Raltegravir Permeability across Blood-Tissue Barriers: Potential Role of Drug Efflux Transporters

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Running Head: Raltegravir Transport across Blood-Tissue Barriers

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Abbreviations

ABC, ATP-binding cassette

ARVs, antiretroviral drugs

BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein

BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester

BCRP, breast cancer resistance protein

CNS, central nervous system

CsA, cyclosporine A

FTC, fumitremorgin C

hCMEC/D3, human cerebral microvessel endothelial cell line

HIV-1, human immunodeficiency virus type 1

Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1′,2′:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester

MK571, 3-[[3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methyl[thio]propanoic acid

MRPs, multidrug resistance-associated proteins

P-gp, P-glycoprotein

PSC833, valspodar

R-6G, rhodamine-6G
Abstract

The objectives of this study were to investigate raltegravir transport across several blood-tissue barrier models and potential interactions with drug efflux transporters. Raltegravir uptake/accumulation/permeability were evaluated in vitro in: i) P-gp, BCRP, MRP1 or MRP4 overexpressing MDA-MDR1 (P-gp), HEK-ABCG2, HeLa-MRP1 or HEK-MRP4 cells, respectively; ii) cell culture systems of the human blood-brain (hCMEC/D3), mouse blood-testicular (TM4) and human blood-intestinal (Caco-2) barriers; and iii) rat jejunum and ileum segments using an in situ single-pass intestinal perfusion model. [3H]Raltegravir accumulation by MDA-MDR1 (P-gp) and HEK-ABCG2 overexpressing cells was significantly enhanced in the presence of PSC833, a P-gp inhibitor, or Ko143, a BCRP inhibitor, suggesting the inhibition of P-gp or BCRP mediated efflux process, respectively. Furthermore, [3H]raltegravir accumulation by human cerebral microvessel endothelial hCMEC/D3 and mouse Sertoli TM4 cells was significantly increased by PSC833 or Ko143. In human intestinal Caco-2 cells grown on Transwell filters, PSC833 but not Ko143 significantly decreased [3H]raltegravir efflux ratios. In rat intestinal segments, [3H]raltegravir in situ permeability was significantly enhanced by concurrent administration of PSC833 and Ko143. In contrast, in transporter inhibition assays, raltegravir (10-500 µM) could not increase the accumulation of substrates for P-gp (rhodamine-6G), BCRP ([3H]mitoxantrone) or MRP1 (BCECF) by MDA-MDR1 (P-gp), HEK-ABCG2 or HeLa-MRP1 overexpressing cells, respectively. Our data suggest that raltegravir is a substrate but not an inhibitor of drug efflux transporters, P-gp and BCRP. These transporters could play a role in restricting raltegravir permeability across the blood-brain, blood-testicular and
blood-intestinal barriers potentially contributing to its low tissue concentrations and/or low oral bioavailability observed in the clinic.
Introduction

Highly active antiretroviral therapy, which combines several classes of antiretroviral drugs (ARVs), has been proven effective in suppressing human immunodeficiency virus type 1 (HIV-1) replication and subsequently can reduce HIV-associated morbidity and mortality (1-3). Despite the success of ARVs in the treatment of HIV-1 infection, the emergence of resistant viral strains, non-adherence to therapy, interindividual variability in ARVs pharmacokinetics/pharmacodynamics, drug-drug interactions and adverse effects continue to pose major therapeutic challenges (4,5). Furthermore, ARVs are also known to be substrates, inhibitors and/or inducers of metabolic enzymes and/or membrane drug transporters, such as ATP binding cassette (ABC) transporters, which actively efflux drugs from the cell, and/or solute carrier transporters, which mediate drug uptake and/or bidirectional transport across cell membranes (3). Overall, drug transporters and metabolic enzymes can regulate intestinal absorption, tissue distribution, renal excretion, and hepatobiliary elimination of ARVs and may also contribute to drug-drug interactions at these sites (3,6).

Lack of an effective vaccine along with increasing incidence of drug resistance have emphasized the need to develop new drugs with novel mechanisms of action (7).

Raltegravir represents a new class of ARVs developed by Merck & Co. Inc. which functions as an inhibitor of HIV-1 integrase, a viral enzyme that catalyzes an essential process in the viral replication cycle i.e., the insertion of HIV-1 proviral DNA into the host genome (8,9). Clinical studies have shown that raltegravir has a sustained antiretroviral effect and good tolerability in both treatment-naive and -experienced HIV-1 infected
patients (10,11). In humans, raltegravir is not a substrate or an inhibitor of cytochrome P450 enzymes and its primary route of metabolism and elimination is glucuronidation via uridine 5’-diphosphate glucuronosyltransferase 1A1 (12,13).

