based on radioactivity associated with the cell pellet and expressed as pmoles of drug/µg protein or fmoles of drug/µg protein. We tried various drug loading and efflux times and found that the experiments produced the optimal efflux activity with 30 min drug loading and 1 h efflux.

**Data Analysis.** IC₅₀ values representing the inhibitory effectiveness of HPIs on BCRP-mediated MX efflux in the flow cytometric efflux assays were calculated by fitting the following model to
the data (Fig. 3) using nonlinear regression (WinNonLin software version 3.2 (Mountain View, CA)):

\[ \Delta F = \frac{\Delta F_{\text{max}}}{\gamma} \left( \frac{C}{\gamma + IC_{50}} \right) \]

where \( \Delta F \) and \( \Delta F_{\text{max}} \) are inhibition and the maximal inhibition, respectively. \( IC_{50} \) is the concentration of HPIs leading to half-maximal inhibition of BCRP activity, \( C \) is the concentration of HPI and \( \gamma \) is the slope factor. To elucidate if the \( IC_{50} \) values of the HPIs (Table 1) for wild-type BCRP (482R) are statistically different from the \( IC_{50} \) values for the BCRP mutants (482T and 482G), we compared the \( IC_{50} \) values for 482R with the values for 482T or 482G, one pair at a time for each HPI, by student’s t-test. A p-value of < 0.05 was considered significant.
Results

Expression of BCRP in HEK cells. Whole cell lysates prepared from various HEK cell lines stably expressing wild-type BCRP (482R) and its two mutants (482T and 482G) were subjected to immunoblotting for analysis of BCRP expression. Fig. 1 shows substantial expression of BCRP in the HEK cells after 30 seconds exposure. 482R and 482G displayed comparable levels of expression. However, 482T was expressed in amounts approximately 20% lower than 482R and 482G. Similar expression pattern of BCRP in HEK cells has been reported earlier (Robey et al., 2003). Since HPIs are known substrates for P-gp, MRP1 or MRP2, to rule out any possible contribution of these transporters, the same protein samples were subjected to immunoblotting for P-gp, MRP1 and MRP2. No P-gp, MRP1 or MRP2 was detected in the samples after a brief exposure for 1 min. Faint bands for these transporters appeared only upon prolonged exposure of the blot for approximately 2 h. The levels of expression of P-gp, MRP1 and MRP2 were comparable in both the BCRP-expressing and vector-control cells (data not shown). These bands represent the endogenous expression levels of these transporters. Thus, relative to the expression of BCRP, the HEK cells express little of endogenous P-gp, MRP1 or MRP2.

Effects of HPIs on BCRP-mediated MX efflux. MX is a fluorescent compound and a well known BCRP substrate. MX is not a substrate of MRP1 (Litman et al., 2000). Although MX is a substrate of both P-gp and BCRP (Litman et al., 2000), we have already shown that the HEK cells express little of endogenous P-gp. Hence, MX can be used as a model substrate for measuring BCRP transport activity in the HEK cells using flow cytometry. We observed that the level of intracellular MX was much greater in HEK cells transfected with pcDNA empty vector than in the BCRP-overexpressing cells (data not shown) and the reduction in intracellular MX in
the BCRP expressing cells could be abrogated by addition of 10 μM FTC, a specific BCRP inhibitor (Fig. 2A). This suggests that the decrease in intracellular MX is mediated by BCRP. Therefore, the change in MX fluorescence (ΔF) upon addition of BCRP inhibitors can be used to express inhibition of BCRP activity as described in “Materials and Methods”. In preliminary studies, we found that 10 μM MX in the presence and absence of 10 μM FTC produced ΔF values that were optimal for determining BCRP activity (Fig. 2A). Hence, concentrations of 10 μM MX and 10 μM FTC were used in all the subsequent flow cytometric efflux assays.

We first examined whether HPIs are BCRP inhibitors. The effect of various concentrations (0 – 150 μM) of five HPIs (ritonavir, saquinavir, nelfinavir, indinavir and amprenavir) on intracellular MX fluorescence in HEK cell lines expressing wild-type BCRP and its two mutants was determined by flow cytometry. A typical inhibition profile for wild-type BCRP (482R) with nelfinavir is shown in Fig. 2B. Ritonavir, saquinavir and nelfinavir significantly increased intracellular MX fluorescence in a concentration-dependent manner in the cells expressing wild-type BCRP and its two mutants (Fig. 3). In contrast, indinavir and amprenavir did not inhibit any isoforms of the BCRP protein, even at a high concentration of 150 μM. The same treatments of the vector control cell line with HPIs did not show any significant shift in the intracellular MX fluorescence (data not shown). These results demonstrate that ritonavir, saquinavir and nelfinavir inhibit BCRP-mediated efflux of MX, whereas indinavir and amprenavir are not BCRP inhibitors.

