Pharmaceutical Research, Vol. 22, No. 8, August 2005 (© 2005)
DOI: 10.1007/s11095-005-5271-y

Research Paper

Quantitative Assessment of HIV-1 Protease Inhibitor Interactions with Drug Efflux Transporters in the Blood–Brain Barrier

Corbin J. Bachmeier,[1] Timothy J. Spitzenberger,[1] William F. Elmquist,[2] and Donald W. Miller[3,4]

Received December 16, 2004; accepted April 5, 2005

Purpose. To quantitatively characterize the drug efflux interactions of various HIV-1 protease inhibitors in an *in vitro* model of the blood–brain barrier (BBB) and to compare that with HIV-1 protease inhibitor stimulated P-glycoprotein (P-gp)-ATPase activity.
Methods. Cellular accumulation of the P-gp sensitive probe, rhodamine 123 (R123), and the mixed P-gp/multidrug resistance–associated protein (MRP) probe, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), were evaluated in primary cultured bovine brain microvessel endothelial cells (BBMEC) in the presence of various concentrations of HIV-1 protease inhibitors. The potency (IC_{50}) and efficacy (I_{max}) of the drugs in the cell accumulation assays for P-gp and/or MRP was determined and compared to activity in a P-gp ATPase assay.
Results. For R123 (P-gp probe), the rank order potency for inhibiting R123 accumulation in the BBMEC was saquinavir = nelfinavir > ritonavir = amprenavir > indinavir. This correlated well with the rank order affinity in the P-gp ATPase assay. The rank order potency for MRP-related drug efflux transporters, was nelfinavir > ritonavir > saquinavir > amprenavir > indinavir.
Conclusions. HIV-1 protease inhibitors potently interact with both P-gp and MRP-related transporters in BBMEC. Characterization of the interactions between the HIV-1 protease inhibitors and drug efflux transporters in brain microvessel endothelial cells will provide insight into potential drug–drug interactions and permeability issues in the BBB.

KEY WORDS: BCECF; blood–brain barrier; HIV-1 protease inhibitors.

INTRODUCTION

The development of more effective therapeutic strategies to treat human immunodeficiency virus (HIV-1) in the central nervous system (CNS) has become an area of focus in recent years. HIV-1 enters the brain early following infection where the virus remains through the duration of the disease (1). The prolonged residence of HIV-1 in the brain can result in AIDS-related neurological complications (i.e., encephalopathy and dementia) and eventually lead to death (1). Antiviral therapy has been successful in reducing the viral load in the blood, but the brain has proven to be a sanctuary site where the virus can reside relatively unchecked by therapeutic interventions (2).

A major obstacle in delivering antiviral therapies to the brain is the blood–brain barrier (BBB). In addition to the tight cellular junctions that reduce paracellular diffusion, a key component of the BBB is the presence of various drug efflux transporters systems that extrude a broad range of chemically unrelated compounds from the brain. These include members of the ATP-binding cassette superfamily of proteins, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance–associated protein (MRP), as well as organic anion transporters including organic anion transporter (OAT) and organic anion transporting polypeptide (OATP) [for recent reviews see (3,4)].

The HIV-1 protease inhibitors are key components of the highly active antiretroviral therapy (HAART) currently used to treat HIV infection. The HIV-1 protease inhibitors demonstrate a reduced penetration into the brain that is, in part, the result of interactions with drug efflux transporters in the BBB (2). Prior studies have established an interaction between the HIV-1 protease inhibitors and P-gp (5–9) and MRP (5,10). Evidence to this point indicates that HIV-1 protease inhibitors are not substrates for BCRP (11–13),

[1] Department of Pharmaceutical Sciences, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, Nebraska, USA.
[2] Department of Pharmaceutics, University of Minnesota, Minneapolis, Minnesota, USA.
[3] Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, MB, R3E 0T6, Canada.
[4] To whom correspondence should be addressed. (e-mail: miller5@cc.umanitoba.ca)

ABBREVIATIONS: BBB, blood–brain barrier; BBMEC, bovine brain microvessel endothelial cells; BCA, bicinchoninic acid; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCRP, brain cancer resistance protein; CNS, central nervous system; HAART, highly active retroviral therapy; HIV-1, human immunodeficiency virus; MRP, multidrug resistance–associated protein; OAT, organic anion transporter; OATP, organic anion transporting peptide.

0724-8741/05/0800-1259/0 © 2005 Springer Science + Business Media, Inc.

although ritonavir, saquinavir, and nelfinavir are effective inhibitors of the transporter (13).

Although interactions between the HIV-1 protease inhibitors and P-gp, MRP, and BCRP drug efflux transporters have been reported, a quantitative assessment as to the extent of these interactions in the BBB is lacking. Since this class of compounds is frequently used in combination with other HIV-1 protease inhibitors or various antiviral agents as part of the HAART regimen, insight into the extent of the interactions between the HIV-1 protease inhibitors and the drug efflux transport systems of the BBB is of interest. The present study examines, in a quantitative manner, the drug efflux interactions of various HIV-1 protease inhibitors in an *in vitro* model of the BBB, using 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) in conjunction with rhodamine 123 (R123) as fluorescent probes to determine relative contributions of P-gp and MRP-related efflux. The IC_{50} and I_{max} values were determined for each HIV-1 protease inhibitor for both P-gp and MRP-related efflux transporter proteins. The interaction of selected protease inhibitors with P-gp was further examined using a P-gp ATPase assay. This information may not only identify potential drug–drug interactions, but may also provide a rationale for modifying current therapies.

MATERIALS AND METHODS

Materials/Reagents

2′,7′-bis(2-Carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and its free acid (BCECF) were purchased from Molecular Probes (Eugene, OR). Rhodamine 123 (R123), indomethacin, bovine fibronectin, and equine serum were purchased from Sigma Chemical Co. (St. Louis, MO). Saquinavir mesylate was supplied by Hoffmann–La Roche (Basel, Switzerland). Nelfinavir mesylate was donated by Pfizer (La Jolla, CA). Ritonavir was obtained from Abbott (Chicago, IL). Indinavir was supplied by Merck (West Point, PA). Amprenavir and GF120918 were provided by GlaxoSmithKline (Research Triangle Park, NC). Cyclosporin A was purchased from Alexis Corporation (San Diego, CA). Tissue culture plates, minimum essential media (MEM), Ham's F-12, Triton-X 100, and rat tail collagen was purchased from Fisher (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Indianapolis, IN). The suspension of membranes expressing human MDR1 used for the P-gp ATPase assay was purchased from Gentest (Woburn, MA).

Cell Isolation and Culturing

Primary bovine brain microvessel endothelial cells (BBMEC) were isolated from the gray matter of fresh bovine cerebral cortices using enzymatic digestion and centrifugal separation methods as previously described (14). The BBMEC were seeded (50,000 cells/cm^2) on collagen-coated, fibronectin-treated, 24-well polystyrene tissue culture plates (2 cm^2/well). The culture media consisted of: 45% minimum essential medium, 45% Ham's F-12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, 50 μg/ml of gentamicin, 10% equine serum, 2.5 μg/ml of amphotericin B, and 100 μg/ml of heparin. The BBMEC were cultured in a humidified incubator at 37°C and 5% CO_2, with media replacement occurring every other day until the monolayers reached confluence (approximately 10–14 days).

