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Role of P-glycoprotein in the efflux of raltegravir from human intestinal cells and CD4+ T-cells as an interaction target for anti-HIV agents

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ABSTRACT

Cellular efflux and uptake transports of several anti-HIV agents are mediated by plasma membrane-localized solute transporters. However, transporters involved in raltegravir disposition have not been fully characterized. Here, we performed in vitro studies to identify transporters mediating transcellular transport of raltegravir. Transepithelial raltegravir transport was examined using porcine kidney epithelial cell line (LLC-PK1) and LLC-PK1 cells stably transfected with P-glycoprotein (also known as Multiple drug resistance 1) (L-MDR1). Transepithelial transport of raltegravir in Caco-2 cell monolayers, and intracellular accumulation of raltegravir in the MT-2 and MT-4 (CD4+ T-) cells were measured in the presence or absence of anti-HIV agents. The uptake of raltegravir was investigated in HEK-293 cells expressing each of several solute carrier family transporters. The apical-to-basal raltegravir transport was significantly decreased in L-MDR1 as compared to that in LLC-PK1 monolayers. In HEK-293 cells expressing breast cancer resistance protein (BCRP), raltegravir accumulation was lower than that in the mock-transfected cells. In Caco-2 cells, protease inhibitors including nelfinavir, ritonavir and lopinavir enhanced the apical-to-basal transport of raltegravir. By contrast, reverse transcriptase inhibitors such as zidovudine, efavirenz, and nevirapine, had no effect on raltegravir transport. The cellular accumulation of raltegravir in MT-2 cells, which express P-glycoprotein, was significantly increased in the presence of protease inhibitors. By contrast, protease inhibitors only marginally increased the accumulation of raltegravir in MT-4 cells, in which P-glycoprotein is not expressed. The present findings suggest that raltegravir is a substrate of both P-glycoprotein and BCRP. Protease inhibitors increase the absorptive transport of raltegravir in Caco-2 cells, and the cellular accumulation in T-cells, at least in part, by P-glycoprotein-mediated interaction.

1. Introduction

The survival of patients with human immunodeficiency virus (HIV) infection has been prolonged by the development of highly active antiretroviral therapy (HAART). This type of therapy comprises a combination of several antiviral agents belonging to different mechanistic classes [1,2]. However, treatment failure can still occur despite earlier long-term HAART treatment. Development of drug-resistant HIV-1 variants and chronic side effects caused by the anti-HIV agents are regarded as the cause of treatment failure [3–6].

Raltegravir (RAL) is an HIV-1 integrase strand transfer inhibitor approved for the treatment of HIV infection as a new alternative class of anti-HIV agents. RAL displays potent activity against HIV strains in treatment-naïve or HIV patients that have undergone

Abbreviations: ABC, ATP-binding cassette; AM, acetoxymethyl ester; APV, amprenavir; ATV, atazanavir; BCRP, breast cancer resistance protein; BSA, bovine serum albumin; CsA, cyclosporine A; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; DRV, darunavir; EFV, efavirenz; FBS, fetal bovine serum; HAART, highly active antiretroviral therapy; LPV, lopinavir; MRP, multidrug resistance-associated proteins; NEAA, non-essential amino acid; NFV, nelfinavir; NVP, nevirapine; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein; RAL, raltegravir; RTV, ritonavir; SLC, solute carrier; UGT, uridine diphosphate glucuronosyltransferase; ZDV, zidovudine.

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long-term treatment. The major mechanism of systemic disposition of RAL in human is glucuronidation mediated by UGT1A1 but not hepatic metabolism by cytochrome P450 (CYP) enzymes [7].