For any given BCRP protein, IC50 values of nelfinavir, ritonavir and saquinavir, were not significantly different (Table 1). However, the IC50 values of all three drugs were significantly lower for the wild-type BCRP (482R) than the mutants (482T and 482G) but not significantly different within the two mutants. Frequently, a decrease in ΔF values at higher HPIs
concentrations was observed (Fig. 3). This decrease was likely due to the cytotoxic effect of the HPIs on the cells exposed at higher concentrations. Interestingly, the reduction was more pronounced for 482R cells than cells expressing the two mutants. This is consistent with the observation that 482R appears to be more sensitive to inhibition by the HPIs than the mutants 482T and 482G.

**Inhibition of [³H]-MX Efflux by HPIs.** To further confirm that ritonavir, saquinavir and nelfinavir are effective inhibitors of BCRP, the effect of these drugs on the direct efflux of [³H]-MX was measured in HEK cell lines transfected with wild-type BCRP cDNA and the control vector. As expected, the BCRP (482R)-expressing cell line showed a significant reduction in intracellular [³H]-MX level compared to the vector control cell line (Fig. 4). This reduction was abrogated by the addition of 10 µM of FTC. In addition, ritonavir, saquinavir and nelfinavir, at concentrations of 25 µM and 100 µM, reversed the reduction in intracellular [³H]-MX (Fig. 4) in BCRP expressing cells, indicating that the three HPIs are indeed inhibitors of BCRP. In contrast, indinavir and amprenavir, even at the high concentration of 100 µM, did not have a significant effect on [³H]-MX efflux in the 482R cells, confirming that they are not inhibitors of BCRP.

**Efflux of Radiolabeled HPIs.** To confirm if the HPIs used in the above studies are substrates for BCRP, direct efflux of these drugs in BCRP (482R)-expressing and vector control cells was measured using radiolabeled HPIs. Indinavir was not studied as it was not available to us in a radiolabeled form. The efflux of the four radiolabeled HPIs was not significantly different in the 482R and the vector control cells, indicating that all four HPIs are not substrates of BCRP (Fig. 5). Interestingly, intracellular [³H]-ritonavir (Fig. 5A) and [¹⁴C]-saquinavir levels (Fig. 5C) were
increased approximately 40% and 200%, respectively, by addition of 10 µM FTC in both the vector control and 482R cells (solid bars). However, addition of FTC did not have significant influence on intracellular levels of [³H]-amprenavir (Fig. 5B) and [³H]-nelfinavir (Fig. 5D) in both the control and BCRP cells (solid bars). These results suggest that there may be an endogenous efflux transporter in the HEK cells for ritonavir and saquinavir which can be inhibited by FTC.
Discussion

HPIs, including saquinavir, indinavir and ritonavir, are high affinity substrates for P-gp which limits their oral absorption and entry into the CNS (Kim et al., 1998a; Kim et al., 1998b; Lee et al., 1998; Polli et al., 1999). Recent studies (Huisman et al., 2002; Williams et al., 2002) have shown that MRP2 also effectively transports saquinavir, ritonavir and indinavir. Thus, low and/or variable oral bioavailability of HPIs could be explained in part by the high level expression of P-gp and MRP2 in the small intestine and the liver, where these transporters reduce absorption and increase intestinal and hepatobiliary clearance of drugs. BCRP shares many similarities with P-gp and MRP2 with respect to substrate specificity and tissue localization. Like P-gp and MRP2, BCRP is highly expressed in the luminal membranes in the small intestine and liver (Maliepaard et al., 2001; Doyle and Ross, 2003). In fact, BCRP transcript has been reported to be expressed in greater amount than P-gp in the intestine (Taipalensuu et al., 2001). Moreover, BCRP is also highly expressed in sites of clinical importance for HIV drug action such as the blood-brain barrier and placental barrier (Maliepaard et al., 2001; Cooray et al., 2002). Thus, BCRP would be expected to alter pharmacokinetic properties of HPIs if these drugs are BCRP substrates. This prompted us to investigate whether the HPIs are substrates and/or inhibitors of BCRP. The results of our study provide the first direct evidence that ritonavir, saquinavir and nelfinavir are effective inhibitors of BCRP but indinavir and amprenavir are not, and that none of the HPIs is a substrate for BCRP.

