Active Apical Secretory Efflux of the HIV Protease Inhibitors Saquinavir and Ritonavir in Caco-2 Cell Monolayers

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Purpose. To investigate *in vitro* the mechanisms involved in the gastrointestinal absorption of the HIV protease inhibitor, saquinavir mesylate (Invirase®), whose oral bioavailability is low, variable, and significantly increased by co-administration with ritonavir, also an HIV protease inhibitor but with higher oral bioavailability.

Methods. Confluent epithelial layers of human Caco-2 cells mimicking the intestinal barrier.

Results. Both saquinavir and ritonavir showed polarized transport through Caco-2 cell monolayers in the basolateral to apical direction (secretory pathway), exceeding apical to basolateral transport (absorptive pathway) by factors of 50-70 and 15-25, respectively. Active efflux was temperature dependent, saturable and inhibited by verapamil and cyclosporin A. Saquinavir and ritonavir decreased each other's secretory permeability and hence elevated their net transport by the absorptive pathway.

Conclusions. Saquinavir and ritonavir are both substrates for an efflux mechanism in the gut, most likely P-glycoprotein, which acts as a counter-transporter for both drugs. Together with sensitivity to gut-wall metabolism by cytochrome P-450 3A, this may partially account for the low and variable oral bioavailability of saquinavir in clinical studies and for its increased bioavailability after co-administration with ritonavir.

KEY WORDS: HIV protease inhibitors; saquinavir; ritonavir; Caco-2 cells; efflux system.

INTRODUCTION

The oral bioavailability of the human immunodeficiency virus (HIV) protease inhibitor saquinavir mesylate (Invirase®) is low and variable in patients (1). Exposure to a new formulation, Fortovase®, is approximately 3-fold higher. Saquinavir bioavailability increases both supraproportionally to dose (1,2) and on coadministration with ritonavir (Norvir®), another HIV protease inhibitor, in rats and in man (3–5).

The reasons for low oral bioavailability are unclear. One explanation is that absorption is decreased by an active efflux pump in the intestine such as p-glycoprotein (P-gp). P-gp is a 170 kDa transmembrane protein member of the ATP binding cassette (ABC) transporter family (6). It is localized at the apical secretory surface of various tissues (e.g. adrenal cortex, liver, kidney, gastrointestinal tract, blood brain barrier) where it mediates the active transmembrane transport of a variety of lipophilic substrates. It appears to act as a general detoxification system protecting tissues from a broad spectrum of lipophilic endogenous or exogenous toxic compounds, most of which tend to be large, aromatic and amphiphilic (7,8). A certain type of multidrug resistance (MDR) in tumor cells is associated with P-gp overexpression; P-gp also limits the oral absorption of a number of drugs, e.g. vinblastine and cyclosporin A, by transporting them back from the intestinal cells into the gut lumen (9,10).

To determine whether saquinavir and ritonavir are also substrates for P-gp and to account for some clinical observations with saquinavir, we studied the *in vitro* transport of both molecules in a human intestinal Caco-2 cell system expressing P-gp on its apical surface (11–13).

MATERIAL AND METHODS

Chemicals

All cell culture media were supplied by Gibco BRL. (G-^{3}H)Vinblastine sulphate (18.1 Ci/mMol) (MW 909) and (mebmt-β-^{3}H)cyclosporin A (8.90 Ci/mMol) (MW 1204) were purchased from Amersham. ^{14}C-saquinavir was supplied by Roche Welwyn (Ro-31-8959/006, MW 766.95, 115 μCi/ml, 85.3 μCi/mg), and saquinavir mesylate by Roche Basle. Ritonavir and ^{14}C-ritonavir (ABT-538, MW 721, 50 μCi/ml, 56.4 μCi) were kindly provided by Abbott. All other chemicals were supplied by Sigma and Gibco BRL.

Caco-2 Cell Model and Cellular Transport

Caco-2 cells were kept frozen in aliquots in liquid nitrogen. After thawing, the cells were maintained at 37°C in flasks in DMEM/MEM/Pen-Strep supplemented with 10% FCS and Glutamax in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every week by trypsinization. The medium was exchanged every second day. After a maximum of 8 passages, cell culture was restarted with the original frozen stock. Cell passage numbers used in this study ranged from 105–112.