As well as metabolic enzymes, cell membrane-located transporters also contribute to the pharmacokinetics of anti-HIV agents and antiretroviral drug–drug interactions. Numerous HIV protease inhibitors are recognized as substrates of P-glycoprotein (P-gp), which functions as a primary active efflux pump and is expressed in many tissues including the small intestine, liver, kidney, brain and lymphocytes [8]. P-gp limits the bioavailability and the transport of HIV protease inhibitors into several viral target sites [8]. Multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) have been suggested to mediate systemic disposition of several anti-HIV agents [9,10], including the ATP-binding cassette (ABC) superfamily. The transporters play key roles in the tissue distribution of anti-HIV agents and can be associated with a couple of drug–drug interactions. The solute carrier (SLC) transporter families, including organic anion-transporting polypeptides (OATPs), organic anion and organic cation transporters (OATs, OCTs), are xenobiotic-recognizing transporters that mediate the transport of some anti-HIV agents [11,12]. Many protease inhibitors are reported to be substrates and inhibitors of OATPs [11]. However, nucleoside reverse transcriptase inhibitors appear to be substrates of OATs and OCTs [12]. The involvement of transporters in the transcellular transport of anti-HIV agents complicates the drug–drug interaction due to pharmacokinetic/pharmacodynamic events [13]. There are few reports describing the transporter(s) involved in the disposition of RAL and its drug–drug interactions.

The purpose of this study was to identify the transporters mediating transcellular transport of RAL in cultured renal and intestinal cells, and to analyze the effect of the co-administration of other anti-HIV agents on absorptive transport of RAL in intestinal Caco-2 cells. Penetration of RAL into T-cells was also examined by measuring its cellular accumulation in the presence of anti-HIV agents.

2. Materials and methods

2.1. Drugs and chemicals

Anti-HIV agents including RAL, nelfinavir (NFV), ritonavir (RTV), lopinavir (LPV), darunavir (DRV), atazanavir (ATV), amprenavir (APV), zidovudine (ZDV), efavirenz (EFV), and nevirapine (NVP), were spared by a kind gift of various pharmaceutical companies and research institutes: Merck & Co., Inc. (Whitehouse Station, NJ), JT Co. (Tokyo, Japan), Abbott Laboratories Co. (Abbott Park, IL), Glaxo-Wellcome Inc. (Durham, NC), Janssen-Cilag A.G. (Baar, Switzerland), and International Medical Center (Tokyo, Japan). Cyclosporine A was obtained from Novartis Pharma (Basel, Switzerland). Calcein-acetoxymethyl (AM) ester was purchased from Molecular Probe Co. (Eugene, OR). All other chemicals used in this study were of the highest purity grade available.

2.2. Cell culture

Porcine kidney epithelial cell line (LLC-PK1) and LLC-PK1 cells stably transfected with P-glycoprotein (also known as Multiple drug resistance 1) (L-MDR1) were maintained in a complete medium consisting of Medium 199 supplemented with 10% fetal bovine serum (FBS) and were subcultured every 3 to 4 days. For the transport studies, LLC-PK1 and L-MDR1 cells were seeded on polycarbonate membrane filters inside Transwell cell culture chambers (24 mm in diameter, 3.0 μm pores; Coster, Cambridge, MA) at a cell density of 2×10^6 cells/well. The cell monolayers were cultured with 2.6 ml of a complete medium on the basolateral side and 1.5 ml of a complete medium with 1% penicillin and streptomycin on the apical side. The medium was replaced after 2 days by fresh medium with no antibiotics. Cells were used in the transport studies on the third day after inoculation. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO) supplemented with 10% FBS and 0.1 mM non-essential amino acid (NEAA; GIBCO, Grand Island, NY). A subculture was set-up every 7 days. Caco-2 cells were seeded on filters inside Transwell plates (24 mm in diameter, 3.0 μm pores; Coster) at a cell density of 3×10^5 cells/well. The cells were grown with 2.6 ml of a complete medium on the basolateral side and 1.5 ml of a complete medium on the apical side and given fresh medium every 2 days. The cells were used in the transport studies on day 14 after inoculation. MT-2 and MT-4 cells were cultured in DMEM supplemented with 10% FBS and subcultured every 3 to 4 days. HEK-293 cells were cultured in DMEM supplemented with 10% FBS and subcultured every 3 to 4 days. All cells were grown under an atmosphere of 5% CO_2–95% air at 37 °C.