Two different analyses suggest that ritonavir, saquinavir and nelfinavir inhibit BCRP. Ritonavir, saquinavir and nelfinavir effectively increased intracellular MX fluorescence in the BCRP-expressing cells in a concentration-dependent manner as would be expected for BCRP inhibitors (Fig. 2 and Fig. 3). Indinavir and amprenavir did not significantly change intracellular
MX fluorescence. Additional evidence that ritonavir, saquinavir and nelfinavir inhibit BCRP activity was obtained in the $[^3]$H-MX efflux assays. Ritonavir, saquinavir and nelfinavir effectively abrogated reduction of intracellular $[^3]$H-MX in the wild-type BCRP-overexpressing cells, whereas indinavir and amprenavir did not (Fig. 4). Given the fact that the five HPIs tested in this paper have extremely diverse chemical structures and molecular sizes (their molecular weights range from 506 to 767), it remains to be investigated why some of the HPIs interact with BCRP but others do not and what determines the binding affinity of these compounds to the transporter.

We also found that the wild-type BCRP (482R) was significantly more sensitive than its mutants 482T and 482G to inhibition by the HPIs, suggesting that position 482 in BCRP may be a critical determinant for binding of the HPIs to BCRP (Table 1). Position 482 has been shown to be critical in determining substrate specificity of BCRP (Honjo et al., 2001; Allen et al., 2002; Volk et al., 2002; Chen et al., 2003; Robey et al., 2003). Thus, our data again support the evidence that mutations at position 482 in BCRP have a significant effect on ligand recognition by the transporter.

To determine if HPIs are substrates of BCRP, we measured their efflux in cells expressing the wild-type BCRP. The efflux of radiolabeled ritonavir, saquinavir, nelfinavir and amprenavir was not significantly different in the vector control and BCRP-overexpressing cells, suggesting that these drugs are not transported by BCRP (Fig. 5). These data are consistent with the results previously reported by other laboratories. Wang et al. (2003) analyzed cytotoxicity of nelfinavir in the drug resistant MT-4/DOX$_{500}$ (BCRP over-expressing cells) and the parental MT-4 cells and reported that cytotoxicity of nelfinavir was not reduced in MT-4/DOX$_{500}$ cells, a result to be expected if nelfinavir is not a substrate of BCRP. However, they did not directly
measure transport of HPIs by the BCRP over-expressing cells. Huisman et al. (2002) also found that Bcrp1, the murine homolog of BCRP, does not transport ritonavir, saquinavir or indinavir. Taken together, our results and the data published by others indicate that the HPIs tested in this paper are not substrates of BCRP. The data presented here are the first report where the inhibitory characteristics of HPIs to BCRP have been characterized in detail. Moreover, this is the first time that transport of HPIs by human BCRP has been measured directly. We noticed that the efflux of radiolabeled ritonavir and saquinavir by both the vector control HEK cells and the 482R cells could be inhibited by incubation with FTC, suggesting that there is an endogenous efflux transporter for ritonavir and saquinavir in the HEK cells that can be inhibited by FTC. Several HPIs investigated in this study are substrates of P-gp, MRP1 or MRP2 (Lee et al., 1998; Williams et al., 2002); however, it is unlikely that any of these pumps is the endogenous transporter for ritonavir and saquinavir in the HEK cells, based on the following evidence: first, we have shown that the HEKs cells express little of endogenous P-gp, MRP1 or MRP2; and second, there is no evidence thus far to suggest that FTC is able to inhibit these transporters. Recently, Imai et al. (2003) also reported the existence of an endogenous transporter for MX in LLC-PK1 cells that can be inhibited by FTC. Whether the endogenous transporter found by Imai et al. (2003) and the one reported in this study are the same transporter is unknown.