Drug Screening

Confluent BBMEC monolayers were pretreated in pH 7.4 Tyrode's balanced salt solution (TBSS) consisting of: 136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 5.56 mM D-glucose, and 5 mM *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), in the presence or absence of various concentrations of HIV-1 protease inhibitor (1–100 μM) or cyclosporin A (3.2 μM) and indomethacin (10 μM) for 30 min at 37°C. Owing to a lack of solubility, saquinavir and nelfinavir could be examined only at concentrations as high as 32 μM. Following the pretreatment period, solutions were removed and the monolayers were incubated with BCECF-AM (1 μM) or R123 (3.2 μM) in the presence or absence of various concentrations of HIV-1 protease inhibitor or cyclosporin A and indomethacin for 60 min at 37°C. The P-gp inhibitor cyclosporin A and the organic anion transport inhibitor indomethacin were used in combination as a positive control for the BCECF accumulation studies, while cyclosporin A alone was used as a positive control for the R123 accumulation studies. After the incubation period, solutions were removed and the monolayers were washed three times in ice-cold phosphate-buffered saline (PBS). The cell monolayers were subsequently solubilized in 1% Triton-X 100. Aliquots (100 μl) of the solubilized cell solutions were removed for determination of intracellular BCECF (λ_{ex} = 505 nm and λ_{em} = 535 nm) or R123 (λ_{ex} = 505 nm and λ_{em} = 535 nm) accumulation using a fluorescence spectrofluorophotometer (Shimadzu RF5000; Shimadzu, Kyoto, Japan). The protein content in the samples was determined using the Pierce BCA method, and the data were expressed as the amount of fluorescent probe (nanomoles) per milligram of cell protein.

Potency and Efficacy Determinations

The potency and efficacy of each HIV-1 protease inhibitor for P-gp and/or MRP-related transport was determined using BCECF-AM and R123 accumulation assays as previously described (15). Briefly stated, confluent BBMEC monolayers were pretreated in the presence or absence of various concentrations of HIV-1 protease inhibitor and/or GF120918 (3.2 μM) for 30 min at 37°C. Following the pretreatment period, solutions were removed and the monolayers were incubated with BCECF-AM (1 μM) in the presence or absence of various concentrations of HIV-1 protease inhibitor either alone to determine total drug efflux activity, or in the presence of GF120918 for 60 min at 37°C to evaluate MRP-related transporter activity. Previous studies have established that 3.2 μM GF120918 is sufficient for complete inhibition of P-gp-mediated transport in the BBMEC (15). The BBMEC were also incubated with R123 (3.2 μM) in the presence or absence of various concentrations of HIV-1 protease inhibitor for 60 min at 37°C to determine P-gp activity. After the incubation period, cell monolayers were washed, solubilized, and analyzed for intracellular

BCECF or R123 and protein content according to the analytical procedure specified above. IC_{50} values and efficacies were determined using a sigmoidal dose-response nonlinear regression curve fit of the experimental data performed by GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA).

P-gp ATPase Assay

The effect of each HIV-1 protease inhibitor on P-gp ATPase activity was determined using a membrane suspension of High Five insect cells expressing human MDR1 (Gentest, Woburn, MA). A 0.06-ml reaction mixture containing 40 μg of membranes, various concentrations of HIV-1 protease inhibitor, and 5 mM MgATP, in buffer containing 50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide, was incubated at 37°C for 20 min in the presence or absence of sodium orthovanadate. Orthovanadate inhibits P-gp ATPase by trapping MgADP in the nucleotide binding site. Thus, ATPase activity measured in the presence of orthovanadate represents non-P-gp ATPase activity and can be subtracted from the activity observed in the absence of orthovanadate to yield vanadate-sensitive ATPase activity. The reaction was stopped by the addition of 30 μl of 10% sodium dodecyl sulfate (SDS) + antifoam A. An additional 20-min incubation at 37°C was performed following the addition of 200 μl of 35 mM ammonium molybdate in 15 mM zinc acetate-10% ascorbic acid (1:4). The liberation of inorganic phosphate resulting from an interaction between the HIV-1 protease inhibitor and P-gp was detected through absorbance at 630 nm and quantitated by comparing the absorbance to a phosphate standard curve (16–18). K_m and V_{max} parameters were determined using a nonlinear regression curve fit of the experimental data performed by WinNonlin (Pharsight Corp., Mountain View, CA).

RESULTS

Initial Drug Screening for Transporter Interactions

BCECF and R123 accumulation in the presence of various HIV-1 protease inhibitors was evaluated to determine the interactions of these agents with the drug efflux transporters expressed in BBMEC. The maximal response of each agent on BCECF and R123 accumulation in BBMEC monolayers is shown in Fig. 1, along with the responses to the positive control, either cyclosporin A (R123) or the combination of cyclosporin A and indomethacin (BCECF). Cyclosporin A caused a twofold increase in R123 accumulation compared to control, while the combination of cyclosporin A and indomethacin significantly enhanced BCECF accumulation (5.5-fold) over control conditions (Fig. 1). In the R123 accumulation assay, which assesses P-gp activity, each HIV-1 protease inhibitor examined increased R123 accumulation to levels equivalent to or greater than the positive control (cyclosporin A). In the BCECF accumulation assay, which examines both P-gp and MRP-related transport systems, saquinavir, nelfinavir, and ritonavir increased cellular BCECF to levels equal to or greater than the positive control, while indinavir and amprenavir stimulated BCECF accumulation to a lesser, albeit statistically significant, extent.

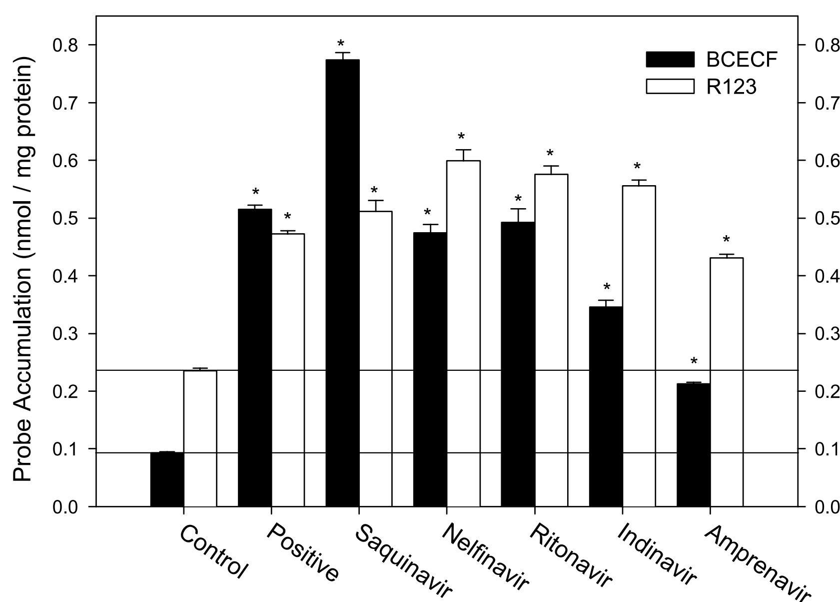

Fig. 1. Intracellular accumulation of BCECF or R123 in the absence or presence of various HIV-1 protease inhibitors at 60 min in BBMEC monolayers. Accumulation studies were performed in the presence of 100 μM drug with the exception of saquinavir and nelfinavir, which were examined at 32 μM. Control represents BCECF or R123 accumulation in the absence of drug. Positive represents the combination of cyclosporin A (3.2 μM) and indomethacin (10 μM) for BCECF and cyclosporin A alone for R123. Values represent the mean ± SEM ($n = 9$). *$p < 0.05$ compared to control monolayers as determined by a one-way ANOVA and Fisher's *post hoc* comparisons.