2.3. Transepithelial transport of RAL using cell monolayers

In the transepithelial transport experiments, after removal of the culture medium from both sides of the cell monolayers, the cells were pre-incubated for 10 min with incubation medium [PBS buffer (pH 7.4) made up of 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 8 mM Na_2HPO_4, 1.5 mM K_2HPO_4, and 5 mM N-(2-hydroxyethyl) piperazine-N′-2- ethanesulfonic acid (HEPES)] [14]. Medium on either the apical or basal side of the monolayers was replaced with a fresh incubation medium containing RAL with or without other anti-HIV agents. The monolayers were incubated in 5% CO_2–95% air at 37 °C for 2 h, and aliquots (200 μl) from the opposite side were taken at 10, 30, 60 and 120 min. Supernatants obtained after centrifugation at 200*g* for 5 min were used for the HPLC assay [15].

2.4. Calcein-AM efflux assay

Efflux assays were performed as described previously [16]. A kinetic fluorometric assay was used to study the interaction of RAL with P-gp. For the calcein-AM efflux assay, L-MDR1 and LLC-PK1 cells were seeded on 96-well tissue culture plates at a cell density of 1×10^5 cells/well. Cells were cultured in 200 μl of Medium 199 supplemented with 10% FBS in each well under an atmosphere of 5% CO_2–95% air at 37 °C for 1 day. Cells were plated in 96-well tissue culture plates in Medium 199 containing RAL. After 30-min incubation period, calcein-AM was added to a final concentration of 2 μM, and the plates were placed into a Fluoroskan Ascent (Thermo Labsystems, Franklin, MA). Fluorescence was measured from 0 to 30 min using an excitation of 485 nm and an emission of 530 nm. The rate of calcein accumulation in the presence and absence of RAL was calculated by linear regression analysis using Ascent software (Thermo Labsystems). The apparent Ki value of P-gp inhibition was calculated using the following equation:

$$\mathrm{Ki} = [\mathrm{I}] \times \frac{(\mathrm{LLC-PK1}[0], 30\ \mathrm{min}) - (\mathrm{L-MDR1}[0], 30\ \mathrm{min})}{(\mathrm{L-MDR1}[\mathrm{I}], 30\ \mathrm{min}) - (\mathrm{L-MDR1}[0], 30\ \mathrm{min})} - [\mathrm{I}] \quad (1)$$

where [I] represents the concentration of RAL, and [0] indicates the absence of RAL.

2.5. Intracellular RAL accumulation in MT-2 and MT-4 cells

MT-2 and MT-4 cells were incubated in 1.5 ml tubes at a cell density of 1×10^6 cells/ml. After removal of the culture medium

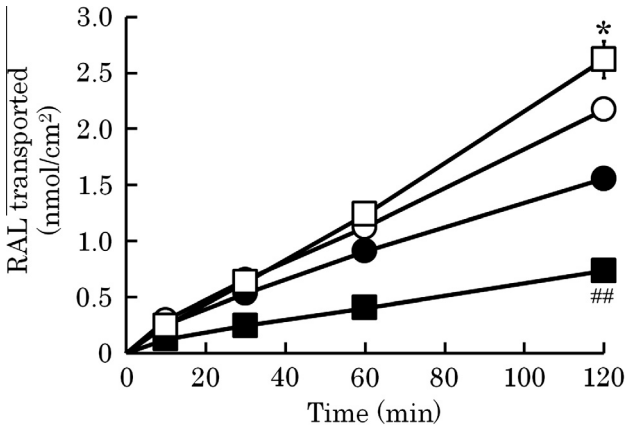

Fig. 1. Transepithelial transport of RAL (raltegravir) in LLC-PK1 cells and L-MDR1 cells. Transport of RAL (40 μM) in the apical-to-basal direction (closed symbol) and in the basal-to-apical direction (open symbol) in LLC-PK1 (○, ●) or L-MDR1 (□, ■) cells. Each value represents the mean ± S.D. ($n = 3$) $^{*}p < 0.05$, significantly different from basal-to-apical transport in LLC-PK1. $^{\#\#}p < 0.01$, significantly different from apical-to-basal transport in LLC-PK1.

from each tube, the cells were incubated for 10 min with incubation medium containing RAL with or without other anti-HIV agents. Following incubation, the cells were centrifuged at 200*g* for 10 min and the pelleted cells washed twice with ice-cold incubation medium. To extract the intracellular RAL, the cells were immersed in 200 μl of 50% methanol for 1 h at room temperature. The extract solutions were centrifuged at 200*g* for 5 min and the supernatants analyzed by HPLC. The pellets were solubilized in 200 μl of 1 N NaOH and the protein content was determined by the method of BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard.