Several anatomically protected sites such as the central nervous system (CNS) and male genital tract have been recognized as sanctuary sites for HIV-1 (14). Viral replication can occur in these sites even in the presence of therapeutic concentrations of ARVs in plasma (15). Clinical studies have shown significant variability in raltegravir penetration in such tissues compared to plasma (16-19). In addition, raltegravir is reported to have low oral bioavailability in humans (20, 21,22). Studies from our group as well as others have shown functional expression of several ABC drug efflux transporters including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs) in blood-brain barrier, blood-testis barrier and blood-intestinal barrier models, which can collectively serve as a biochemical barrier to drug entry/distribution in these tissues. (25-29). Raltegravir interactions with P-gp and BCRP transporters have been investigated recently in a few studies; however, inconsistent results have been reported depending on the methods used (30-32). To the best of our knowledge, raltegravir interactions with drug efflux transporters at critical blood-tissue barriers have not been addressed previously.

The aim of this study was to investigate raltegravir interactions with drug efflux transporters and identify the role of these transporters in the permeability of raltegravir at blood-tissue barrier sites, such as the blood-brain barrier, blood-testis barrier and blood-intestinal barrier using in vitro and in situ cell/organ systems.
Materials and Methods

Materials

Raltegravir and [3H]raltegravir (0.5 mCi/mmol) were kindly provided by Merck Canada Inc. (Kirkland, Canada). [3H]Mitoxantrone (12.7 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Rhodamine-6G (R-6G) was purchased from Sigma-Aldrich (Oakville, Canada). PSC833/valspodar (6-[2S,4R,6E]-4-Methyl-2-(methylamino)-3-oxo-6-octenoic acid)-7-L-valine-cyclosporin A) and fumitremorgin C (FTC) were a gift from Novartis Pharma (Basel, Switzerland) and Dr. Susan Bates (Bethesda, MD), respectively. Cyclosporine A (CsA), (3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino [1′,2′:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethyl ester (i.e., Ko143) and (E)-3-[[3-[(1E)-2-(7-chloro-2-quinolinyl) ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid (i.e., MK571) were purchased from Tocris Biosciences (Ellisville, MO). All cell culture reagents as well as 2′,7′-bis-(carboxyethyl)-5(6′-carboxyfluorescein acetoxy methyl ester (i.e., BCECF-AM) were purchased from Invitrogen (Carlsbad, CA).

Cell culture systems

All cell lines were grown and maintained in a humidified incubator at 37°C, 5% CO2, 95% air atmosphere with fresh media replaced every 2-3 days. The human cell lines stably transfected with human cDNA for 1) MDR1/P-gp (MDA-MDR1), 2) ABCC1/MRP1 (HeLa-MRP1), 3) ABCC4/MRP4 (HEK-MRP4) and 4) ABCG2/BCRP (HEK-ABCG2) were kindly provided by Drs. Robert Clarke (Georgetown University, Washington, DC), Susan Cole (Queen’s University, Kingston, Canada), Piet Borst (Netherlands Cancer Institute, Amsterdam, Netherlands) and Susan Bates (Bethesda, MD), respectively, and...
were cultured according to our previously published protocols (28). The immortalized human cerebral microvessel endothelial cell line (hCMEC/D3) was kindly provided by Dr. P.O. Couraud (Institut Cochin, Departement Biologie Cellulaire and INSERM, Paris, France). The mouse Sertoli testicular (TM4) and Caco-2 cell lines were obtained from the American Tissue Culture Collection (Manassas, VA). These cells were cultured as described in our previous publications and others (26,28,29).

Functional studies

Cellular uptake/accumulation of fluorescent substrates of P-gp (R-6G, 1 µM) and MRP1 (BCECF-AM, 5 µM), radiolabelled substrate of BCRP [³H]mitoxantrone (20 nM, 0.1 µCi/ml) or [³H]raltegravir (1 µM, 0.1 µCi/ml) were determined by standard transport assays protocols well established in our laboratory (33,34) in the absence or presence of P-gp inhibitors (CsA, 25 µM and/or PSC833, 5 µM), BCRP (Ko143, 5 µM and/or FTC, 10 µM) or MRP1 (MK571, 10 µM) or in the presence of raltegravir, 25-500 µM (35). Because BCECF-AM and mitoxantrone are also known substrates of P-gp, all assays involving these two substrates were performed in the presence of the P-gp inhibitor PSC833 (2 µM).