Table I. BCECF:R123 Ratio Values in the Presence of Various HIV-1 Protease Inhibitors

Drug	BCECF : R123[a]	Predominant Interaction
Saquinavir	3.81	P-gp and MRP-related transport
Ritonavir	2.16	⇕
Nelfinavir	1.99	
Indinavir	1.57	
Amprenavir	1.24	P-gp transport

[a] Ratio of BCECF accumulation in the presence of drug over respective control to R123 accumulation in the presence of drug over respective control.

Using the data generated from Fig. 1, the maximum fold-increase in R123 and BCECF accumulation over that of control BBMEC monolayers was determined (Table I). As the R123 enhancement reflects P-gp mediated interactions and BCECF enhancement is influenced by both P-gp and MRP drug efflux processes, expression of the data as the BCECF/R123 enhancement ratio provides a qualitative indication of P-gp and MRP transporter interactions. Thus, agents such as indinavir and amprenavir whose BCECF/R123 enhancement ratio were near 1, display predominantly P-gp mediated transporter interactions. In contrast, saquinavir, nelfinavir and ritonavir all had BCECF/R123 ratios much greater than 1 (Table I). This suggests that, in addition to a significant P-gp interaction, these agents also interact to a significant extent with MRP.

Potency and Efficacy Determinations

For quantitative analysis of the drug efflux transporter interactions in BBMEC monolayers, R123 and BCECF accumulation was examined using a greater concentration range

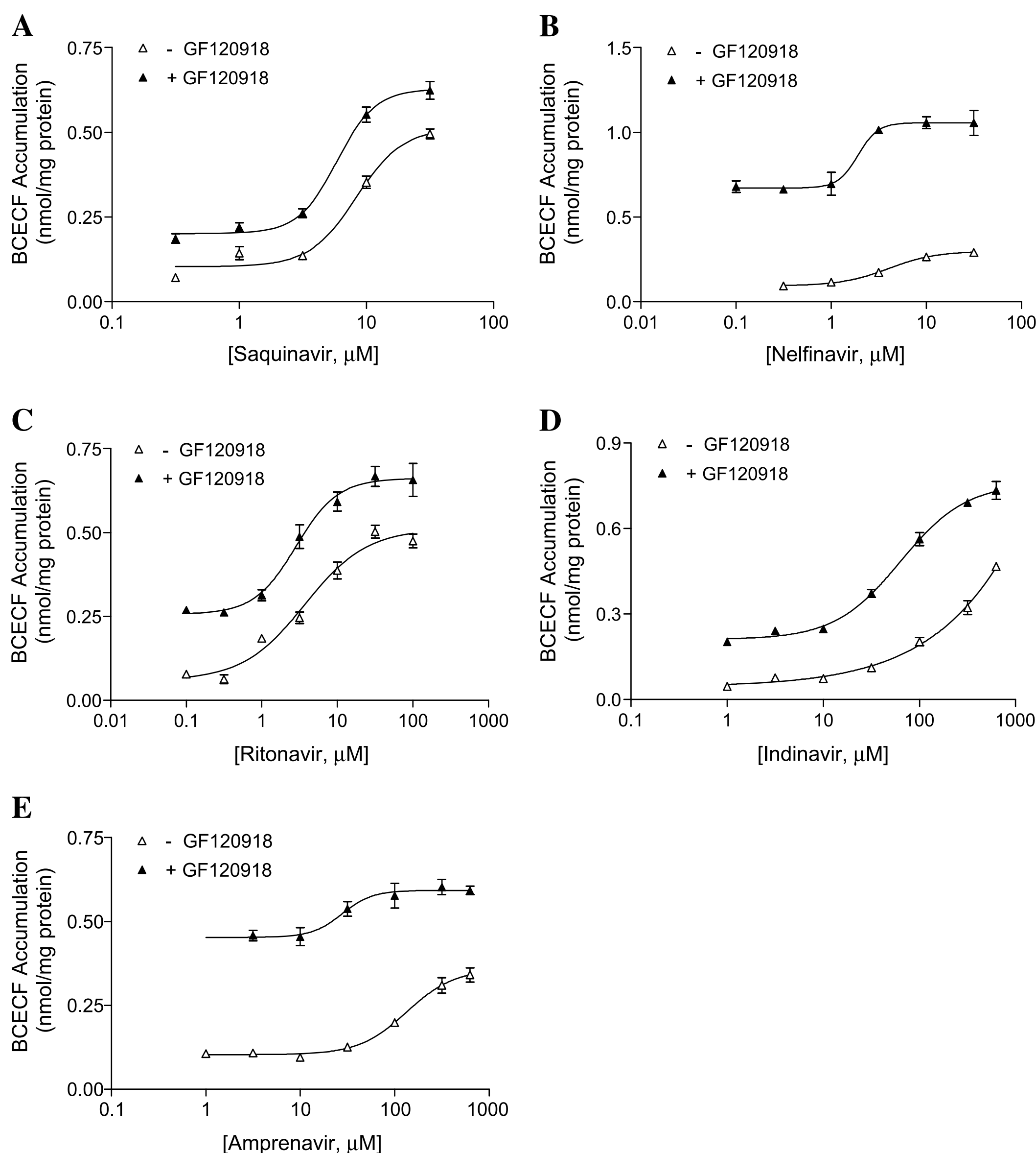

Fig. 2. Intracellular accumulation of BCECF (open triangles) or BCECF in combination with 3.2 μM GF120918 (closed triangles) in the presence of various concentrations of (A) saquinavir, (B) nelfinavir, (C) ritonavir, (D) indinavir, and (E) amprenavir at 60 min in BBMEC monolayers. Values represent the mean ± SEM (n = 3).