2.6. Cellular uptake of RAL in HEK-293 cells

HEK-293 cells were seeded on poly-D-lysine-coated 24 well plates (Iwaki, Tokyo, Japan) at a cell density of 2×10^5 cells/well with 500 μl of a complete medium. After 24 h cultivation, 0.8 μg vector was transfected by adding 2 μl/well of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). HEK-293 cells were transfected with the pCMV6XL5 vectors (OATP1A2 and OCTN2), the pCMV6XL4 vectors (OATP1B1, OATP1B3, OATP3A1 and OCT1), and pCMVneo vectors (MRP2) purchased from Origene Technologies (Rockville, MD). The pcDNA3.1 vectors (BCRP) were generous gifts from Dr. Sugiyama Y. (Tokyo University, Japan). At 48 h after gene transfection, the culture medium was replaced and the cells were incubated in 500 μl of incubation medium containing RAL with or without inhibitors. After incubation, cells were washed once in ice-cold PBS containing 1% BSA and twice in BSA-free ice-cold PBS. The cells were then lysed by incubation in 200 μl of 50% methanol/HPLC mobile phase for 24 h. The resultant solution was centrifuged at 200*g*, 4 °C for 5 min. The supernatant was used for quantification of RAL using HPLC. The pellet was mixed with 1 N NaOH to solubilize the cells. The protein content of the solubilized cells was determined using a BCA protein assay kit.

2.7. HPLC analysis

The concentration of RAL was determined using HPLC (model LC-6A; Shimadzu, Kyoto, Japan). A 100 μl aliquot of sample was injected onto the HPLC column. Separation was performed on a C18 reversed-phase column (Symmetry C18, 5 μm, 3.9 mm × 150 mm) at 40 °C. The mobile phase was 50 mM phosphate buffer (pH 3.0) and acetonitrile (60:40). The flow rate was 1.0 ml/min. The fluorescence detector was set at excitation and emission wavelengths of 299 nm and 396 nm, respectively.

2.8. Statistical analysis

Statistical analysis was performed by *Student's t*-test, or the one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test, where multiple comparisons against the control were necessary.

3. Results

3.1. Transepithelial transport of RAL

Transepithelial transport of RAL was examined by using LLC-PK1 and L-MDR1 cell monolayers cultured in Transwell chambers. Fig. 1 shows the time profiles of transepithelial transport of RAL across the LLC-PK1 and L-MDR1 monolayers. The basal-to-apical transport in L-MDR1 cells exceeded that observed in LLC-PK1 cells, whereas the apical-to-basal transport was significantly lower in L-MDR1 cells. The ratio of basal-to-apical transport versus apical-to-basal transport of RAL at 2 h was 3.6 in L-MDR1 cells.

3.2. Inhibitory effect of RAL on P-gp-mediated calcein-AM efflux

In order to evaluate inhibitory effects of RAL on P-gp transport activity, calcein-AM extrusion test was performed in LLC-PK1 and L-MDR1 cells. As shown in Fig. 2, cellular accumulation of calcein was time dependent. The value of calcein-AM fluorescence in L-MDR1 cells in the absence of drugs was significantly lower than that for LLC-PK1 cells. Cyclosporine A, a potent inhibitor of P-gp, significantly increased calcein fluorescence in L-MDR1 cells, while RAL did not affect calcein-AM accumulation in L-MDR1 cells even at high concentrations. The apparent Ki value of cyclosporine A was 1.09 μM. RAL exhibited a high Ki value of >2000 μM.