In situ single-pass perfusion of rat intestine

Open-loop in situ perfusion studies were carried out in male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) according to our previously published method (29) in accordance with the study protocol approved by the University of Toronto Animal Ethics Committee. The method was adapted from previously published protocols from our group (29). Briefly, animals were anaesthetized with the use of isoflurane inhalant anaesthetic and maintained at 37°C throughout surgery and perfusion procedures. The abdominal cavity was opened by midline incision (3-4 cm) to expose the proximal jejunum (6-8 cm in length,
starting 2 cm after the ligament of Treitz) and distal ileum (6-8 cm in length, ending 1 cm before the cecum) segments. These segments were selected for perfusion studies because of their known importance in the absorption of drugs (36,37). Each segment was connected to the inlet and outlet tubing via cannulas, and perfused at a constant flow rate of 0.2 ml/min using a syringe infusion pump (Harvard Apparatus syringe pump, Model 22) with a pre-incubation solution containing unlabeled raltegravir (10 μM) and D-mannitol (10 μM), a marker of paracellular permeability, for a 30 min period to reach the steady state. To initiate the measurement of raltegravir permeability, the perfusion buffer containing [³H]raltegravir and [¹⁴C]-D-mannitol, supplemented with unlabeled raltegravir and D-mannitol to achieve 10 μM concentrations, was introduced into the segment (at time 0) and the perfusate exiting from each segment through outlet tubing was collected into vials in consecutive 10 min intervals (control conditions). After 1h, the perfusion buffer was replaced with identical [³H]raltegravir (10 μM) and [¹⁴C]-D-mannitol (10 μM) solution also containing PSC833 (5 μM) or Ko143 (5 μM) or both PSC833 and Ko143 (5 μM/each), and the perfusate was collected in consecutive 10 min intervals over a 60 min period. All collected perfusate samples were weighed in order to determine the change in weight between perfusion buffer entering and exiting each segment. Raltegravir and D-mannitol concentrations in perfusate were quantified by liquid scintillation counting by measuring ³H/¹⁴C radioactivity. These concentrations were corrected for water reabsorption by gravitational method (using the ratio of inflow/outflow weight). In each animal, raltegravir steady-state effective permeability (P$_{eff}$) value was determined during the first hour (0-60 min) in the absence of inhibitor (control) and compared to P$_{eff}$ obtained in the same animal during the second hour, when PSC833 (5 μM) or Ko143 (5 μM) or both (5 μM each)
inhibitors were present in the perfusate buffer (60-120 min), in order to use each animal as its own control and allow paired data analysis.

**Data analysis**

Each set of *in vitro* experiments was repeated at least three times in cells pertaining to different passages. In an individual experiment, each experimental point was performed in triplicate. Statistical analysis was performed using GraphPad Prism® (version 5.01 for Microsoft Windows, Graph Pad Software, San Diego, California) and significance was assessed by applying the unpaired or paired two-tailed Student’s *t*-test for unpaired or paired experimental values, or the one-way analysis of variance (ANOVA) followed by Bonferroni correction for test of repeated measures, as appropriate. A *p* value < 0.05 was considered statistically significant.

To determine raltegravir permeability across the Caco-2 monolayers grown on Transwell inserts, the data for time dependent raltegravir flux in the apical-to-basolateral or basolateral-to-apical direction was fitted into equation 1 (38):

\[
P_{\text{app}} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}
\]

where \(P_{\text{app}}\): apparent permeability coefficient (cm/s); \(\Delta Q/\Delta t\): solute flux rate (mol/s) from donor into the receiver compartment at steady state; \(A\): surface area of the filter insert (cm\(^2\)); \(C_0\): initial solute concentration (mol/cm\(^3\)) in the donor compartment. To evaluate the role of active transport in raltegravir directional permeability, the efflux ratio defined as the quotient of the secretory permeability and the absorptive permeability was determined according to equation 2 (38):

\[
ER = \frac{P_{\text{app}}(B-A)}{P_{\text{app}}(A-B)}
\]
where ER: efflux ratio; $P_{\text{app}}$ (A-B): apparent permeability coefficient measured for drug permeability in the apical-to-basolateral by introducing drug into the donor compartment (apical) and measuring its appearance in the receiver compartment (basolateral); $P_{\text{app}}$ (B-A): apparent permeability coefficient measured in the basolateral-to-apical by introducing the drug into the donor compartment (basolateral) and measuring its appearance in the receiver compartment (apical).

The effective perfusion coefficient for raltegravir permeability across the rat intestinal segment at steady state ($P_{\text{eff}}$) was measured according to equation 3 (39):

$$P_{\text{eff}} = \frac{-Q \ln(C_{\text{out}}/C_{\text{in}})}{2 \pi RL}$$

where $Q$: flow rate (0.2 ml/min, converted to cm$^3$/s), $C_{\text{out}}/C_{\text{in}}$: ratio of the outlet raltegravir concentration (corrected for water reabsorption) and the inlet raltegravir concentration in the perfusion buffer (i.e., 10 μM), $R$: radius of rat intestine (0.18 cm); $L$: length of intestinal segment (approximately 8 cm). Data fitting into each model was performed by nonlinear least-squares analysis using GraphPad Prism®.
Results

**ABC transporter inhibition assays**

We investigated if raltegravir can serve as an inhibitor for the drug efflux transporters, P-gp, BCRP or MRP1. Initially, we examined the accumulation of the P-gp substrate R-6G by MDA-MDR1 (P-gp) overexpressing cells in the presence of different concentrations of raltegravir. In contrast to the effect observed with P-gp inhibitor CsA, no statistical significant differences were detected in R-6G accumulation in the presence of 10-500 µM raltegravir, compared to control. These data suggest that raltegravir does not inhibit P-gp mediated transport of R-6G (Fig. 1A).