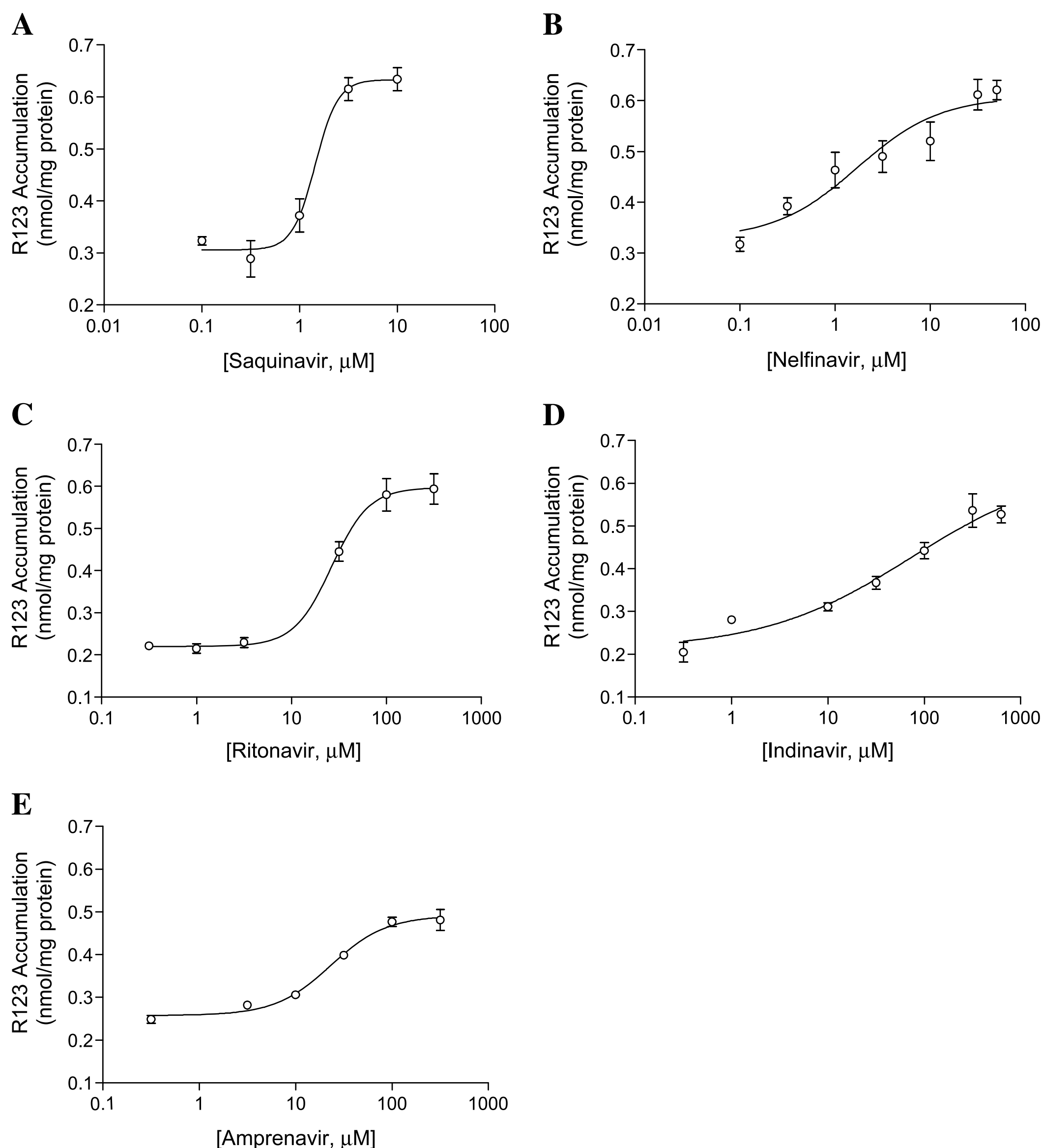

Fig. 3. Intracellular accumulation of R123 in the presence of various concentrations of (A) saquinavir, (B) nelfinavir, (C) ritonavir, (D) indinavir, and (E) amprenavir at 60 min in BBMEC monolayers. Values represent the mean ± SEM (n = 3).

Table II. Potency and Efficacy Determinations of Various HIV-1 Protease Inhibitors for P-gp, MRP-Related, or Total Efflux in BBMEC Monolayers

HIV-1 Protease Inhibitor	P-gp (R123)			MRP-related (BCECF + GF120918)			P-gp and MRP-related (BCECF)		
	Potency		Efficacy	Potency		Efficacy	Potency		Efficacy
	IC_{50} (μM)	95% CI	Imax[a]	IC_{50} (μM)	95% CI	Imax[a]	IC_{50} (μM)	95% CI	Imax[a]
Saquinavir	1.4	0.8 to 5.1	39.8	5.9	1.1 to 11.1	54.9	8.4	3.8 to 18.3	28.1
Nelfinavir	1.7	0.3 to 11.1	34.0	1.9	1.5 to 2.5	49.7	4.1	1.2 to 13.1	13.9
Ritonavir	26.4	22.2 to 31.5	45.9	2.9	1.9 to 4.3	52.2	3.7	1.2 to 11.9	30.9
Indinavir	54.6	46.9 to 110	52.4	71.8	8.5 to 150	70.7	449	178 to 932	46.5
Amprenavir	23.1	10.2 to 52.7	28.4	27.8	12.8 to 60.7	18.1	134	92.7 to 194	17.4

[a] Values represent the maximal response of drug expressed as a percentage of the maximal inhibition obtained with GF120918 (P-gp), indomethacin (MRP-related), or the combination (P-gp and MRP-related).