3.3. Accumulation of RAL in HEK-293 cells transfected with transporters

Time profiles of RAL accumulation into HEK-293 cells expressing SLC or ABC transporters are illustrated in Fig. 3. None of the six SLC transporters (OATP1A2, OATP1A3, OATP1B1, OATP1B3, OCT1 and OCTN2) facilitated RAL accumulation. By contrast,

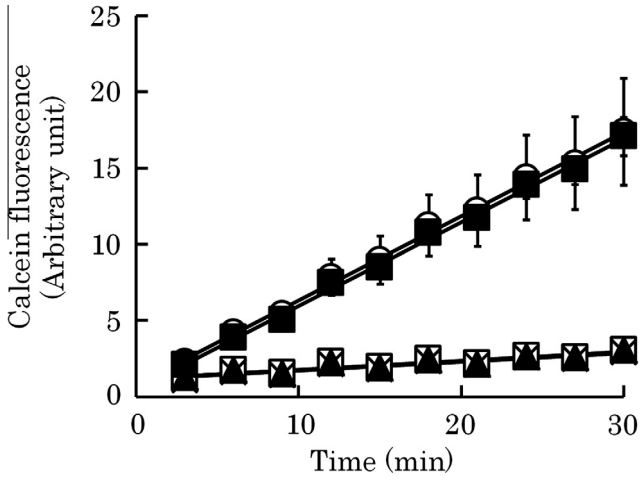

Fig. 2. Effect of RAL (raltegravir) and CsA (cyclosporine A) on calcein-AM flux in L-MDR1 and LLC-PK1 cell monolayers. LLC-PK1 cells without RAL (○), L-MDR1 cells without RAL (□), L-MDR1 cells with 10 μM CsA (■) and RAL at concentrations of 10 μM (▲) and 100 μM (×). Each point represents the mean ± S.D. ($n = 5$).