For transport and cellular uptake studies, Caco-2 cells were seeded on microporous polycarbonate filter inserts (Costar Transwells, 1.13 cm^2, mean pore diameter 3 μm) at a density of 60,000 cells per cm^2. The inserts were placed in wells in 12-well cell culture plates and medium was changed every 2–3 days. For transport studies 21–28-day-old cell monolayers were used.

For apical to basolateral (AP > BL) transport experiments, compound, alone or in the presence of inhibitors, was dissolved in the apical compartment in 0.5 ml sample buffer (HEPES-buffered Hank's balanced salt solution, pH 7.4 containing 0.4 mM glucose, 0.1 mM sodium hydrogen carbonate, and 10% FCS). After 20 min, 40 min, and 80 min at 37°C, cell culture inserts were moved to other basolateral chambers containing 1.5 ml of fresh prewarmed sample buffer. Drug concentrations

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ABBREVIATIONS AP, apical (luminal side); AP > BL, drug transport from the apical to the basolateral compartment of Caco-2 cells; BL, basolateral (serosal side); BL > AP, drug transport from the basolateral to the apical side of Caco-2 cells; P-gp, p-glycoprotein; CYP3A, cytochrome P450 3A; HIV, human immunodeficiency virus.

in starting samples, collected samples and residual levels in the donor compartment at the end of the experiment were determined by LSC. Drug recovery was generally > 90% of applied material. For inhibition experiments, 10 mM stock solutions of compounds in DMSO were prepared and diluted at least 1:100 (prior to the experiment) in sample buffer. The final concentration of ≤1% DMSO did not alter cell viability or drug permeability significantly (data not shown) (14).

To investigate basolateral to apical (BL > AP) transport, drug was added to the basolateral compartment (in 1.5 ml sample buffer) and, after the indicated time intervals, 400 μl of the apical 500 μl in the cell culture inserts were replaced by fresh, prewarmed sample buffer. Some transport studies were performed at 4°C in order to identify active transport mechanisms. The integrity of the cell monolayers during the experiment was monitored by adding Lucifer yellow (LY) (2 mg/ml stock solution) to the donor chamber at a final concentration of 20 μg/ml. Cell-associated radioactivity was determined after washing the Caco-2 cells twice with 500 μl of ice-cold sample buffer on both sides of the filter.

P-gp substrate permeability coefficients varied up to 30% between experiments and between days although intra-assay variation in triplicate samples was relatively small (10%). Variation appeared dependent on Caco-2 passage number and cell monolayer age. Similar observations have been reported by others (12,13). As a consequence, only data from individual experiments (identical passage number and same day after seeding) were used for direct compound comparison.

Sample Analysis

Labeled compounds were quantified by carefully mixing 40 μl of sample with 200 μl of Microscint™ 20 (Packard) in 96-well OptiPlate™ microtiter plates (Packard) and measuring the cpm in a Packard Top Count System. No appreciable quenching was observed.

The integrity of the cell monolayer during the experiment was monitored by determining the amount of LY appearing in the receiver compartment. 100 μl of sample were added to MicroFLUOR™ 96-well plates (Dynatech) and fluorescence (excitation: 420 nm, emission: 515 nm) determined with a Perkin Elmer luminescence spectrophotometer LS 50B and a Perkin Elmer plate reader.

Calculations of Permeability Coefficients (Pe)

Apparent permeability coefficients (Pe) (cm/sec) were calculated using the equation:

$$Pe = (1/A*C_D)*dQ_A/dt$$

where dQ_A (mg) is the amount of drug transported within a given time period dt ($*s^{-1}$), A the Caco-2 cell surface area exposed to the compound (1.13 cm^2 in these experiments), and C_D the initial concentration of solute in the donor chamber (mg/ml).

Since Pe values calculated from this equation are only correct under experimental conditions that provide constant concentration gradients, all transport experiments were conducted under sufficient "sink" conditions (the experiment was stopped before the concentration in the acceptor compartment exceeded 10% of the donor concentration) (15,16).

Data are presented as mean ± SD. Statistical comparisons between two groups were made using t-tests. Values of $P < 0.05$ were considered significant.