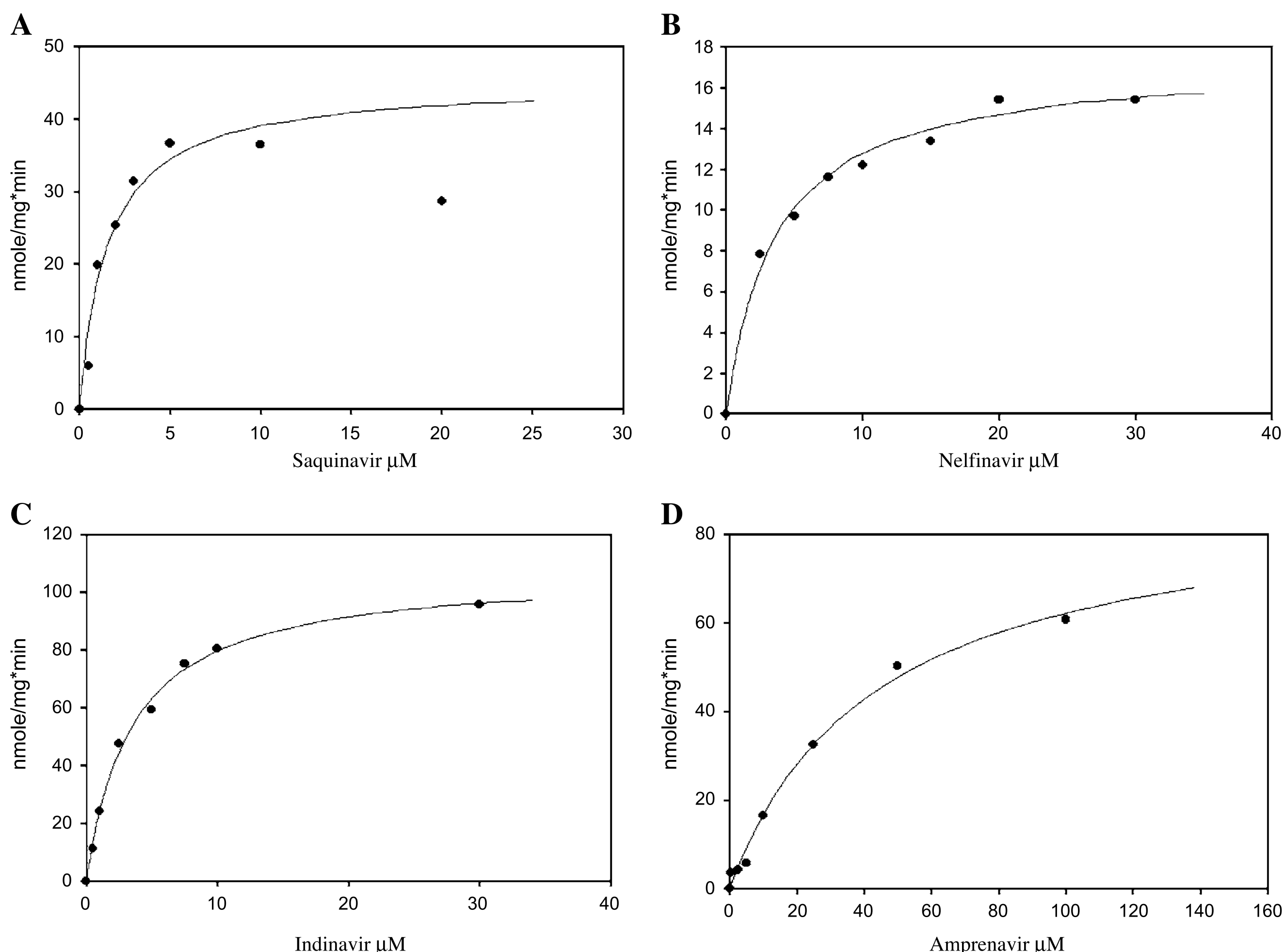

Fig. 4. Representative data sets depicting the stimulation of P-gp ATPase activity by various concentrations of (A) saquinavir, (B) nelfinavir, (C) indinavir, and (D) amprenavir.

of the various protease inhibitors. The effects of the protease inhibitors on BCECF accumulation provided a quantitative assessment of both P-gp and MRP-related transporter interactions (Fig. 2A–E). The addition of GF120918 along with the protease inhibitors allowed for the assessment of MRP-related drug efflux transporter interactions in BBMEC monolayers (Fig. 2A–E). These results were compared to the effects of the protease inhibitors on P-gp activity as assessed using R123 accumulation in the BBMEC (Fig. 3A–E). The potencies (IC_{50}) and probe accumulation efficacies (I_{max}) for each HIV-1 protease inhibitor on P-gp, MRP-related, or total combined drug efflux transporters are summarized in Table II. From these studies, the rank order of potency for P-gp in BBMEC monolayers was saquinavir = nelfinavir > ritonavir = amprenavir > indinavir. The rank order potency for MRP-related transport systems was nelfinavir > ritonavir > saquinavir > amprenavir > indinavir.

P-gp ATPase Assay

The effect of each HIV-1 protease inhibitor on P-gp ATPase activity was determined using a membrane suspension of High Five insect cells expressing human *MDR1*. Using various concentrations of each drug, all of the HIV-1 protease inhibitors significantly increased the liberation of inorganic phosphate above that of control (no drug). A representative data set of the ATPase activation by each HIV-1 protease inhibitor along with their respective kinetic parameters is presented in Fig. 4. Averages of the kinetic parameters derived from at least three separate studies are displayed in Table III. Saquinavir, nelfinavir, and indinavir expressed high affinity for P-gp with K_m values of 1.4, 3.6, and 2.1 µM, respectively. The K_m for amprenavir was greater than 10 times that of the other HIV-1 protease inhibitors (46.9 µM). Saquinavir and nelfinavir demonstrated similar V_{max} values (30.7 and 20.8 nmol/min*mg protein, respective-

Table III. K_m and V_{max} Determinations for HIV-1 Protease Inhibitors in the P-gp ATPase Assay

HIV-1 protease inhibitor	K_m (µM)[a]	V_{max} ($nmol*min^{-1}*mg^{-1}$)[a]
Saquinavir	1.4 ± 0.4	30.7 ± 18.4
Nelfinavir	3.6 ± 0.2	20.8 ± 6.2
Indinavir	2.1 ± 1.1	99.9 ± 25.1
Amprenavir	46.9 ± 3.1	96.4 ± 12.9

[a] Values represent mean ± standard deviation (*n* = 3 or 4).

ly), while the V_{max} values of indinavir and amprenavir were over threefold larger at 99.9 and 96.4 nmol/min*mg protein, respectively.

DISCUSSION

Numerous studies have established an interaction between the HIV-1 protease inhibitors and drug efflux transporters. Using an *in vitro* co-culture model of the BBB, amprenavir, ritonavir, and indinavir demonstrated a polarized transport, which was effectively blocked by various inhibitors of P-gp (5). In both bovine and porcine brain capillary endothelial cells, P-gp-dependant transport of saquinavir was observed (6,19). With regard to drug efflux interactions *in vivo*, rodent brain concentrations of amprenavir (7,20) and nelfinavir (8) significantly increased in the presence of a P-gp modulator. Similarly, following i.v. administration, the brain penetration of indinavir (9,21) and saquinavir (9,22) was greater in P-gp-deficient mice compared to mice expressing P-gp. For MRP, saquinavir and ritonavir were potent inhibitors of MRP1 and MRP2 in porcine brain microvessels (10). MRP1 has also been linked to the polarized transport of indinavir (5). In contrast, nelfinavir (23) and amprenavir (5) do not appear to be strong substrates for MRP1.

The brain microvessel endothelial cells that form the BBB express several different drug efflux transporters (3,4); thus the poor brain penetration of the HIV-1 protease inhibitors likely results from a combination of different drug efflux transporter interactions. The BBMEC express many of the same transporters found *in vivo*, including P-gp, MRP1, MRP4, MRP5, and MRP6 (24,25). Recently, we reported a method for identifying and quantitatively assessing drug efflux interactions for a variety of therapeutic agents in BBMEC monolayers (15). The present study quantitatively examined the drug efflux interactions of the HIV-1 protease inhibitors in an *in vitro* model of the BBB and compared that with HIV-1 protease inhibitor stimulated P-gp ATPase activity.