We then examined the accumulation of the BCRP substrate [³H]mitoxantrone by HEK-ABCG2 overexpressing cells in the absence or presence of BCRP inhibitor Ko143 or several concentrations of raltegravir (10-100 µM). [³H]Mitoxantrone accumulation was significantly enhanced in the presence of Ko143 but not in the presence of raltegravir, suggesting that raltegravir does not interfere with BCRP-mediated transport processes (Fig. 1B).

In order to investigate if raltegravir is an inhibitor of MRP1, we determined the accumulation of the MRP substrate BCECF in HeLa-MRP1 overexpressing cells in the presence or absence of MRP inhibitor MK571 (10 µM) or raltegravir (10-500 µM). MK571 significantly enhanced the accumulation of BCECF whereas raltegravir did not exert any effect, suggesting that raltegravir does not serve as an inhibitor of this efflux transporter (Fig. 1C).
We investigated if raltegravir can serve as a substrate for the drug efflux transporters, P-gp, BCRP, MRP1 and MRP4. We characterized the accumulation of [3H]raltegravir by MDA-WT and MDA-MDR1 (P-gp) overexpressing cells in the absence or presence of P-gp inhibitors PSC833 or CsA. [3H]Raltegravir accumulation was significantly lower in P-gp overexpressing MDA-MDR1 (P-gp) compared to MDA-WT cells. In addition, its accumulation was significantly enhanced in the presence of the two inhibitors only in MDA-MDR1 (P-gp) but not in MDA-WT cells. Together, these data suggest that raltegravir is a P-gp substrate (Fig. 2A).

[3H]Raltegravir accumulation was also measured in both HEK-WT and HEK-ABCG2 overexpressing cells in the absence or presence of BCRP inhibitors, Ko143 or FTC. Compared to HEK-WT, [3H]raltegravir accumulation was significantly lower in HEK-ABCG2 cells. In addition, [3H]raltegravir accumulation was significantly enhanced in the presence of Ko143 or FTC, but remained unchanged in HEK-WT cells. These data suggest that [3H]raltegravir can serve as a BCRP substrate (Fig. 2B).

We further determined [3H]raltegravir accumulation in HeLa-WT and HeLa-MRP1 overexpressing cells in the absence or presence of the MRP inhibitor MK571. No difference in [3H]raltegravir accumulation was observed between HeLa-WT and HeLa-MRP1 cells, suggesting that [3H]raltegravir is not a substrate for MRP1 (Fig. 2C). We also investigated if raltegravir can serve as a substrate for MRP4 using HEK-WT and HEK-MRP4 overexpressing cells. However, [3H]raltegravir did not interact with this transporter (data not shown).
We investigated the time-dependent uptake of $[^3]$Hraltegravir in hCMEC/D3 and TM4 cells, two cell systems representative of human brain microvessel endothelial cells and rodent testicular Sertoli cells, respectively. As shown in Figures 3A and 4A, $[^3]$Hraltegravir cellular uptake at 37°C gradually increased over time until a plateau was reached at approximately 60 min.

In order to determine if P-gp, BCRP or MRPs can recognize $[^3]$Hraltegravir as a substrate in hCMEC/D3 and TM4 cells, we performed $[^3]$Hraltegravir accumulation assays in the absence or presence of P-gp, BCRP or MRPs selective inhibitors PSC833, Ko143 or MK571, respectively. The accumulation of $[^3]$Hraltegravir was significantly increased when cells were incubated in the presence of PSC833 or Ko143 in both hCMEC/D3 (Fig. 3B) and TM4 cells (Fig. 4B). Taken together, these data suggest that $[^3]$Hraltegravir permeability can be regulated by P-gp and/or BCRP-mediated efflux processes in an in vitro human blood-brain barrier and mouse blood-testis barrier cell culture systems.

$[^3]$HRaltegravir accumulation in TM4 cells was also significantly enhanced by MK571; however, in hCMEC/D3 cells MK571 addition did not exert any effect.