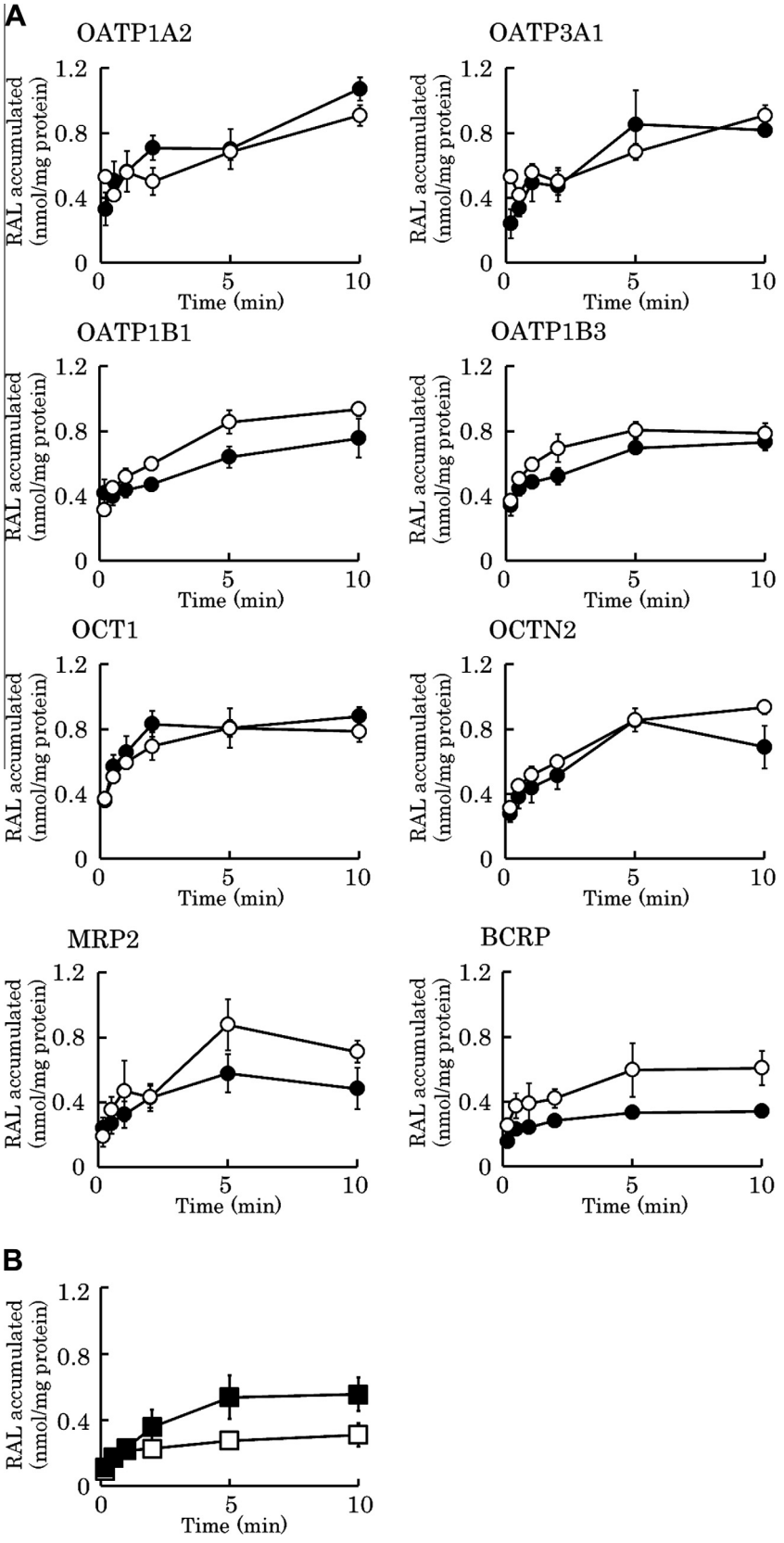

Fig. 3. Time course of intracellular accumulation of RAL (raltegravir) in HEK-293 cells expressing several transporters. (A) RAL (5 μM) accumulation in HEK-293 cells expressing transporters (●), OATP1A2, OATP3A1, OATP1B1, OATP1B3, OCT1, OCTN2, MRP2 and BCRP, and control (○). (B)RAL (5 μM) accumulation in HEK-293 cells expressing BCRP in the presence (■) or absence (□) of fumitremorgin C. Each point represents the mean ± S.D. (n = 3).