The initial screening studies in BBMEC monolayers displayed strong interactions of the HIV-1 protease inhibitors with both the P-gp probe (R123) and the mixed P-gp/MRP-related probe (BCECF). In the R123 assay, all five HIV-1 protease inhibitors examined significantly increased R123 accumulation, indicative of P-gp drug efflux transporter inhibition. This correlates well with the literature in that all of these agents effectively interact with P-gp (5,20). For MRP-related transport, numerous studies in the literature demonstrated saquinavir and ritonavir have the strongest interactions with MRP of any of the HIV-1 protease inhibitors (10,23,26–30). In the initial screening, saquinavir and ritonavir gave the largest responses in the BCECF assay (Fig. 1) and also had the two highest BCECF:R123 enhancement ratios (Table I), supporting the findings in the literature. On the other hand, prior studies have indicated nelfinavir and amprenavir do not appear to interact with MRP1. Yet, these compounds produced a significant effect in the BCECF assay. Since BCECF interacts with multiple transporters and the BBMEC express many forms of MRP, the observed responses may be due to interactions with P-gp and/or MRP transporters other than MRP1.

While these initial studies were in good agreement with previous studies done in either transfected or drug resistant cell lines, a more quantitative assessment of these drug efflux transporter interactions in the BBB would be of more value in terms of predicting BBB permeability, potential CNS drug interactions, or BBB-specific mechanisms of transport. Thus, more quantitative experiments were undertaken to ascertain the potency and efficacy of each of the HIV-1 protease inhibitors for the P-gp and MRP-related drug efflux transporters expressed in the BBB.

Examining the effects of the HIV-1 protease inhibitors on BCECF accumulation provided a means to determine total (i.e., P-gp and MRP-related) drug efflux interactions in the BBMEC. All of the HIV-1 protease inhibitors examined displayed significant drug efflux interactions in this assay. However, from a potency standpoint, there was a clear delineation within the protease inhibitors with ritonavir, nelfinavir, and saquinavir having IC_{50} values in the low micromolar range, compared to amprenavir and indinavir with high μM IC_{50} values. Like other accumulation assays (31), these studies cannot determine whether the HIV-1 protease inhibitors are substrates or merely inhibitors of the drug efflux transporters. However, from a drug efflux transporter perspective, one would predict that amprenavir and indinavir might have better BBB permeability.

The potency data obtained in the present study also offer some insight into potential drug–drug interactions involving the drug efflux transporters in the BBB. From a clinical perspective, drug interactions with the efflux transporters are a cause for concern whenever the systemic concentration approaches or surpasses the IC_{50} for drug efflux transport inhibition. For saquinavir, normal serum levels range from 6 to 85 nM (32). These concentrations are approximately 100 times lower than the IC_{50} determined in the present study for saquinavir and drug efflux transport. This indicates that although saquinavir does potently interact with the drug efflux transporters in the BBMEC, normal therapeutic concentrations of saquinavir may not significantly impact drug efflux in the BBB. Conversely, the maximum serum concentrations typically achieved for nelfinavir and ritonavir (4.38 and 7.35 μM, respectively) (33,34) are higher than the respective IC_{50} values determined in the present study (4.1 and 3.7 μM). This would indicate the potential for a drug–drug interaction. Because of the high binding of these agents to plasma proteins (98–99%), however, the concentration of free drug in the blood is likely to be significantly lower than the IC_{50} values obtained in the present study, thus minimizing the possibility that an interaction would occur (34,35).

Although the BCECF accumulation assay can provide information regarding total drug efflux interactions (i.e., P-gp and MRP-related), it does not indicate which particular drug efflux transporter the HIV-1 protease inhibitors may interact with or to what extent. A method of quantitatively determining P-gp and MRP-related interactions in the BBMEC was reported previously (15). Values obtained from the fluorometric assay in the present study were compared to values determined in a P-gp ATPase assay in order to quantitate the interactions of each HIV-1 protease inhibitor with P-gp. The IC_{50} values obtained in R123 fluorometric assay for saquinavir and nelfinavir were 1.4 and 1.7 μM, respectively, which is comparable to the K_m values obtained

in the P-gp ATPase assay for these agents (1.4 and 3.6 μM, respectively). However, the IC_{50} for amprenavir was only half that of the K_m obtained in the P-gp ATPase assay. It should be noted that results from the fluorometric assay represent the ability of the HIV-1 protease inhibitors to block P-gp function, whereas the P-gp ATPase assay measures the transport affinity of each HIV-1 protease inhibitor for P-gp. This may explain potential differences in response between the two assays.

Indinavir demonstrated the largest discrepancy between the two assays. The K_m of indinavir for P-gp was about 25 times lower than the observed IC_{50}. As mentioned above, a possible explanation for this discrepancy may be that indinavir is a good substrate for P-gp, but a weak inhibitor (31). This phenomenon was previously observed for indinavir and other therapeutic agents including erythromycin, emetine, and trimethoprim (31). The authors suggested that these compounds, including indinavir, have a low binding affinity and were therefore unable to displace other substrates from P-gp (31). However, results from the P-gp ATPase assay in the present study indicate a relatively high affinity of indinavir for human P-gp (K_m = 2.1 μM). Along these same lines, there is evidence to suggest P-gp may have multiple binding sites for transported substrates (36). R123, in particular, has been associated with a binding site separate from sites occupied by other transported compounds such as vinblastine and Hoechst 33342 (36,37). Thus, indinavir may lack an inhibitory effect because it does not compete for the same transport site as R123, allowing efflux of R123 to proceed despite the binding of indinavir to P-gp. Alternatively, the differences observed between the two assays may be a product of species differences in P-gp (human *vs.* bovine) and variation in the binding affinity of indinavir to each.

Less is known regarding the interactions of the HIV-1 protease inhibitors and the various organic anion transport systems (e.g., MRP, OAT, and OATP). The few studies that have been done primarily focused on the interactions of the HIV-1 protease inhibitors with MRP1 and MRP2. The assay used in the present study can determine the total transport of all of the proteins that transport BCECF, but cannot determine the interactions or contributions of individual MRP transporters. Regardless, this is to our knowledge the first study to examine multiple organic anion transporters in such a quantitative manner. Based on studies present in the literature thus far, saquinavir and ritonavir appear to have the strongest interactions, at least with respect to MRP1 and MRP2 (10,23,26–30). These studies correlate well with the results from the present study as both saquinavir and ritonavir not only had low IC_{50} (high potency) values, but were highly efficacious in their inhibition of MRP-related transport (approximately threefold above the absence of drug). This indicates that these two compounds may significantly influence the ability of MRP-related transporters to eliminate compounds from the brain.

Studies suggest that indinavir is transported by MRP1 and MRP2 (5,11), but does not appear to be an effective inhibitor of MRP1 function (23,38), a relationship similar to the one indinavir has with P-gp. In our study, indinavir had the highest IC_{50} (lowest potency) for the various organic anion transporters present. However, indinavir did demonstrate the strongest efficacy of any of the HIV-1 protease inhibitors examined. Results from the literature suggest amprenavir and nelfinavir have no interaction with MRP1 (5,23,38). The MRP-related transporter assay in the present study suggests a relationship does exist; however, the I_{max} values for these two compounds were the lowest of any of the compounds examined, especially for amprenavir. This indicates that these two compounds are only able to weakly inhibit the efflux action of the MRP-related transport systems in the BBMEC despite a relatively strong ability to bind these transporters.

