Role of P-Glycoprotein on the CNS Disposition of Amprenavir (141W94), an HIV Protease Inhibitor

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Purpose. To determine the role of P-glycoprotein (Pgp) on the CNS penetration of the HIV protease inhibitor (PI) amprenavir (141W94) and to test the hypothesis that co-administration of a second HIV PI (ritonavir) could enhance amprenavir's brain penetration in vivo.
Methods. Pgp-mediated efflux was investigated in vitro with Caco-2 cells and in vivo by whole-body autoradiography (WBA). "Genetic" *mdr1a/1b* double knockout mice, "chemical" Pgp knockout mice generated by administration of the Pgp inhibitor GF120918, and mice pretreated with ritonavir were used in WBA studies to investigate the effects of Pgp modulation on the CNS penetration of amprenavir.
Results. Amprenavir, indinavir, ritonavir, and saquinavir had 2-to 23-fold higher transport rates from the basolateral to apical direction than from the apical to basolateral direction across Caco-2 monolayers. Incubation with GF120918 negated this difference, suggesting that the efflux was Pgp-mediated. WBA studies demonstrated a 13- and 27-fold increase in the brain and a 3.3-fold increase in the CSF concentrations of amprenavir in mice pretreated with GF120918 and in *mdr1a/1b* double knockout mice. In contrast, pretreatment with ritonavir did not alter the CNS exposure of amprenavir.
Conclusions. These results provide evidence that amprenavir and other HIV PIs are Pgp substrates and that co-administration of a specific Pgp inhibitor will enhance amprenavir's CNS penetration in vivo. These results will have an important therapeutic impact in the treatment of AIDS dementia.

KEY WORDS: ritonavir; whole-body autoradiography; blood-brain barrier; cytochrome P450; Caco-2.

INTRODUCTION

The antiviral compound amprenavir (141W94) is a potent, selective HIV-1 protease inhibitor (PI) being developed for the treatment of Acquired Immune Deficiency Syndrome (AIDS) (1). Amprenavir inhibits the type-1 protease that is a product of the *pol* gene required for processing of the *gag-pol* or *gag* polypeptide. Inhibition of the protease results in immature and non-infectious virus particles (2). The development of protease inhibitors has been a major advancement in the treatment of AIDS and has proven to dramatically reduce plasma viral load (2,3). It is hoped that HIV PIs will not only eliminate virus from the periphery, but also reduce the incidence of central nervous system (CNS) complications which may lead to the development of HIV-1 Associated Dementia or AIDS Dementia Complex (ADC) (4,5). The cause of HIV-1 Associated Dementia is believed to be the sheltering of virus-infected brain microglia and macrophages from current antiviral agents, which often have poor blood-brain barrier (BBB) penetration. This has resulted in an effort to increase the CNS penetration of the next generation of anti-HIV drugs.

One major limitation to the CNS penetration of a number of clinically important drugs is active efflux by P-glycoprotein (Pgp), the product of the multidrug resistance (MDR) gene (6). Pgp is expressed on the luminal side of brain capillaries where it can actively exclude agents such as ivermectin, digoxin, vinca alkaloids, and cyclosporin A. Potent and selective MDR reversing agents, such as the acridone carboxamide GF120918, have been shown to enhance brain levels of Pgp substrates (7,8). Previously, we have shown that amprenavir has a direction- and concentration-dependent transport across a primary bovine brain endothelial cell (BBEC) blood-brain barrier model, suggesting active efflux from brain capillaries (9). Recently, it has been found that Pgp limits the CNS penetration of the HIV PIs ritonavir, indinavir, saquinavir and nelfinavir (10–13). These studies provided a mechanistic basis for the limited CNS penetration of HIV PIs. The following work was initiated to compare the membrane penetration properties of amprenavir and other HIV PIs, to determine the role of Pgp on the CNS penetration of amprenavir in vivo, and to examine if co-administration of a second HIV PI could enhance the CNS concentrations of amprenavir.

MATERIALS AND METHODS

Chemicals and Materials

Amprenavir, [^{14}C]-amprenavir, and GF120918 were obtained from Glaxo Wellcome Compound Registry. Samples of indinavir sulfate, ritonavir, and saquinavir mesylate were gifts from Merck & Co., Inc., Abbott Laboratories, and Roche Research Centre, respectively. All other chemicals except radiolabelled compounds and cell culture reagents were purchased from Sigma Chemical Co., St Louis, MO. Cell culture reagents were purchased from Gibco-BRL, Grand Island, NY. DL-[4-^{3}H]-propranolol (15–30 Ci/mmol) and D-[1-^{14}C]-mannitol (50–63 mCi/mmol) were purchased from Amersham Life Sciences, Arlington Heights, IL. Transwells™ (12-well, 11 mm diameter, 3.0 μm pores) were purchased from Corning Costar, Cambridge, MA.