The molecular mechanism by which HPIs inhibit BCRP transport is unknown. However, the finding that a molecule that binds to and inhibits a drug transporter but itself is not transported is not unexpected. Zhang et al. (2000) found that saquinavir inhibits transport of tetrathylammonium by the human organic cation transporter OCT1, but itself is not transported by OCT1. Similarly, the nucleoside analogue chloroadenosine was reported to inhibit transport of nucleoside substrates by the human intestinal hCNT2 and hCNT1 Na⁺-nucleoside transporters,
but itself is not a substrate of these transporters (Lum et al., 2000; Patil et al., 2000). Most recently, Nakanishi et al. (2003a) studied reciprocal inhibition of BCRP transport by its substrates including daunorubicin, mitoxantrone and flavopiridol and found that, in any pair of two substrates, none of the substrate caused mutual inhibition of the transport of the other. For example, flavopiridol significantly inhibited BCRP (482T)-mediated transport of daunorubicin, but daunorubicin did not reciprocally inhibit BCRP (482T) transport of flavopiridol. This led them to propose that BCRP may contain multiple ligand interaction sites. Thus, it is possible that MX and the HPIs have distinct or overlapping binding sites on BCRP.

The finding that ritonavir, saquinavir and nelfinavir are effective inhibitors but not substrates of BCRP is clinically significant with respect to drug-drug interactions with HPIs. Although all the HPIs are potent inhibitors of CYP3A enzymes and their inhibitory effectiveness to these enzymes is similar, they produce profoundly different degrees of clinically significant drug interactions (Unadkat and Wang, 2000). One possible explanation of these differences is the possibility that the HPIs inhibit multiple and different transporters in the intestine and liver with different potencies. As BCRP is highly expressed in the intestine, one such transporter could be BCRP. The current recommended doses of ritonavir, saquinavir and nelfinavir for monotherapy are, respectively, 600 mg twice daily, 600 mg three times daily, and 750 mg three times daily. Thus, assuming 100 percent dissolution of these drugs in the intestine, the estimated intestinal luminal concentrations of these HPIs on consuming these doses would be around 500 – 1500 µM. Such concentrations far exceed the IC₅₀ values of the HPIs for BCRP. Thus, given the IC₅₀ values observed here (12 – 20 µM for wild-type BCRP), inhibition of intestinal BCRP activity by the orally administered HPIs could be achieved. Since HPIs extensively bind to protein in plasma, the IC₅₀ values reported in this study may be overestimated as they were determined in
the presence of FBS. However, it is unlikely that the blood/plasma concentrations of HPIs will be sufficient to inhibit BCRP systemically since the average steady-state plasma concentrations of the HPIs are approximately 1 – 5 µM (Unadkat and Wang, 2000). Further studies are needed to determine if drugs routinely administered to HIV patients are substrates of BCRP.

In summary, the present study demonstrates that ritonavir, saquinavir and nelfinavir are clinically significant inhibitors of BCRP but indinavir and amprenavir are not. None of the HPIs investigated in this study is a substrate for BCRP. Further studies are needed to determine if the HPIs are also able to inhibit BCRP-mediated transport of a model substrate drug in in vivo studies using animal models. Such studies are in progress in our laboratory. Inhibition of BCRP transport by HPIs may prove beneficial in increase of the bioavailability of poorly bioavailable drugs that are BCRP substrates.
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Footnotes:

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**Figure Legends**

**Figure 1**: BCRP expression, as determined by immunoblotting, in whole cell lysates (20 µg) of HEK cells stably expressing wild-type BCRP(482R) and its two mutants (482T and 482G) or transfected with the control vector (pcDNA). The relative expression of BCRP was 1.0, 1.18 and 1.27 (arbitrary unit) for 482T, 482R and 482G, respectively.

**Figure 2**: Flow cytometric MX efflux assays. Efflux assays were performed with 30 min accumulation of MX and 1 h efflux as described in “Materials and Methods”. Representative histograms are shown for the HEK cells expressing BCRP (482R). The solid histograms represent autofluorescence of the cells. **A**, Intracellular MX fluorescence in the absence (black bold line) or presence (grey line) of 10 µM FTC. **B**, Intracellular MX fluorescence in the absence (black bold line) and presence of various concentrations (10 – 150 µM) of nelfinavir.