**Raltegravir permeability across Caco-2 monolayers**

We also investigated the time-dependent accumulation of $[^3]$Hraltegravir by the human intestinal Caco-2 monolayer cells. $[^3]$HRaltegravir uptake gradually increased over time until a plateau was reached at approximately 60 min (Fig. 5A). In addition, $[^3]$Hraltegravir accumulation (60 min) by Caco-2 monolayer cells was significantly enhanced in the presence of the P-gp inhibitor PSC833 but not the BCRP inhibitor Ko143 (Fig. 5B).
further evaluated in vitro raltegravir permeability across Caco-2 cell monolayers grown on Transwell filters (29). In the absence of inhibitors, $[^3]H$raltegravir permeability in the apical-to-basolateral direction ($P_{\text{app}}(A-B)$) was significantly lower compared to its basolateral-to-apical permeability ($P_{\text{app}}(B-A)$) (Fig. 5C), with an efflux ratio of 3.1. Addition of P-gp inhibitor PSC833 resulted in decreased basolateral-to-apical raltegravir $P_{\text{app}}$ with an efflux ratio approaching 1.43. In contrast, $[^3]H$raltegravir permeability remained unchanged in the presence of BCRP inhibitor, Ko143 (Fig. 5C). This could be attributed to the fact that BCRP protein expression is undetectable in Caco-2 cells (40). Together, these results suggest that raltegravir efflux at the apical membrane of Caco-2 cells is P-gp-mediated.

**Effect of P-gp and/or Bcrp inhibitors on raltegravir in situ intestinal permeability in rat intestinal segments**

The effect of P-gp and BCRP inhibitors, PSC833 and Ko143, respectively, on raltegravir in situ intestinal permeability was investigated by single-pass intestinal perfusion of proximal jejunum and distal ileum segments of rat intestine. These segments were selected because of their significant role in drug absorption (36,37). Raltegravir steady-state permeability ($P_{\text{eff}}$) was measured over a two-hour period (0-60 min, without inhibitors; 60-120 min, in the presence of PSC833 or Ko143 or both inhibitors) and the $P_{\text{eff}}$ values obtained during the two periods in each animal were compared by paired Student’s $t$-test using five animals per treatment group. The inhibition of P-gp or BCRP alone did not increase raltegravir $P_{\text{eff}}$ in the jejunum or ileum (Fig. 6A, 6B). However, when both PSC833 and Ko143 inhibitors were used together, significant increases in raltegravir permeability were observed both in
the jejunum (average increase of 78% [47-153%), \( p<0.0001 \)) and ileum (average increase of 79% [46%-127%], \( p<0.0001 \)) segments (Fig. 6C). In a set of control experiments, raltegravir \( P_{eff} \) was measured in the absence of inhibitors during both periods or in the presence of PSC833 or Ko143 or both inhibitors during both perfusion periods (data not shown). No significant differences were observed between raltegravir permeability during the 0-60 min and 60-120 min periods when experimental conditions were maintained the same.
Discussion

Raltegravir is a preferred drug regimen used along with other ARVs for the treatment of HIV-1 infection in HIV-naive and -experienced patients (41). It is known that ARVs can be substrates, inhibitors and/or inducers of drug efflux transporters which can play an important role in ARVs permeability in tissues having barrier function, such as blood-brain barrier, blood-testis barrier and blood-intestinal barrier (3). Although several recent studies have examined raltegravir interactions with drug transporters, these data remain limited and provide contradicting outcomes depending on the methods used (30-32,42-44).

Furthermore, the role that these drug transporters play in raltegravir permeability across blood-tissue barriers and its distribution into sanctuary sites of HIV infection (e.g., CNS, male genital tract) is not fully understood.

We initially investigated if raltegravir can serve as an inhibitor of drug efflux transporters. The results obtained from R-6G accumulation assay using P-gp-overexpressing cells suggest that raltegravir (up to 500 μM) is not an inhibitor of P-gp. Our observation is consistent with findings reported in other studies showing no inhibition of P-gp function by raltegravir (31,32,44). In mitoxantrone accumulation assay using BCRP-overexpressing cells, we were unable to detect any functional inhibition of BCRP by raltegravir (up to 100μM). These findings are in agreement with other studies reporting that raltegravir does not inhibit BCRP-mediated efflux of pheophorbide A in MDCKII-BCRP overexpressing cells (31) and only slightly inhibits BCRP-mediated transport of [3H]mitoxantrone in membrane vesicles expressing human BCRP (42). This slight inhibition reported by Rizk et al. may be due to the different method used to study BCRP function (i.e. membrane
vesicles) and/or interaction of raltegravir with P-gp-mediated transport of [3H]mitoxantrone in these vesicles, since mitoxantrone is a known substrate of P-gp. In our experiments, PSC833 was used to inhibit P-gp activity in order to only evaluate raltegravir interactions with BCRP-mediated transport of mitoxantrone. In BCECF accumulation assay, using MRP1-overexpressing cells, we did not detect MRP1 inhibition by raltegravir (up to 500 µM). To the best of our knowledge, this is the first study reporting that raltegravir does not inhibit MRP1-mediated transport. Collectively, our results suggest that raltegravir does not inhibit transport function of P-gp, BCRP or MRP1, and most likely will not exert drug-drug interactions with other concurrently administered drugs that are known to be substrates for these transporters.