In Vitro Caco-2 Transport Studies

Caco-2 cells were grown as described (14) and were chosen because they have been shown to have robust expression of Pgp (15). For transport studies, cells were seeded onto polycarbonate Transwell™ filter membranes at a density of 60,000 cell/cm^2 and monolayers were ready for studies 21 days later. Compounds were dissolved at 20 mM in 100% DMSO and dilutions for studies prepared in transport buffer (8.1 mM Na_2HPO_4, 138 mM NaCl, 0.5 mM $MgCl_2$, 1.47 mM KH_2PO_4, 2.67 mM KCl, 0.9 mM $CaCl_2$, 5.6 mM glucose, and 0.33 mM sodium pyruvate). Compounds were tested at one concentration (25 μM)

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and in two directions (apical to basolateral [A → B] and basolateral to apical [B → A]) in order to determine mechanism of transport across the epithelial monolayer. Transport studies were conducted at 37°C in a humidified incubator over 90 minutes. Transendothelial electrical resistance (TEER) was measured for each well with an Endohm Meter (World Precision Instruments, Sarasota, FL). Markers for paracellular ([^{14}C]-mannitol) and transcellular ([^{3}H]-propranolol) transport were included in each experiment as controls and were analyzed by liquid scintillation counting with Ready Safe Liquid Scintillation Fluid (Beckman, Foster City, CA). The HIV PIs (amprenavir, indinavir, ritonavir, saquinavir) were analyzed by HPLC-UV with a 3.0 × 250 mm BDS-Hypersil C18 reverse phase column (Keystone, State College, PA), and a mobile phase of 40% 20 mM ammonium acetate (pH 6.8) and 60% acetonitrile.

The apparent permeability (Papp) was calculated with the equation: Papp = $1/AC_0$ (dQ/dt) where Papp = apparent permeability; A = membrane surface area; C_0 = donor drug concentration at t = 0; dQ/dt = amount of drug transported within a given time period. Data are presented as the average Papp (nm/sec) ± standard deviation from three monolayers. A ratio of the B → A/A → B Papp values was calculated. Involvement of a Pgp-mediated efflux mechanism is suggested if the B → A/A → B ratio is greater than 1.5.

Animals

"Chemical" Pgp Knockout Animals

Male CD-1 mice were purchased from Charles Rivers Labs (Raleigh, NC). Mice were given a single oral 250 mg(base)/kg dose of GF120918 (dosing vehicle of 0.5% hydroxypropylmethylcellulose and 1% Tween 80; dosing volume 5 mL/kg) or 250 mg (base)/kg ritonavir (dosing vehicle of 90% propylene glycol:10% ethanol; dosing volume 5 mL/kg) once a day for four consecutive days. As a control, a separate group of mice was administered a single oral dose of dosing vehicle once a day for four consecutive days. Mice were not fasted prior to dosing. Two hours after the last dose of GF120918, ritonavir, or dosing vehicle, a single oral 50 mg(base)/kg dose of [^{14}C]-amprenavir (specific activity: 22–27 μCi/mg) in a dosing vehicle of D-alpha-tocopherol polyethylene glycol 1000 succinate: polethylene glycol 400:propylene glycol was administered to each mouse (dosing volume 5mL/kg). At two-hours after the [^{14}C]-amprenavir dose, animals were euthanized and processed for whole-body autoradiography (WBA). The two-hour time point was selected because previous studies had shown this to be T_{max}, that amprenavir had significant body distribution at this time, and the majority of circulating radioactivity was still parent compound (16).

Genetic Knockout Mice

Pgp deficient "knockout" male FVB *mdr1a/1b* (−/−) mice and genetically matched *mdr1a/1b* (+/+) controls expressing Pgp were purchased from Taconic Farms, Inc, (Germantown, NY) (17). These mice are deficient in both the *mdr1a* and *mdr1b* gene products; hence, they are double knockout animals. Mice received a single oral 50 mg(base)/kg dose of [^{14}C]-amprenavir (specific activity: 19.3 μCi/mg; dosing volume: 5 mL/kg). Mice were not fasted prior to dosing. The animals were processed for WBA two hours after the [^{14}C]-amprenavir dose.