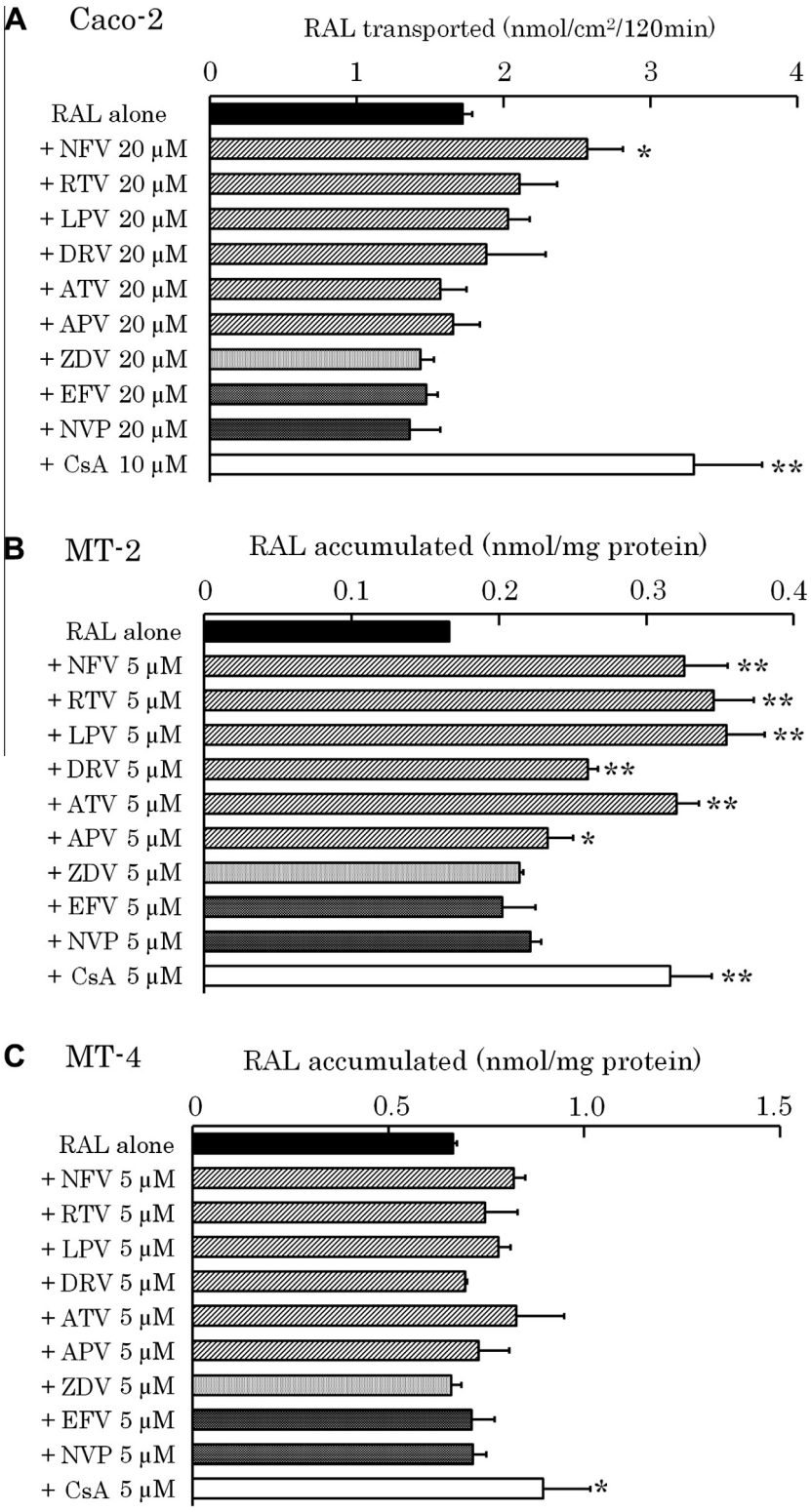

Fig. 4. Effect of anti-HIV agents on the transepithelial transport of RAL in a Caco-2 cell monolayer and intracellular accumulation of RAL in MT-2 cells and MT-4 cells. Transport of RAL (50 μM) in the apical-to-basal direction (corresponding to the intestinal absorption) in Caco-2 cell monolayer (A) and intracellular accumulation of RAL (5 μM) in the MT-2 (B) or MT-4 (C) cells in the absence or presence of anti-HIV agents or CsA. Each point represents the mean ± S.D. ($n = 3$) $^{*}p < 0.05$; $^{**}p < 0.01$, significantly different from control (RAL alone). RAL, raltegravir; NFV, nelfinavir; RTV, ritonavir; LPV, lopinavir; DRV, darunavir; ATV, atazanavir; APV, amprenavir; ZDV, zidovudine; EFV, efavirenz; NVP, nevirapine; CsA, cyclosporine A.

HEK-293 cells transfected with ABC efflux transporters, BCRP or MRP2, appeared to accumulate reduced levels of RAL compared to the control cells. In HEK-293 cells expressing BCRP, the accumulation of RAL was not suppressed in the presence of fumitremorgin C, a potent inhibitor for BCRP.

3.4. Effect of anti-HIV agents on the transepithelial transport of RAL in Caco-2 cell monolayers

The apical-to-basal transport of RAL was significantly increased in the presence of cyclosporine A (Fig. 4A). NFV markedly enhanced

the apical-to-basal transport of RAL, suggesting NFV had a potent inhibitory effect on P-gp-mediated transport of RAL when compared to the other protease inhibitors examined in this study. By contrast, reverse transcriptase inhibitors such as ZDV, EFV and NVP, had little effect on transepithelial transport of RAL.

3.5. Effects of anti-HIV agents on the intracellular accumulation of RAL in MT-2 and MT-4 cells

The cellular accumulation of RAL in MT-2 cells was significantly higher in the presence of protease inhibitors by comparison to experiments conducted in the absence of these inhibitors (Fig. 4B). However, the cellular accumulation of RAL in MT-4 cells was unaffected in the presence of other anti-HIV agents (Fig. 4C). Western blot analysis revealed that both P-gp and BCRP immunoreactive proteins were expressed in MT-2 cells (data not shown). However, P-gp could not be detected in MT-4 cells.