We further evaluated raltegravir transport in ABC transporter-overexpressing cell lines and found that raltegravir serves as a substrate of P-gp and BCRP but not of MRP1 and MRP4. Our results are in agreement with a recent study where raltegravir was reported to be a substrate of both P-gp and BCRP using overexpressed LLC-PK1 (L-MDR1) and HEK293 (ABCG2) cells, respectively (32). Similarly, Zembrusk et al. reported that raltegravir is a substrate of P-gp but not of MRP1 or other MRP isoforms (MRP2 and MRP3) using cell proliferation assays in MDCKII overexpressing cell lines for each ABC transporter; however, they could not detect raltegravir transport by BCRP in MDCKII/BCRP cell line system [22]. Raltegravir transport by P-gp is also controversial. Most previous studies are in agreement with our findings that raltegravir is a substrate for P-gp (30-32,43). However, a recent study by Dupuis et al. reported that raltegravir is a weak P-gp substrate in P-gp-overexpressing cell line and Caco-2 monolayers, but at the same time shows no interactions
with P-gp in UIC2 shift assay, highlighting that different assays and methods used to investigate transporter interactions can provide contradictory results (44). Based on our results, raltegravir tissue distribution into sanctuary sites of HIV infection (e.g., CNS, male genital tract) and its oral bioavailability could be potentially regulated by drug transporters (e.g., P-gp and BCRP) expressed at these blood-tissue barriers.

We examined the role of P-gp and BCRP in raltegravir permeability across the blood-brain barrier using hCMEC/D3 cells, a well characterized in vitro cell line known to display morphological and biochemical properties of human brain microvessel endothelial cells representative of the blood-brain barrier. We found that P-gp or BCRP inhibitors, PSC833 or Ko143, respectively, significantly increased raltegravir accumulation by hCMEC/D3 cells. However, raltegravir accumulation was not enhanced in the presence of MRPs inhibitor, MK571. To the best of our knowledge these are the first observations showing the role of P-gp and BCRP in raltegravir transport in an in vitro human blood-brain barrier model. These data suggest that P-gp and BCRP localized at the blood-brain barrier could play a role in limiting raltegravir permeability in the brain. Our data support the recent observation of Yilmaz et al. who found significantly lower raltegravir concentration in cerebrospinal fluid (2.0-126 ng/ml, median 18.4 ng/ml) compared to plasma (37-5180 ng/ml, median 448 ng/ml) in 16 HIV-1 infected individuals receiving raltegravir at the recommended therapeutic dose (400mg, twice daily) (16). Furthermore, raltegravir cerebrospinal fluid-to-plasma concentration ratio varies significantly between individuals (16-18) suggesting that its permeability across the blood-brain barrier may be dependent on the expression/activity of drug efflux transporters such as P-gp or BCRP at this blood-tissue barrier.
barrier. Poor drug penetration into the CNS, a known sanctuary site for HIV-1 replication, has been linked to suboptimal clinical response to ARVs therapy and decline in neurocognitive function (45,46). Although a recent study by Johnson et al. could not demonstrate a link between raltegravir cerebrospinal fluid concentrations and ABCB1 (P-gp) polymorphism in healthy volunteers, this observation could be due to compensatory efflux mechanisms at the blood-brain barrier (e.g., BCRP-mediated efflux) or other interindividual differences which were not accounted for in this sample population (18).

Further studies are needed to elucidate the role of P-gp and BCRP in limiting raltegravir penetration into the CNS.

Male genital tract is another recognized HIV sanctuary site which is protected by the blood-testis barrier primarily composed of Sertoli epithelial cells which form tight junctions near the basement membrane. Raltegravir accumulation by mouse Sertoli cell line (TM4), an in vitro cell culture model of blood-testis barrier, was significantly enhanced by P-gp, Bcrp or Mrp inhibitors, PSC833, Ko143 or MK571, respectively, demonstrating that raltegravir permeability across the blood-testis barrier could be regulated by drug efflux transporters.