Whole-Body Autoradiography (WBA)

Mice were euthanized by CO_2 asphyxiation, and immediately frozen in a bath of hexane and dry ice. Animals were prepared for WBA by embedding in a 2% carboxymethylcellulose solution and freezing at −60°C. Using a Leica cryomacrocut microtome, sagittal sections (40 μm) were taken from each animal, freeze-dried, and along with Amersham ^{14}C standards, exposed to [^{14}C]-sensitive phosphor-imaging plates for 2–5 days. The imaging plates were scanned and digitized using a Fuji BAS 2000 Bio-Imaging scanner and the resulting digital images were quantitated with Imaging Research MCID/M2 image analysis software (Imaging Research, St. Catharine's, Canada).

RESULTS

Transport of Amprenavir and HIV PIs Across Caco-2 Monolayers

The permeability of amprenavir, ritonavir, saquinavir and indinavir was measured in two directions (apical to basolateral [A → B] and basolateral to apical [B → A]), at a concentration of 25 μM, and in the presence or absence of 500 nM GF120918, a potent, specific inhibitor of Pgp (7,8,18,19). The HIV PIs had a 2- to 23-fold higher transport rate for the B → A direction than the rate for the A → B direction suggesting efflux of these compounds across Caco-2 monolayers (Table 1). The addition of GF120918 abolished any significant directionality in transport rates, confirming that amprenavir, ritonavir, saquinavir and indinavir are substrates for Pgp. GF120918 is specific for Pgp and does not inhibit MDR3 or the multidrug resistance-associated protein, MRP (8,19). An apparent permeability × surface area coefficient (Papp) was calculated for each compound. The rank order of epithelial cell permeability based on the Papp value was: amprenavir = ritonavir >indinavir = saquinavir which suggests that amprenavir and ritonavir have better permeation properties and will be the most effective agents at penetrating membrane barriers.

Table 1. Effect of GF120918 on the Transport of HIV Protease Inhibitors Across Caco-2 Cell Monolayers

Compound	Papp A → B (nm/sec)	Papp B → A (nm/sec)	BA/AB ratio	Pgp substrate
Amprenavir	62.7 ± 1.7	177.3 ± 7.8	2.8	Yes
+GF120918	125.6 ± 6.3	131.6 ± 1.6	1.0	
Indinavir	4.4 ± 1.2	61.4 ± 7.0	14.0	Yes
+GF120918	27.1 ± 1.7	36.3 ± 0.9	1.3	
Ritonavir	67.7 ± 7.7	144.7 ± 3.1	2.1	Yes
+GF120918	79.3 ± 8.5	106.2 ± 6.3	1.3	
Saquinavir	2.0 ± 0.2	46.4 ± 2.6	23.3	Yes
+GF120918	8.3 ± 0.4	16.6 ± 1.2	2.0	

Note: HIV PIs were assayed with Caco-2 cells at 25 μM, in two directions (A → B and B → A) and in the presence or absence of 500 nM GF120918, a potent, specific inhibitor of Pgp. Data are the average ± standard deviation from three monolayers. Involvement of a Pgp-mediated efflux mechanism is suggested if the BA/AB ratio is greater than 1.5.

Whole-Body Autoradiography Studies

WBA was used to establish the role of Pgp in affecting the CNS disposition of amprenavir in vivo. The CNS penetration of amprenavir in "chemical" and "genetic" Pgp knockout animals was compared to control animals.

"Chemical" Pgp Knockouts

Chemical Pgp knockouts were generated by pretreatment of mice with the Pgp inhibitor GF120918 for four days prior to administration of a single oral dose of [^{14}C]-amprenavir. Whole-body images and concentrations of amprenavir-related material in the brain, CSF, blood, testes and muscle following oral administration of [^{14}C]-amprenavir in the absence and presence of GF120918 are shown in Fig. 1 and Table 2. Co-administration with GF120918 led to a 13-fold increase in the brain and a 3.3-fold increase in the CSF concentrations of amprenavir-related material. Blood and muscle levels of amprenavir-related material increased 2-fold, whereas testes, a tissue that expresses Pgp, had a 4-fold increase.