**Figure 3**: Effects of HPIs (0 – 150 µM) on MX efflux in HEK cells. Graphs represent shift in intracellular MX fluorescence (ΔF) versus concentrations of various HPIs in the HEK cell expressing wild-type BCRP (482R) (A), and its two mutants 482T (B) and 482G (C). Ritonavir is represented by solid squares (■), saquinavir by solid triangles (▲), nelfinavir by solid circles (●), indinavir by open circles (○) and amprenavir by open squares (□). The data points are mean ± SD of 3-4 independent experiments.

**Figure 4**: Effects of HPIs on efflux of [3H]-MX (20 nM) in the BCRP(482R) and vector control cells. The assays were carried out with 30 min accumulation of [3H]-MX and 1 h efflux as
described in “Materials and Methods”. Bars represent intracellular levels of \(^{3}\text{H}\)-MX as % of intracellular \(^{3}\text{H}\)-MX in the vector control cells. Intracellular \(^{3}\text{H}\)-MX levels were measured in the presence of various HPIs at concentrations of 25 µM (slashed bars) and 100 µM (solid bars). The chequered bar represents intracellular \(^{3}\text{H}\)-MX in 482R cells in the presence of 10 µM FTC and the open bar represents intracellular \(^{3}\text{H}\)-MX in 482R cells incubated with vehicle only (DMSO). Intracellular \(^{3}\text{H}\)-MX in the vector control cells incubated with MX and the vehicle was set as 100% (dotted bar). The data are mean ± SD of 3 independent experiments. Statistical significance of the differences between the levels of intracellular MX in 482R cells with vehicle alone and the rest of the data was determined by student’s t-test (* p < 0.05).

**Figure 5**: Efflux of radio-labeled HPIs in the vector control and 482R cells incubated with (A) 0.5 µM of \(^{3}\text{H}\)-ritonavir, (B) 0.25 µM of \(^{3}\text{H}\)-amprenavir, (C) 1 µM of \(^{14}\text{C}\)-saquinavir and (D) 1 µM of \(^{3}\text{H}\)-nelfinavir. The detailed assay conditions with 30 min accumulation of radiolabeled HPIs and 1 h efflux are described in “Materials and Methods”. The open bars represent intracellular levels of HPIs alone. The solid bars represent intracellular levels of HPIs in the presence of 10 µM FTC. The data are as mean ± SD of 3-5 independent experiments. Statistical significance was determined by student’s t-test (* p < 0.05 and ** p < 0.01).
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Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1

by multidrug resistance-associated protein (MRP1) and canalicular multispecific organic

Interactions of HIV protease inhibitors with a human organic cation transporter in a
Table 1: Capacity of HPIs (IC$_{50}$) to inhibit BCRP.

<table>
<thead>
<tr>
<th></th>
<th>Ritonavir</th>
<th>Saquinavir</th>
<th>Nelfinavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type, 482R</td>
<td>19.5 ± 0.8*</td>
<td>19.5 ± 6.7*</td>
<td>12.5 ± 4.1*</td>
</tr>
<tr>
<td>Mutant, 482T</td>
<td>40.4 ± 2.8</td>
<td>36.3 ± 7.2</td>
<td>31.1 ± 6.8</td>
</tr>
<tr>
<td>Mutant, 482G</td>
<td>43.1 ± 11.1</td>
<td>41.3 ± 6.4</td>
<td>26.7 ± 4.3</td>
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IC$_{50}$ values (mean ± SD, n = 3 - 4 independent experiments) were determined in the presence of a range of ritonavir, saquinavir and nelfinavir concentrations (0 - 150 µM) as described in “Materials and Methods”. * indicates that the IC$_{50}$ values for 482R are significantly different (p < 0.05) from 482T or 482G as calculated by student’s t-test.
Figure 1

PCDNA 482T 482R 482G

72 kDa BCRP
Figure 2

A

MTX (10 µM)  MTX + FTC (10 µM)

autofluorescence

ΔF

B

Nelfinavir (0-150 µM)

0 10 17.5 25 37.5 50 75 100 150

Counts

FL3-FLX

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Figure 3

Concentration of HPIs (µM)
Figure 4

Intracellular [3H]-MX, % of control cells

- pcDNA-DMSO
- 482R+DMSO
- 482R+FTC 10µM
- 482R+Ritonavir 25µM
- 482R+Saquinavir 25µM
- 482R+Nelfinavir 25µM
- 482R+Nelfinavir 100µM
- 482R+Indinavir 100µM
- 482R+Amprenavir 100µM
Figure 5