Using human in vitro cell culture systems overexpressing MRP1 and MRP4, we did not detect increased accumulation of \(^{3}H\)raltegravir in the presence of Mrp established inhibitor, MK571, suggesting that raltegravir is not a substrate of MRP1 or MRP4. However, in a recent publication from our group, we have demonstrated that BCECF (a fluorescent substrate of several Mrp isoforms including Mrp1 and Mrp4) accumulation in TM4 cells was significantly enhanced in the presence of MK571, showing that Mrp efflux transporters are functional in Sertoli cells (28). These results suggest that raltegravir might
interact with other MRP isoforms (e.g., MRP2, MRP3 or MRP5) at the blood-testis barrier. While Zembruski et al. reported that raltegravir is not a substrate of human MRP1, MRP2, or MRP3 using growth inhibition assays in MDCKII cells overexpressing these transporters (31), Hashiguchi et al. provided contradictory evidence that raltegravir is a substrate of MRP2 in HEK293-MRP2 overexpressing cells (32). Hence, further studies are needed to explore this hypothesis. Intestinal epithelium is another blood-tissue barrier characterized by expression of tight junctions, which restrict paracellular transport of drugs, and cytochrome P450 enzymes and drug transporters, which collectively act as a biochemical barrier restricting transcellular passage of drugs into the circulation. In humans raltegravir is reported to have low oral bioavailability (approximately 30%), which suggests that its permeability across the intestinal epithelium is low (21,22). Since raltegravir is not metabolized by cytochrome P450 enzymes, drug transporters localized to the intestinal barrier may restrict raltegravir oral absorption. Using Caco-2 cell monolayers grown on Transwell filters, an in vitro model for human intestinal epithelium, we found that PSC833, a P-gp inhibitor, significantly reduced raltegravir basolateral-to-apical permeability, demonstrating that P-gp mediates raltegravir efflux at the apical membrane of Caco-2 cells, in agreement with findings from other studies (30,32,43). However, we did not observe similar effects in the presence of Ko143, the BCRP inhibitor. This could likely be due to the low BCRP protein expression in this cell system (40). These results demonstrate that P-gp is the primary efflux transporter mediating raltegravir efflux from Caco-2 cells and restricting its apical membrane permeability. We further investigated the role of P-gp and BCRP in raltegravir
permeability across rat intestinal barrier using an *in situ* perfusion technique. Raltegravir $P_{eff}$ was not changed in the presence of P-gp or Bcrp inhibitor, PSC833 or Ko143, respectively, when used individually. However, in the presence of both P-gp and Bcrp inhibitors, raltegravir $P_{eff}$ increased significantly in both jejenum and ileum segments, suggesting cooperative activity of these transporters in restricting raltegravir intestinal permeability. The cooperative function of P-gp and Bcrp transporters in the brain accumulation of the anti-cancer drug, sunitinib, has been reported previously (47). In addition, previous studies have reported that raltegravir accumulation and permeability in Caco-2 cells is dependent on intestinal lumen pH (43,48), suggesting that oral absorption of raltegravir could be altered by changes in luminal pH as well as the expression and/or activity of drug efflux transporters at the blood-intestinal barrier.

Both P-gp and BCRP are known to play a significant role in the permeability of several drugs including ARVs in HIV sanctuary sites such as the brain and testis (3,6). Herein, we show that raltegravir can serve as a substrate of both P-gp and BCRP in respective transporter overexpressing cells as well as in *in vitro* cell culture models of human blood-brain barrier (hCMEC/D3), blood-testicular barrier (TM4), blood intestinal barrier (Caco-2) and in an *in situ* intestinal perfusion rat model. Together, our results suggest that P-gp and/or BCRP may play a significant role in restricting the permeability of raltegravir in these tissues.
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**Figure legends**

**FIG 1** Raltegravir interactions with ABC transporters. (A) Effect of raltegravir on R-6G accumulation by MDA-MDR1 (P-gp) monolayer cells. Cellular accumulation of R-6G (1 µM) was determined at 60 min by exposing confluent MDA-MDR1 (P-gp) cells to R-6G in assay buffer with or without P-gp inhibitor CsA (25 µM) or raltegravir (10-500 µM). (B) Effect of raltegravir on [³H]mitoxantrone accumulation by HEK-ABCG2 overexpressing cells. Accumulation of [³H]mitoxantrone (20 nM) at 120 min was measured in the absence (control) or presence of BCRP inhibitor (Ko143, 5 µM) or raltegravir (10-100 µM), all in the presence of 2 µM PSC833. (C) Effect of raltegravir on BCECF accumulation by HeLa-MRP1 monolayer cells. Cellular accumulation (120 min) of BCECF was determined by exposing confluent HeLa-MRP1 cells to 5 µM BCECF-AM, with or without MRP inhibitor MK571 (10 µM) or raltegravir (10-500 µM), all in the presence of 2 µM PSC833. Results are expressed as mean ± S.E.M. of at least three independent experiments with each data point in an individual experiment representing triplicate measurements. Statistical significance was assessed by applying the unpaired two-tailed Student’s t-test analysis. *, p<0.05.