"Genetic" Pgp Knockouts

Whole-body images and concentrations of amprenavir-related material in the brain, CSF, blood, testes, and muscle following a single oral administration of [^{14}C]-amprenavir are shown in Fig. 2 and Table 2. A 27-fold increase in the brain concentration of amprenavir-related material was seen in *mdr1a/1b* (−/−) knockout mice compared to *mdr1a/1b* (+/+) control animals. The levels of amprenavir in the CSF of *mdr1a/1b* (−/−) animals could not be determined because concentrations of amprenavir in brain tissue were high enough that the ventricles could not be differentiated from surrounding tissue. Concentrations of amprenavir in testes increased 4-fold, while blood and muscle had increases of 1.3- and 1.5-fold.

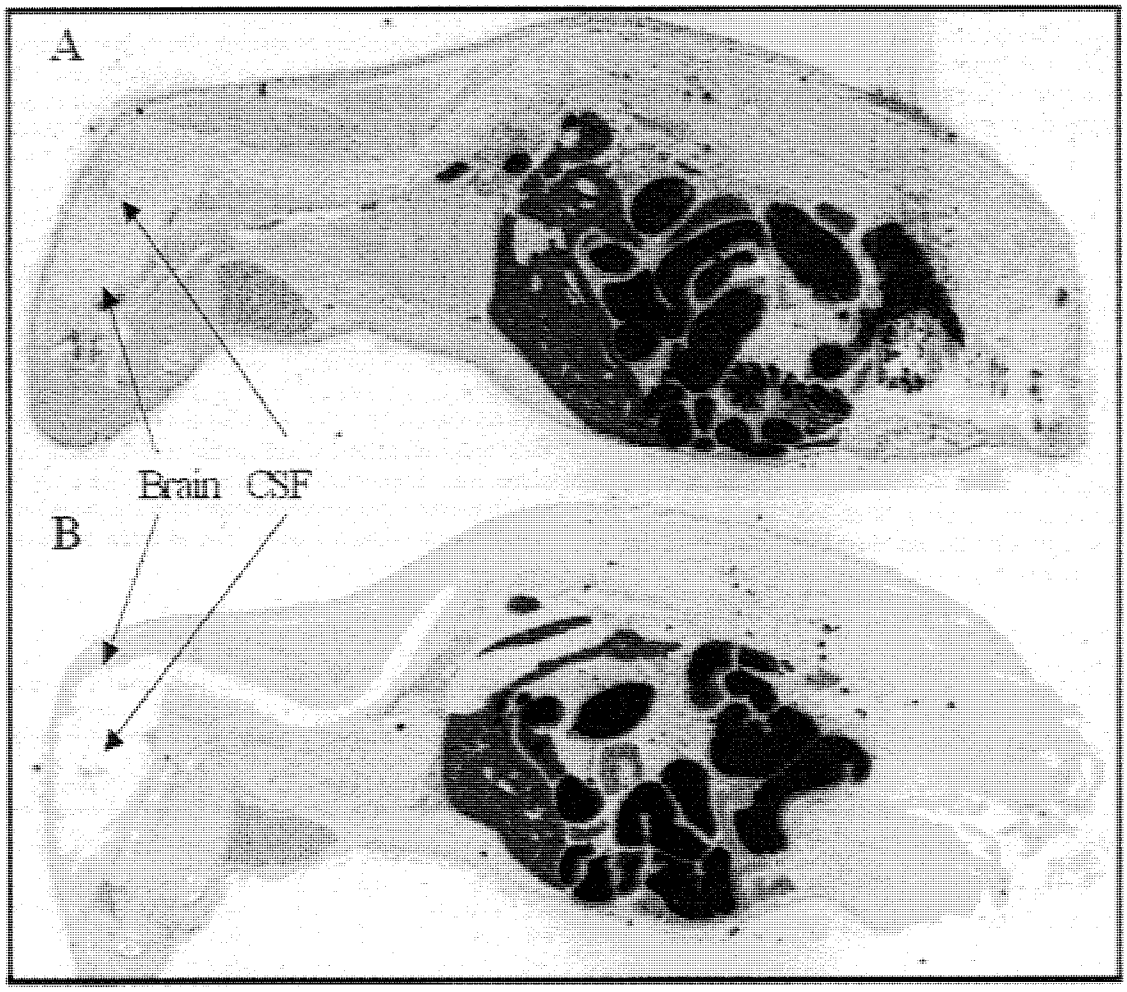

Fig. 1. Distribution of [^{14}C]-amprenavir in male CD-1 mice pretreated with the Pgp inhibitor GF120918. Male CD-1 mice were administered either 250 mg(base)/kg of GF120918 or dosing vehicle once a day for four consecutive days. Two-hours after the last dose of GF120918 or vehicle, a single oral 50 mg/kg dose of ^{14}C-amprenavir was administered to each mouse. Two hours post-dosing of amprenavir, mice were euthanized and processed for whole-body autoradiography. Sections were exposed to [^{14}C]-sensitive phosphor-imaging plates for 4 days. Animals treated with GF120918 (Panel A) had a 13-fold increase in brain and 3.3-fold increase in CSF levels of amprenavir-related material over vehicle treated mice (Panel B).

Table 2. Concentrations (nCi/g) of [^{14}C]-Amprenavir in CD-1 Mice Pretreated with GF120918 and in FVB *mdr1a/1b* Double Knockout Mice

	GF120918 chemical knockout			*mdr 1a/1b* genetic double knockout		
Tissue	Vehicle	GF120918	Ratio	(+/+)	(−/−)	Ratio
Blood	46.3 ± 18.4	90.7 ± 25.7	2.0	112 ± 40.6	147 ± 8.3	1.3
Brain	3.33 ± 0.6[a]	43.8 ± 14.6	13.2	5.4 ± 2.4	146 ± 17.1	27.0
CSF	23.3 ± 11.2	75.6 ± 27.4	3.3	58.4 ± 6.8	NV	ND
Testes	15.2 ± 3.55	60.9 ± 17.9	4.0	37.8 ± 5.47	160 ± 25.7	4.2
Muscle	33.0 ± 9.09	70.4 ± 20.2	2.1	117 ± 5.47	179 ± 52.6	1.5

Note: Data are the average from 3–4 animals ± the standard deviation. NV = not visible. ND = not determined. Limit of Quantitation (LOQ): 2.447 nCi/g.

[a] Sections had >40% of pixels below quantification limit (BQL).