RESULTS

Transport of ^{3}H-vinblastine and ^{3}H-cyclosporin A

Caco-2 cells are reported to express a P-gp efflux pump on the apical side of cell monolayers after reaching the differentiation stage (11–13). To confirm the functional expression of this efflux pump in our Caco-2 cell passage, ^{3}H-vinblastine (10 μM) and ^{3}H-cyclosporin A (1 μM), two known substrates of the P-gp efflux pump, were added to either the apical or basolateral side of the cell monolayer and radioactivity appearing in the receiver compartment was determined. Drug concentrations were selected to be below the reported saturation concentrations of vinblastine and cyclosporin A in the active transport system of Caco-2 cells (20 μM and 1 μM, respectively) (9,11,18). Results showed directional transport of both vinblastine and cyclosporin A, with BL > AP exceeding AP > BL approximately 60-fold and 7-fold, respectively (Table 1). No significant difference between the two directions was observed with paracellular markers such as LY or mannitol or with transcellularly transported midazolam (data not shown). Verapamil, a known P-gp inhibitor, reduced BL > AP transport and increased AP > BL transport in agreement with the literature data (12,17). In addition, verapamil increased the intracellular concentrations of both drugs. Average secretory Pe values (vinblastine: 3809 x 10^{-6} cm/min; cyclosporin A: 1105 x 10^{-6} cm/min) were generally higher than previously reported [vinblastine: 2712 x 10^{-6} cm/min (12); 731 x 10^{-6} cm/min (9); 465 x 10^{-6} cm/min (11); cyclosporin A: 600 x 10^{-6} cm/min (18); 106 x 10^{-6} cm/min (13)].

Table 1. Cumulative Transport of Verapamil and Cyclosporin A Through Caco-2 Cell

	Vinblastine			
	AP > BL		BL > AP	
	Transported	Intracellular	Transported	Intracellular
Buffer	64 ± 5	120 ± 7	3860 ± 322	522 ± 43
Verapamil	210 ± 19	300 ± 20	1890 ± 149	783 ± 69
	Cyclosporin A			
	AP > BL		BL > AP	
	Transported	Intracellular	Transported	Intracellular
Buffer	15 ± 3	52 ± 4	108 ± 8	114 ± 9
Verapamil	27 ± 2	125 ± 10	32 ± 3	245 ± 14

Note: 10 μM of ^{3}H-vinblastine or 1μM ^{3}H-cyclosporin A in buffer alone (control) or in the presence of 100 μM verapamil were added to the apical (AP > BL) or basolateral side (BL > AP) of Caco-2 cell monolayers. Cumulative drug transport (pMol) into the receiver compartment and intracelullar drug concentrations were determined after 80 min at 37°C (n = 3, ±SD).

Transport of ^{14}C-saquinavir and of ^{14}C-ritonavir

Results with ^{14}C-saquinavir and ^{14}C-ritonavir were similar to those with ^{3}H-vinblastine and ^{3}H-cyclosporin A. BL > AP transport of ^{14}C-saquinavir was, on average, 25-fold higher than AP > BL transport; for ritonavir the ratio was about 15-fold (Fig. 1). Pe values in the BL > AP direction ranged from about 1900 to 3100 x 10^{-6} cm/min for saquinavir and from 3000 to 4000 x 10^{-6} cm/min for ritonavir, depending on Caco-2 passage number and cell monolayer age; in the absorptive direction, they ranged from 50 to 100 x 10^{-6} cm/min and from 200 to 300 x 10^{-6} cm/min, respectively. Saquinavir polarized transport was temperature sensitive, indicating the presence of an energy-dependent active transport mechanism (Fig. 2). Similar results were obtained for ritonavir (not shown).

Effect of P-gp Inhibitors on ^{14}C-saquinavir and ^{14}C-ritonavir Transport

The effects of two known P-gp substrates, verapamil and cyclosporin A, on ^{14}C-saquinavir and ^{14}C-ritonavir transport through Caco-2 cells were also tested. Both substrates, acting as competitive inhibitors, increased AP > BL and decreased BL > AP transport (Fig. 3) while increasing the intracellular concentrations of both ^{14}C-saquinavir and, to a lesser extent, ^{14}C-ritonavir (Fig. 4). The presence of 10-fold excess cold compound enhanced the absorptive transport of ^{14}C-saquinavir and ^{14}C-ritonavir about 5-fold and 10-fold, respectively (Fig. 3), but decreased secretory transport by 30% and 50%, respectively. Saquinavir and ritonavir also affected each other's transport: saquinavir increased the AP > BL influx and decreased the BL > AP efflux of ritonavir and *vice versa.*