4. Discussion

It has been reported that several tissue-localized membrane transporters are involved in the uptake of anti-HIV agents into cells [13]. As such, the pharmacokinetics of these agents is affected by the characteristics of the transporter proteins. The ABC membrane-associated efflux transporters, such as P-gp, MRPs and BCRP, have been characterized with regard to the upper limit of anti-HIV agents transport into the cells [8–10]. The SLC transporters, such as OATPs, OATs and OCTs, play an important role in determining the pharmacokinetics of some anti-HIV agents [11,12]. However, the affinity of these transporters toward RAL, which is classified as a novel class of anti-HIV agents, has not been fully characterized. Identification of drug transporters involved in the movement of RAL will be required to delineate its pharmacokinetics and for predicting drug–drug interactions of the anti-HIV agents used in HAART therapy.

Using cell monolayers of renal LLC-PK1 and L-MDR1, we confirmed that P-gp is involved in the transcellular transport of RAL. P-gp is localized in the apical membranes of L-MDR1 cells and therefore mediates the extrusion of RAL from the cells. However, RAL did not exert an inhibitory effect on the P-gp-mediated calcein efflux. The results using HEK-293 cells transfected with each of several transporters suggest that BCRP mediates the efflux of RAL, whereas the SLC transporters examined could not be involved in the transcellular transport of RAL.

According to the HIV therapy guideline (http://www.aidsinfo.nih.gov), it is recommended that RAL be combined with tenofovir/emtricitabine. RAL is also administered with protease inhibitors DRV/RTV in clinical practice. The bioavailability of DRV is improved by oral co-administration with RTV: from 37% for DRV alone to 82% when combined with RTV [17]. The boosting effect of RTV is due to the inhibition of oxidative metabolism in the intestine and liver by specifically targeting CYP3A4 activity [17]. In addition, it was reported that RTV improved the bioavailability of DRV due to inhibition of the efflux transport systems in the intestinal lumen [18]. Because RAL is not metabolized by CYP3A4, drug–drug interactions between RAL and other agents *via* this enzyme are unlikely to occur. However, the penetration of RAL into cells was shown to be relatively low (i.e., 5.3% of the plasma level) in a study using peripheral blood mononuclear cells and plasma from HIV-infected patients [19]. Our results indicated that RAL is a substrate of P-gp and BCRP. In human intestinal cells and CD4+ T-cells, some protease inhibitors enhanced the transcellular transport and cellular accumulation of RAL. These results suggest a combination of protease inhibitors, such as DRV/RTV, could improve the accumulation of RAL in T cells by suppressing its active extrusion *via* efflux transporters. Thus, RAL is less likely to cause drug–drug interactions *via* metabolic enzymes, but does appear to mediate P-gp or BCRP-mediated interaction with other anti-HIV agents.

Patients undergoing HAART for an extended period of time are subject to various side effects including elevated levels of blood cholesterol [20]. Lipid-lowering agents, including statins are often used in combination with anti-HIV agents. Such a combination can cause drug–drug interactions because most statins are metabolized by CYP3A [21]. Moreover, many statins and protease inhibitors have been identified as substrates for the OATP family [22,23]. The present study suggested that RAL was not recognized by OATP1B1 and OATP1B3, which are predominant OATP family members mediating statin uptake into hepatocytes [24,25]. Therefore, the concomitant use of RAL and antilipemic agents may not cause drug–drug interactions *via* these OATP transporters. However, in a previous study, it was revealed that RAL induced *MRP1* mRNA expression in the intestine model cells [26], and RAL was suggested to be a substrate of OAT1 [27]. There may well be more unidentified transporters that determine the distribution of RAL, making it difficult to verify the involvement of transporter-mediated interaction in HIV therapy.

In conclusion, the present study has revealed that RAL is a transport substrate of P-gp. Moreover, our results show that BCRP may be involved in the efflux of RAL out of cells expressing this transporter. SLC family members, OCT1 and OCTN2, have no affinity for RAL. Protease inhibitors increase the absorptive transport of RAL in human intestinal cells as well as the cellular accumulation in CD4+ T-cells, which involve P-gp-mediated interaction. Further investigation is required in order to explore the potential involvement of transporters in clinical RAL treatment.

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