**FIG 2** Interactions of raltegravir with ABC transporters in overexpressing cell lines. Accumulation of [³H]raltegravir by (A) MDA-WT/MDA-MDR1 (P-gp), (B) HEK-WT/HEK-ABCG2 and (C) HeLa-WT/HeLa-MRP1 cells. Accumulation of 1µM [³H]raltegravir (120 min) was measured in cell monolayers in the absence or presence of inhibitors for P-gp (PSC833, 5 µM; CsA, 25 µM); BCRP (Ko143, 5 µM; FTC, 10 µM) or MRP1 (MK571, 5 µM). Results are expressed as mean ± S.E.M. of at least three
independent experiments with each data point in an individual experiment representing triplicate measurements. Statistical significance was assessed applying one-way analysis of variance (ANOVA) followed by Bonferroni correction for test of repeated measures. *

FIG 3 (A) Time profile of $[^{3}\text{H}]$raltegravir in hCMEC/D3 cells. Uptake of 1 μM $[^{3}\text{H}]$raltegravir by the hCMEC/D3 confluent monolayer cells was measured over time at 37°C. Inset shows the linearity of initial uptake up to three minutes. (B) Accumulation of $[^{3}\text{H}]$raltegravir (1 μM, 120 min) by hCMEC/D3 cells in the absence or presence of inhibitors of P-gp (PSC833, 5 μM), BCRP (Ko143, 5 μM) or MRPs (MK571, 5 μM). Results are expressed as mean ± S.E.M. of at least three independent experiments with each data point in an individual experiment representing triplicate measurements. *, $p<0.05$.

FIG 4 (A) Time profile of $[^{3}\text{H}]$raltegravir in TM4 cells. Uptake of 1 μM $[^{3}\text{H}]$raltegravir by the TM4 confluent monolayer cells was measured over time at 37°C. Inset shows the linearity of initial uptake up to three minutes. (B) Accumulation of $[^{3}\text{H}]$raltegravir (1 μM, 120 min) by TM4 cells in the absence or presence of inhibitors of P-gp (PSC833, 5 μM), BCRP (Ko143, 5 μM) or MRPs (MK571, 5 μM). Results are expressed as mean ± S.E.M. of at least three independent experiments with each data point in an individual experiment representing triplicate measurements. *, $p<0.05$.

FIG 5 Raltegravir accumulation and permeability in Caco-2 cells – Interactions with ABC transporters. (A) Time profile of $[^{3}\text{H}]$raltegravir in Caco-2 cells. Uptake of 1 μM $[^{3}\text{H}]$raltegravir by the Caco-2 confluent monolayer cells was measured over time at 37°C.
Inset shows the linearity of initial uptake up to two minutes. (B) Accumulation of $[^{3}H]$raltegravir (1uM, 120 min) by Caco-2 cells in the absence or presence of inhibitors of P-gp (PSC833, 5 µM), BCRP (Ko143, 5 µM) or MRPs (MK571, 5 µM). (C) Raltegravir intestinal permeability across Caco-2 monolayer cells in the apical-to-basolateral and basolateral-to-apical directions. The flux of raltegravir was determined by introducing $[^{3}H]$raltegravir (1 µM) into the donor compartment (apical or basolateral) and measuring its appearance over time in the receiver compartment (basolateral or apical, respectively) in the absence or presence of PSC833 (5 µM) or Ko143 (5 µM) to calculate the apparent permeability coefficients ($P_{app}$). Results are expressed as mean ± S.E.M. of at least three independent experiments with each data point in an individual experiment representing triplicate measurements. Statistical significance was assessed applying the one-way ANOVA followed by Bonferroni correction for test of repeated measures. *, $p<0.05$.

**FIG 6** Raltegravir *in situ* intestinal permeability and interactions with ABC transporters. Effect of P-gp inhibitor (PSC833) (A), Bcrp inhibitor (Ko143) (B) or both P-gp and Bcrp inhibitors (PSC833 + Ko143) (C) on raltegravir intestinal permeability measured by *in situ* single-pass perfusion of Sprague-Dawley rat intestinal segments. The effective permeability ($P_{eff}$) of raltegravir was measured in the jejunum or ileum segment of rat intestine in each animal by *in situ* single-pass perfusion. The intestinal segment was perfused with 10 µM $[^{3}H]$raltegravir at pH 6.5 first without inhibitor (first hour, control) and then in the presence of 5 µM PSC833 or 5 µM Ko143 or both (second hour) to determine the effect of these inhibitors on raltegravir permeability. Raltegravir $P_{eff}$ values measured in the presence of P-gp inhibitor, PSC833; or Bcrp inhibitor, Ko143; or both P-gp and Bcrp inhibitors,
PSC833 + Ko143 were compared to control period in the same animal by paired two-tailed Student's $t$-test analysis using five Sprague-Dawley rats per group.
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