In contrast, ^{14}C-saquinavir and ^{14}C-ritonavir transport was not affected by midazolam (100 μM) (not shown). Midazolam is a known substrate for cytochrome P450 3A4 and was neither metabolized by the cells (24 h, 37°C) nor showed polarized transport across Caco-2 cells (Pe values for midazolam AP > BL: 1196 x 10^{-6} cm/min; BL > AP: 1298 x 10^{-6} cm/min) (data not shown), suggesting that the Caco-2 cell system did not express significant amounts of functional CYP3A4 enzyme. This agrees with published data (19,20). Other metabolic processes also did not seem to affect the transport of ^{14}C-saquinavir, ^{14}C-ritonavir or ^{3}H-cyclosporin A through Caco-2 cell monolayers, since TLC and HPLC analysis showed no degradation of transported compounds.

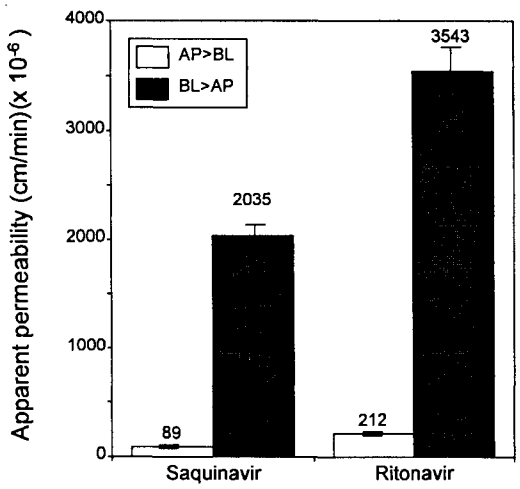

Fig. 1. Saquinavir permeability across Caco-2 cell monolayers. 10 μM of ^{14}C-saquinavir or ^{14}C-ritonavir in sample buffer (control) or in the presence of 100 μM verapamil were added to the apical (AP > BL) or basolateral side (BL > AP) of Caco-2 cell monolayers. After 80 min at 37°C, drug transported into the receiver compartment was determined and the apparent permeability coefficients calculated as described in Materials and Methods (n = 3, ± SD).

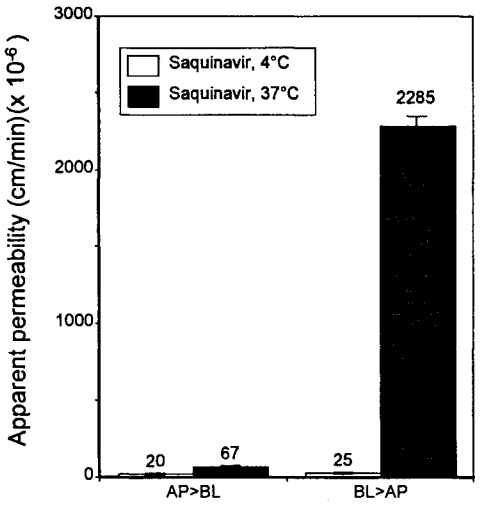

Fig. 2. Temperature dependence of saquinavir transport across Caco-2 cells. ^{14}C-saquinavir (10 μM solution) was added to either the apical or basolateral side of Caco-2 cell monolayers for 80 min at 4°C or 37°C. Apparent permeability coefficients were calculated from the radioactivity appearing in the receiver compartment as described in Materials and Methods. Bars represent the mean ± SD of at least 3 experiments.

DISCUSSION

Known P-gp substrates were used to demonstrate active efflux mechanisms in the Caco-2 cell line used in this study. Both vinblastine and cyclosporin A exhibited polarized transport across Caco-2 cells: BL > AP transport rates were approximately 60-fold and 7-fold higher, respectively, than in the AP > BL direction (Table 1). Average secretory Pe values for both substrates were as high as or higher than those previously described (9,11,19). Net secretory transport of both compounds was affected by verapamil, a known P-gp inhibitor, indicating the functional expression of a polarized efflux system, most likely P-gp, at high levels in our Caco-2 cell line (passages 105–112).