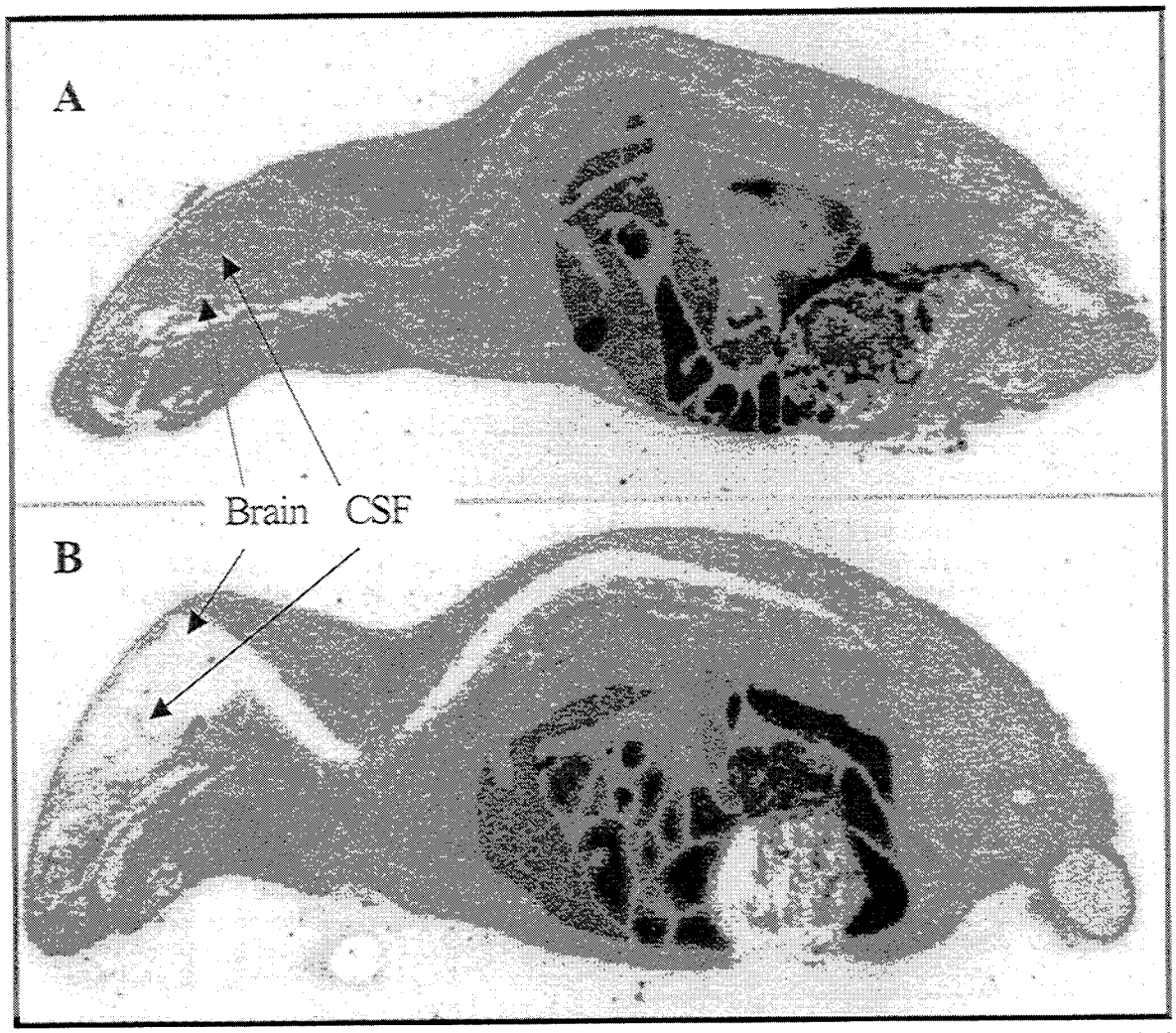

Fig. 2. Distribution of [^{14}C]-amprenavir in male FVB *mdr1a/1b*(−/−) double knockout or *mdr1a/1b*(+/+) control mice. A single oral 50 mg/kg dose of ^{14}C-amprenavir was administered to each mouse. Two hours post-dosing, mice were euthanized and processed for whole-body autoradiography. Sections were exposed to [^{14}C]-sensitive phosphor-imaging plates for 2 days. The *mdr1a/1b*(−/−) double knockout animals (Panel A) had 27-fold higher brain concentrations of amprenavir-related material compared to genetically matched *mdr1a/1b*(+/+) mice (Panel B).

Table 3. Effect of Ritonavir on the Caco-2 Permeability of Amprenavir

Compound	Papp A → B (nm/sec)	Papp B → A (nm/sec)	BA/AB ratio
Amprenavir	128.4 ± 3.6	273.1 ± 10.9	2.13
+Ritonavir	185.4 ± 3.5	197.1 ± 2.5	1.06
+GF120918	175.1 ± 13.4	203.5 ± 4.8	1.16

Note: Amprenavir was assayed with Caco-2 cells at 25 μM, in two directions (A → B and B → A) and in the presence or absence of 500 nM GF120918, a potent, specific inhibitor of Pgp or 25 μM Ritonavir. Data are the average ± standard deviation from three monolayers.

Can Co-administration of Ritonavir Enhance the CNS Penetration of Amprenavir?

Increases in plasma levels of HIV PIs as a result of drug:drug interactions between this class of compounds is well established and is believed to be due to inhibition of cytochrome P450-mediated metabolism (20). Deletion