In the context of this study, the potency of a drug for P-gp- or MRP-related transport is the concentration of drug required to half-maximally enhance the cellular accumulation of fluorescent probe above that observed in the absence of drug, and is indicated by the IC_{50} value. The efficacy of a drug for P-gp- or MRP-related transport is the magnitude by which the drug is able to maximally enhance the cellular accumulation of fluorescent probe above that of monolayers exposed to the probe alone, and is represented by I_{max}. In general, the potency of a drug can estimate whether an interaction will occur by comparison to the drug concentration present in the blood. The efficacy or I_{max} may be more indicative of interactions occurring when high blood concentrations of drug are observed such as following an overdose or when toxicities arise. In the case of indinavir, for example, the IC_{50} (449 μM) is well above the peak therapeutic blood concentration (12.6 μM, based on the 2004 product information for indinavir by Merck and Co.); therefore little impact is likely. However, if the drug concentration exceeds the IC_{50}, then the strong efficacy of indinavir suggests a large degree of inhibition is possible. It should be noted that the indinavir data set in the BCECF assay did not reach saturation due to a lack of solubility. Therefore, the IC_{50} and I_{max} values obtained for indinavir may be overestimated in the BCECF assay. Nevertheless, indinavir appears to be the weakest inhibitor of drug efflux function of any of the HIV-1 protease inhibitors examined based both on the present study and reports in the literature (23,31,38,39).

The present study may be particularly useful in the determination of drug–drug interactions common with the use of these compounds. As part of HAART, the HIV-1 protease inhibitors are administered in the presence of various nucleoside and/or non-nucleoside antiviral agents. Drug efflux interactions that can occur with combination therapy may explain the drug resistance, sub therapeutic concentrations, and unexpected toxicities that occasionally arise with the use of these treatments (40). Furthermore, the HIV-1 protease inhibitors are often used in tandem to overcome their lack of oral bioavailability resulting from extensive metabolism by CYP450 enzymes (41,42). This same strategy may also be of benefit for enhancing HIV protease inhibitor accumulation in the brain by blocking drug efflux transporter activity at the blood–brain barrier. Indeed, previous studies performed in mice demonstrated a significant increase in the brain concentration of various HIV-1 protease inhibitors when P-gp was absent or inhibited (9,20,43).

The current study presents a further understanding of the interactions between the HIV-1 protease inhibitors and drug efflux transporters in the brain. The information

provided by the present study may serve as a guide toward identifying compatible combinations of HAART in HIV-1 animal models. These studies may also help identify better delivery strategies that result in higher brain distributions, decreased toxicity, and improved efficacy for HIV protease inhibitors.

ACKNOWLEDGMENTS

These studies were supported by funds from NIH Grant R01-NS42549 (WFE) and R01-CA93558 (DWM). C. J. Bachmeier was supported through the McDonald/Bukey and Blanche Widaman graduate studies fellowships.