Both HIV protease inhibitors also showed polarized apical efflux with an average 25-fold and 15-fold higher apparent permeability in the BL > AP than in the AP > BL direction for saquinavir and ritonavir, respectively. Secretory transport of both compounds through Caco-2 cells was highly temperature-dependent, suggesting the involvement of an active energy-dependent transport system operating in the BL > AP direction (Fig. 2). Moreover, cyclosporin A and verapamil, two known competitive inhibitors of P-gp, both increased the net absorption of saquinavir and ritonavir, by decreasing efflux and increasing influx (Fig. 3). Similarly, ritonavir and saquinavir increased

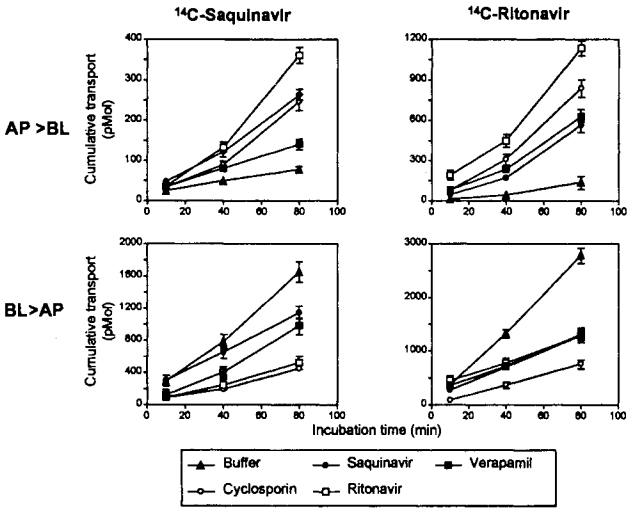

Fig. 3. Transport of saquinavir and ritonavir in the presence of other P-gp substrates. 10 μM of ^{14}C-saquinavir or ^{14}C-ritonavir in sample buffer alone or in the presence of 100 μM cyclosporin A, verapamil, unlabeled saquinavir or ritonavir was added to the apical (AP > BL) or basolateral side (BL > AP) of Caco-2 cell monolayers. Cumulative radioactivity appearing in the receiver compartment was determined and the amount of compound calculated from its specific activity. The data represent the mean ± SD of 3 experiments.

each other's net absorption and intracellular concentration, the effect of ritonavir being more pronounced than *vice versa* (Figs. 3 and 4). Taken together, these results indicate that both saquinavir and ritonavir are substrates for the same active efflux system present on Caco-2 cells, most likely P-gp.

Although our *in vitro* studies revealed that both HIV protease inhibitors are substrates for efflux mechanisms present in Caco-2 cells, the overall influence of efflux systems in the gut

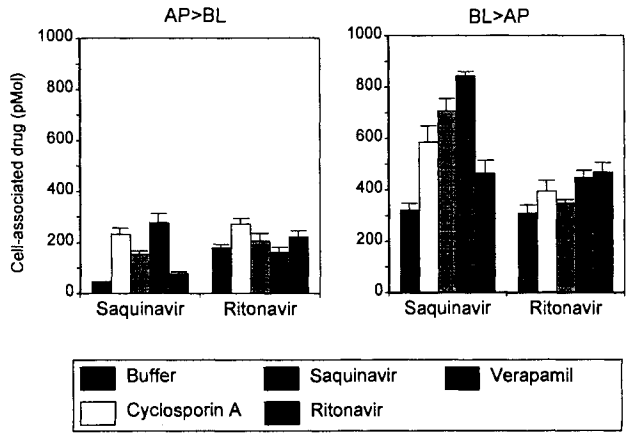

Fig. 4. Saquinavir and ritonavir internalized by Caco-2 cells during drug transport studies. 10 μM of ^{14}C-saquinavir or ^{14}C-ritonavir in sample buffer or in the presence of 100 μM cyclosporin A, verapamil, unlabeled saquinavir or ritonavir were added to the apical (AP > BL) or basolateral (BL > AP) side of Caco-2 cell monolayers. After 80 min at 37°C, Caco-2 cells on filters were washed and cell-associated radioactivity was determined. Bars represent the mean ± SD of 3 experiments.

wall on the oral bioavailability of the two compounds *in vivo* is not clear. In clinical studies, saquinavir oral bioavailability is low, variable and food dependent (1). In contrast, the oral bioavailability of ritonavir is relatively high (3,25). Hence factors other than efflux systems must exist which account for or contribute to the observed differences in bioavailability between the two compounds.