of the *mdr1a* gene also results in increased plasma levels of several HIV PIs and it has been postulated that Pgp-mediated efflux along with P450 metabolism limits the oral bioavailability of HIV PIs (9,12). Therefore, it seemed reasonable that a drug:drug interaction involving Pgp expressed in the brain microvasculature may be one strategy to increase the CNS penetration of HIV PIs. Incubation of Caco-2 cells with 25 μM ritonavir resulted in an increased permeability of amprenavir across the epithelial monolayer (Table 3) suggesting that co-administration of ritonavir increased the membrane permeability of amprenavir by competing for Pgp. To investigate the possibility that addition of ritonavir may increase the brain penetration of amprenavir in vivo, male CD-1 mice were dosed with 250 mg (base)/kg of ritonavir for 4 days prior to administration of a single oral dose of [^{14}C]-amprenavir. Pretreatment with ritonavir did not alter the CNS levels of amprenavir-related material (Table 4). However, concentrations of amprenavir in testis, blood, and muscle increased 1.8- to 2.4-fold, demonstrating that ritonavir did affect the absorption, distribution, and/or metabolism of amprenavir in peripheral tissues.

DISCUSSION

The finding that Pgp influences the CNS disposition of amprenavir provides a mechanistic explanation for the limited in vivo brain penetration of this compound in rats and mice.