REFERENCES

1. F. Gimenez, C. Fernandez, and A. Mabondzo. Transport of HIV protease inhibitors through the blood–brain barrier and interactions with the efflux proteins, P-glycoprotein and multidrug resistance proteins. *J. Acquir. Immune Defic. Syndr.* **36**:649–658 (2004).
2. S. A. Thomas. Anti-HIV drug distribution to the central nervous system. *Curr. Pharm. Des.* **10**:1313–1324 (2004).
3. H. Sun, H. Dai, N. Shaik, and W. F. Elmquist. Drug efflux transporters in the CNS. *Adv. Drug Deliv. Rev.* **55**:83–105 (2003).
4. P. L. Golden and G. M. Pollack. Blood-brain barrier efflux transport. *J. Pharm. Sci.* **92**:1739–1753 (2003).
5. I. C. van der Sandt, C. M. Vos, L. Nabulsi, M. C. Blom-Roosemalen, H. H. Voorwinden, A. G. Boerde, and D. D. Breimer. Assessment of active transport of HIV protease inhibitors in various cell lines and the *in vitro* blood–brain barrier. *AIDS* **15**:483–491 (2001).
6. J. Drewe, H. Gutmann, G. Fricker, M. Torok, C. Beglinger, and J. Huwyler. HIV protease inhibitor ritonavir: a more potent inhibitor of P-glycoprotein than the cyclosporine analog SDZ PSC 833. *Biochem. Pharmacol.* **57**:1147–1152 (1999).
7. J. E. Edwards, K. R. Brouwer, and P. J. McNamara. GF120918, a P-glycoprotein modulator, increases the concentration of unbound amprenavir in the central nervous system in rats. *Antimicrob. Agents Chemother.* **46**:2284–2286 (2002).
8. J. Savolainen, J. E. Edwards, M. E. Morgan, P. J. McNamara, and B. D. Anderson. Effects of a P-glycoprotein inhibitor on brain and plasma concentrations of anti-human immunodeficiency virus drugs administered in combination in rats. *Drug Metab. Dispos.* **30**:479–482 (2002).
9. R. B. Kim, M. F. Fromm, C. Wandel, B. Leake, A. J. Wood, D. M. Roden, and G. R. Wilkinson. The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J. Clin. Invest.* **101**:289–294 (1998).
10. D. S. Miller, S. N. Nobmann, H. Gutmann, M. Toeroek, J. Drewe, and G. Fricker. Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol. Pharmacol.* **58**:1357–1367 (2000).
11. M. T. Huisman, J. W. Smit, K. M. Crommentuyn, N. Zelcer, H. R. Wiltshire, J. H. Beijnen, and A. H. Schinkel. Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs. *AIDS* **16**:2295–2301 (2002).
12. X. Wang, T. Furukawa, T. Nitanda, M. Okamoto, Y. Sugimoto, S. Akiyama, and M. Baba. Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors. *Mol. Pharmacol.* **63**:65–72 (2003).
13. A. Gupta, Y. Zhang, J. D. Unadkat, and Q. Mao. HIV protease inhibitors are inhibitors but not substrates of the human breast cancer resistance protein (BCRP/ABCG2). *J. Pharmacol. Exp. Ther.* **310**:334–341 (2004).
14. D. W. Miller, K. L. Audus, and R. T. Borchardt. Application of cultured bovine brain endothelial cells in the study of the blood–brain barrier. *J. Tissue Cult. Methods* **14**:217–224 (1992).
15. C. J. Bachmeier and D. W. Miller. A fluorometric screening assay for drug efflux transporter activity in the blood–brain barrier. *Pharm. Res.* **22**:113–121 (2005).
16. P. Drueckes, R. Schinzel, and D. Palm. Photometric microtiter assay of inorganic phosphate in the presence of acid–labile organic phosphates. *Anal. Biochem.* **230**:173–177 (1995).
17. B. Sarkadi, E. M. Price, R. C. Boucher, U. A. Germann, and G. A. Scarborough. Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J. Biol. Chem.* **267**:4854–4858 (1992).
18. R. L. Shepard, M. A. Winter, S. C. Hsaio, H. L. Pearce, W. T. Beck, and A. H. Dantzig. Effect of modulators on the ATPase activity and vanadate nucleotide trapping of human P-glycoprotein. *Biochem. Pharmacol.* **56**:719–727 (1998).
19. S. L. Glynn and M. Yazdanian. *In vitro* blood–brain barrier permeability of nevirapine compared to other HIV antiretroviral agents. *J. Pharm. Sci.* **87**:306–310 (1998).
20. J. W. Polli, J. L. Jarrett, S. D. Studenberg, J. E. Humphreys, S. W. Dennis, K. R. Brouwer, and J. L. Woolley. Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. *Pharm. Res.* **16**:1206–1212 (1999).
21. I. Megard, A. Garrigues, S. Orlowski, S. Jorajuria, P. Clayette, E. Ezan, and A. Mabondzo. A co-culture-based model of human blood–brain barrier: application to active transport of indinavir and *in vivo–in vitro* correlation. *Brain Res.* **927**:153–167 (2002).
22. C. B. Washington, H. R. Wiltshire, M. Man, T. Moy, S. R. Harris, E. Worth, P. Weigl, Z. Liang, D. Hall, L. Marriott, and T. F. Blaschke. The disposition of saquinavir in normal and P-glycoprotein deficient mice, rats, and in cultured cells. *Drug Metab. Dispos.* **28**:1058–1062 (2000).
23. R. V. Srinivas, D. Middlemas, P. Flynn, and A. Fridland. Human immunodeficiency virus protease inhibitors serve as substrates for multidrug transporter proteins MDR1 and MRP1 but retain antiviral efficacy in cell lines expressing these transporters. *Antimicrob. Agents Chemother.* **42**:3157–3162 (1998).
24. Y. Zhang, H. Han, W. F. Elmquist, and D. W. Miller. Expression of various multidrug resistance–associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res.* **876**:148–153 (2000).
25. Y. Zhang, J. D. Schuetz, W. F. Elmquist, and D. W. Miller. Plasma membrane localization of multidrug resistance–associated protein homologs in brain capillary endothelial cells. *J. Pharmacol. Exp. Ther.* **311**:449–455 (2004).
26. K. Jones, P. G. Bray, S. H. Khoo, R. A. Davey, E. R. Meaden, S. A. Ward, and D. J. Back. P-Glycoprotein and transporter MRP1 reduce HIV protease inhibitor uptake in CD4 cells: potential for accelerated viral drug resistance? *AIDS* **15**:1353–1358 (2001).
27. K. Jones, P. G. Hoggard, S. D. Sales, S. Khoo, R. Davey, and D. J. Back. Differences in the intracellular accumulation of HIV protease inhibitors *in vitro* and the effect of active transport. *AIDS* **15**:675–681 (2001).
28. G. C. Williams, A. Liu, G. Knipp, and P. J. Sinko. Direct evidence that saquinavir is transported by multidrug resistance-–associated protein (MRP1) and canalicular multispecific organic anion transporter (MRP2). *Antimicrob. Agents Chemother.* **46**:3456–3462 (2002).
29. E. R. Meaden, P. G. Hoggard, P. Newton, J. F. Tjia, D. Aldam, D. Cornforth, J. Lloyd, I. Williams, D. J. Back, and S. H. Khoo. P-glycoprotein and MRP1 expression and reduced ritonavir and saquinavir accumulation in HIV-infected individuals. *J. Antimicrob. Chemother.* **50**:583–588 (2002).
30. H. Gutmann, G. Fricker, J. Drewe, M. Toeroek, and D. S. Miller. Interactions of HIV protease inhibitors with ATP-dependent drug export proteins. *Mol. Pharmacol.* **56**:383–389 (1999).
31. J. W. Polli, S. A. Wring, J. E. Humphreys, L. Huang, J. B. Morgan, L. O. Webster, and C. S. Serabjit-Singh. Rational use of *in vitro* P-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **299**:620–628 (2001).
32. P. Williams, G. Muirhead, and M. Madigan, *et al*. Disposition and bioavailability of the HIV-proteinase inhibitor, Ro 31-8959, after single oral doses in healthy volunteers. *Br. J. Clin. Pharmacol.* **34**:155–156 (1992).

33. P. Krogstad, A. Wiznia, K. Luzuriaga, W. Dankner, K. Nielsen, M. Gersten, B. Kerr, A. Hendricks, B. Boczany, M. Rosenberg, D. Jung, S. A. Spector, and Y. Bryson. Treatment of human immunodeficiency virus 1-infected infants and children with the protease inhibitor nelfinavir mesylate. *Clin. Infect. Dis.* **28**:1109–1118 (1999).
34. D. J. Kempf, K. C. Marsh, J. F. Denissen, E. McDonald, S. Vasavanonda, C. A. Flentge, B. E. Green, L. Fino, C. H. Park, and X. P. Kong, *et al.* ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. *Proc. Natl. Acad. Sci. USA* **92**:2484–2488 (1995).
35. G. Moyle and B. Gazzard. Current knowledge and future prospects for the use of HIV protease inhibitors. *Drugs* **51**:701–712 (1996).
36. C. Martin, G. Berridge, C. F. Higgins, P. Mistry, P. Charlton, and R. Callaghan. Communication between multiple drug binding sites on P-glycoprotein. *Mol. Pharmacol.* **58**:624–632 (2000).
37. A. B. Shapiro, K. Fox, P. Lam, and V. Ling. Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Evidence for a third drug-binding site. *Eur. J. Biochem.* **259**:841–850 (1999).
38. D. P. Olson, D. T. Scadden, R. T. D'Aquila, and M. P. De Pasquale. The protease inhibitor ritonavir inhibits the functional activity of the multidrug resistance related-protein 1 (MRP-1). *AIDS* **16**:1743–1747 (2002).
39. C. B. Washington, G. E. Duran, M. C. Man, B. I. Sikic, and T. F. Blaschke. Interaction of anti-HIV protease inhibitors with the multidrug transporter P-glycoprotein (P-gp) in human cultured cells. *J. Acquir. Immune Defic. Syndr. Human Retrovirol.* **19**:203–209 (1998).
40. T. Ridky and J. Leis. Development of drug resistance to HIV-1 protease inhibitors. *J. Biol. Chem.* **270**:29621–29623 (1995).
41. J. R. King, H. Wynn, R. Brundage, and E. P. Acosta. Pharmacokinetic enhancement of protease inhibitor therapy. *Clin. Pharmacokinet.* **43**:291–310 (2004).
42. R. K. Zeldin and R. A. Petruschke. Pharmacological and therapeutic properties of ritonavir-boosted protease inhibitor therapy in HIV-infected patients. *J. Antimicrob. Chemother.* **53**:4–9 (2004).
43. M. T. Huisman, J. W. Smit, H. R. Wiltshire, J. H. Beijnen, and A. H. Schinkel. Assessing safety and efficacy of directed P-glycoprotein inhibition to improve the pharmacokinetic properties of saquinavir coadministered with ritonavir. *J. Pharmacol. Exp Ther.* **304**:596–602 (2003).