In the case of the P-gp substrate cyclosporin A, poor oral bioavailability has been attributed to both gut counter-transport processes and high metabolism by cytochrome P-450 3A (CYP3A), an enzyme which is relatively abundant in the gut wall (10,20,22). It has been suggested that the extensive gut metabolism of cyclosporin A is enhanced by P-gp which actively transports the compound back into the lumen, thereby increasing its residence time and hence the chance for metabolic transformation (22,23). The situation is probably similar for saquinavir, which also undergoes substantial metabolism by CYP3A (1). In contrast, ritonavir, which is only slowly metabolized by CYP3A, acts as an extremely potent inhibitor of CYP3A (26). Thus oral absorption of ritonavir could be a function of efflux much more than of metabolism. However, its oral bioavailability is high, indicating that substrate specificity for an efflux mechanism in the gut is not by itself predictive of low oral biovailability; other factors must combine to produce this effect, such as gut-wall metabolism, low drug solubility and/or a slow dissolution rate.

The concept that at least two processes work in concert to affect saquinavir oral absorption has consequences for *in vivo* studies with HIV protease inhibitors. First, if drug dissolution and solubility in the gut are not limiting factors, counter-transport and/or metabolic processes limiting drug bioavailability can be expected to be overcome, at least partially, by increasing the dose. Thus *in vitro* saturation of efflux mechanisms increases net compound absorption through Caco-2 cells (Fig. 3). In clinical studies this also seems to occur. The absorption pharmacokinetics of saquinavir are nonlinear: absorption increases supraproportionally to dose (1). Similar observations have been reported for cyclosporin A (24). Thus there seems to be a threshold above which transport/metabolic systems are saturated and passive diffusion across the gut wall becomes the rate limiting step. The threshold for a given compound will be difficult to predict since in addition to its affinity for efflux mechanisms and/or metabolic enzymes, it will also depend on a number of other factors such as solubility and dissolution rate or the degree of counter-transporter expression in an individual. Moreover, prediction of the total dose required to reach therapeutic levels might be further complicated by the recent finding that, apart from the absorption step, related efflux mechanisms seem to impact also on other areas of pharmacokinetics such as distribution and elimination (27,28).

Second, coadministered drugs and/or excipients which compete for efflux mechanisms and/or metabolic processes will affect the bioavailability of saquinavir and ritonavir. For example, CYP3A enzyme inducers (i.e. rifampicin, nevirapine) and inhibitors (i.e. ketoconazole) were found in clinical studies to decrease and increase saquinavir/ritonavir bioavailability, respectively (1,25). Such drug interactions are of particular interest in view of recent combination trials with HIV protease inhibitors, which were initiated to reduce disease progression (risk of developing key resistance mutations) and to prolong survival. In several of these studies, blood levels of one drug

were affected by coadministration of another protease inhibitor (1,25,29,30). For example, ritonavir increased the AUC of saquinavir more than 20-fold and that of nelfinavir (Viracept®) 2.5-fold. Similarly nelfinavir increased the saquinavir AUC 5-fold. The AUC effects have been attributed to inhibition of the metabolism of one protease inhibitor by another. However, in view of our *in vitro* competition studies with ritonavir and saquinavir (Fig. 3), the clinical effects observed with protease inhibitor combinations can also be attributed at least partially, to competition for efflux mechanisms during drug absorption and/or elimination. Consistent with this idea would be the clinical observation that increases in saquinavir blood levels are much higher in the presence of ritonavir than of ketoconazole, although both compounds are potent CYP3A inhibitors.

To clarify this issue further, all HIV protease inhibitors should be screened for inhibition/induction of CYP3A metabolism and competition/induction for efflux mechanisms. This information, together with the known patterns of resistance-associated mutation and individual compound side effect profiles, should allow more rational identification of favorable HIV protease combinations in terms of delaying the emergence of viral resistance, lowering doses, replacing thrice by twice daily dosing, and reducing adverse effects. More research is also needed to explore drug combinations for their effect on the blood-brain-barrier, since HIV is believed to be concealed within the central nervous system and P-gp-mediated efflux mechanisms restrict drug penetration into the brain (17,29).

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