Table 4. Concentrations (nCi/g) of [^{14}C]-Amprenavir in CD-1 Mice Pretreated with Ritonavir

Tissue	Vehicle	Ritonavir	Ratio
Blood	23.2 ± 6.50	41.3 ± 13.9	1.8
Brain	1.88 ± 0.42[a]	1.41 ± 0.61[a]	0.75
CSF	20.2 ± 9.85	17.2 ± 4.28	0.85
Testes	7.03 ± 5.13	19.2 ± 6.33	2.7
Muscle	17.0 ± 3.86	39.8 ± 13.2	2.3

Note: Data are the average from 3–4 animals ± the standard deviation. Limit of Quantitation (LOQ): 1.056 nCi/g.
[a] Sections had >40% of pixels below quantification limit (BQL).

The Caco-2 and WBA studies provide unequivocal evidence that amprenavir has limited CNS penetration in vivo due to efflux by Pgp associated with the blood-brain barrier. Our results are in agreement with a number of previous publications demonstrating that HIV PIs are substrates for Pgp and the importance of this multidrug transporter on the disposition of these agents (9–13). Kim *et al.* demonstrated that indinavir, nelfinavir and saquinavir were Pgp substrates in LLC-PK1 cells overexpressing MDR1, and that administration of these compounds to *mdr1a* (−/−) knockout mice resulted in a 7- to 36-fold increase in brain levels compared to genetically matched animals (10). They also showed that plasma levels were 2- to 5-fold higher in the *mdr1a* (−/−) knockout mice, suggesting a role of Pgp in the intestinal absorption of these molecules. Lee *et al.* demonstrated that inhibition of HIV replication by HIV PIs in KB-3-1 (drug sensitive) and KB-V1 (drug resistant due to Pgp overexpression) cells was dependent on the endogenous expression of Pgp (11). Using reverse transcriptase (RT) activity as a marker of viral production, the authors demonstrated that 1 μM ritonavir, indinavir, or saquinavir reduced RT activity by >75% in the drug sensitive KB-3-1 cells. In contrast, this concentration could only reduce RT activity by 40–50% in the KB-V1 drug-resistant cell line. Addition of Pgp inhibitors such as PSC 833, quinidine, and rapamycin, reversed the drug-resistance of KB-V1 so that the RT inhibition profile was similar to that seen in KB-3-1 cells. These results, in conjunction with our studies, clearly demonstrate the importance of Pgp in affecting the body disposition and efficacy of HIV PIs.

The physico-chemical properties of amprenavir, i.e., lipophilic with clogP = 3.31 and a molecular weight = 505.6, in conjunction with the experimentally determined Papp value in Caco-2 (Table 1) and BBEC (9) monolayers suggest that this compound has good membrane permeation properties. Amprenavir and ritonavir had better membrane permeation properties than saquinavir and indinavir. Therefore, the limited in vivo CNS penetration seen in our early WBA studies could not be explained by the inability of amprenavir to pass through cellular membranes (21).

The limited CNS penetration of amprenavir and HIV PIs may also be attributed to high plasma protein binding. Similar to other HIV PIs, amprenavir is highly protein bound in human plasma, with a fractional binding of 93% (22,23). Amprenavir has a binding constant (K_d) to alpha 1-acid glycoprotein of 4 μM and a dissociation rate constant of 100 s^{-1}. This interaction is weak and has only minor effects on antiviral activity (22). The effect of protein binding limiting the CNS penetration of amprenavir would be expected to be minor as well, and is supported by WBA results. There is the possibility that administration of another compound may displace amprenavir from proteins in blood resulting in an increased free fraction of drug and higher brain levels of amprenavir. This is unlikely with GF120918, which is also extensively bound, and has been shown not to displace compounds including phenytoin, propranolol, diazepam, digitoxin, paclitaxel, daunorubicin or doxorubicin from alpha 1-acid glycoprotein or albumin (24). Further, increased brain concentrations of amprenavir were seen in *mdr1a/1b* (−/−) knockout mice compared to genetically matched controls. These results suggest that protein binding was not rate limiting and that drug:drug interactions can not account for the increased brain levels seen in genetic knockout

mice. Based on these findings, the limited CNS penetration of amprenavir was not the result of high protein binding in blood.

Amprenavir has good CSF penetration in control or Pgp-modulated mice (Figs. 1 and 2). This demonstrates that amprenavir can penetrate the blood-CSF barrier located at the choroid plexus (an epithelial barrier) and that Pgp does not influence amprenavir's permeation of this tissue as greatly as that for the brain capillary endothelial cells. It has been previously demonstrated that the capillaries of the choroid plexus do not express Pgp (25). However, it is not clear whether the choroid plexus itself expresses Pgp. The CSF:blood ratio of amprenavir was less than unity in normal and GF120918-treated mice and the increase in CSF levels of amprenavir were larger than that seen for blood, 3.3-fold compared to 2.0-fold (Table 2). These data suggest that Pgp is expressed in choroid plexus. CSF levels of amprenavir could not be determined in *mdr1a/1b* (−/−) mice due to the high brain tissue concentrations. However, if one assumes that the brain and CSF concentrations are equal in the Pgp deficient mice, then one can calculate a 2.5-fold increase in the CSF concentrations of amprenavir. This is larger than the 1.3-fold increase seen in blood (Table 2). This result further supports expression of Pgp in the choroid plexus and suggests that reducing the expression of Pgp by genetic disruption or chemical inhibition yields concentrations of amprenavir in CSF equal to those in blood. Further characterization of the choroid plexus needs to be completed to understand the role of Pgp in this structure and the importance on the disposition of HIV PIs.

Drug:drug interactions are an important consideration for HIV PIs because multiple medications are given to patients, including combinations of protease inhibitors. It has been documented that addition of ritonavir enhances the bioavailability of saquinavir, indinavir, and nelfinavir (20). Because ritonavir is such a potent inhibitor of CYP3A4 and other CYP450 isozymes, one explanation for the increased exposure of co-administered HIV PIs with ritonavir is inhibition of CYP450 metabolism. Recent reports have demonstrated overlapping substrate specificity of CYP450 3A and P-glycoprotein substrates (26,27). It is be possible that ritonavir increases the absorption of other HIV PIs by inhibiting intestinal Pgp as well as CYP450 metabolism. Therefore, one could envision a "beneficial" drug:drug interaction enhancing the CNS penetration of another HIV PI if given in combination with ritonavir. A WBA study was completed to see if pretreatment with ritonavir could enhance the CNS penetration of amprenavir. Ritonavir was chosen because it is a potent inhibitor of CYP450 3A. This in conjunction with the overlapping substrate specificity with Pgp suggested that it might also be effective at "inhibiting" Pgp activity. Even though amprenavir's exposure in peripheral tissues was increased 2-fold, ritonavir had no effect on the brain or CSF levels of amprenavir. There are several possible explanations for this result. First, the blood concentrations of ritonavir and amprenavir may have been too low to saturate the enzyme at the blood-brain barrier, and Pgp was able to efficiently exclude amprenavir from the CNS. Second, ritonavir may have a lower binding affinity for Pgp than amprenavir, resulting in ritonavir being ineffective as a "MDR modulating agent". Finally, it is possible that upon multiple dosing, ritonavir induces Pgp expression in brain capillaries. Induction of Pgp has been demonstrated for several substrates (28) . HIV PIs may induce Pgp expression as well. These hypotheses require further investigation.

A recent report by Glynn and Yazdanian suggested that amprenavir is not a substrate for Pgp (29). The authors used a primary bovine brain endothelial cell (BBEC) model to screen compounds for brain permeation and transport. We also have used this model previously and observed that amprenavir had a 1.5-fold greater rate of transport from the basolateral to apical (B → A) direction compared to that for the A → B direction, and that the transport rate was concentration-dependent (9). An explanation for this difference in the findings is the limitation of the BBEC model to study Pgp-mediated efflux. The expression of Pgp in BBECs is variable from isolation to isolation, and expression levels of Pgp are very low, making it difficult to quantitatively characterize Pgp substrates with this model. This, along with the inherent good membrane permeation of amprenavir and the "leakiness" of the BBEC system, make it difficult to detect amprenavir's efflux across BBEC monolayers. Therefore, we chose to use Caco-2 cells to examine the Pgp-mediated transport of amprenavir.

In summary, amprenavir is a Pgp substrate in bovine brain endothelial cells, Caco-2 cells, and in vivo. Pgp plays an important role in limiting the CNS penetration of HIV PIs. The challenge is to separate the chemical attributes required for protease inhibitory activity from those required for Pgp binding. If accomplished, it would enable the design of the next generation of HIV PIs that would avoid Pgp and be able to penetrate the blood-brain barrier.

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