Short Communication

In Situ Intestinal Perfusion in Knockout Mice Demonstrates Inhibition of Intestinal P-Glycoprotein by Ritonavir Causing Increased Darunavir Absorption

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ABSTRACT:
Darunavir is a second-generation protease inhibitor designed to have antiviral efficacy against HIV-1 with multiple resistance mutations to protease inhibitors. It is always coadministered with a subtherapeutic dose of ritonavir. It has been shown that darunavir and ritonavir are substrates of P-glycoprotein (P-gp). We explored the contribution of P-gp to the transport characteristics of darunavir (up to 100 μM) using Caco-2 monolayers and the recently developed in situ intestinal perfusion technique using wild-type and mdr1a/1b(−/−) mice. We observed that, in vitro, P-gp has a modulatory effect on the absorption of darunavir, even at a concentration of 100 μM (efflux ratio = 25). Simulated intestinal fluids partially inhibited P-gp functionality, which was further inhibited by adding the P-gp inhibitors verapamil, 6-[(2S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]cyclosporine D (PSC833), N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinoliny1)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918), or ritonavir. Using the in situ intestinal perfusion technique, we demonstrated that coperfusion with ritonavir resulted in a similar apparent permeability coefficient to that observed using P-gp knockout mice, which was 2.7-fold higher than in control mice. We conclude that, in mice, even at a relevant intraluminal concentration of darunavir, P-gp has a modulatory effect on the absorption of darunavir. However, this P-gp-mediated darunavir transport is inhibited when it is combined with ritonavir.

Introduction

The combination of multiple antiretroviral drugs with different modes of action (highly active antiretroviral therapy) has contributed to a spectacular progress in the fight against HIV (Hammer et al., 2008). The standard regimen for newly diagnosed patients usually includes two nucleoside reverse transcriptase inhibitors combined with either a protease inhibitor [boosted with ritonavir (RTV)] or a non-nucleoside reverse transcriptase inhibitor. Darunavir [DRV Prezista; Tibotec Pharmaceuticals, Cork, Ireland] is a second-generation protease inhibitor (PI) designed to have antiviral efficacy against HIV-1 with multiple resistance mutations to protease inhibitors (Busse and Pensa, 2008; Tremblay, 2008). RTV is always coadministered in a subtherapeutic dose as a pharmacokinetic booster of DRV.

In vitro and in vivo studies have demonstrated that all HIV PI are high-affinity substrates for P-glycoprotein [(P-gp) multidrug resistance 1 (MDR1)], which can significantly influence the disposition of antiretroviral drugs (Kis et al., 2009). P-gp is widely distributed with a high level of expression in the small intestine, liver, kidney, and brain (Ho and Kim, 2005). Presently, however, there is still uncertainty about the role of intestinal P-gp in vivo. It is not sure whether P-gp plays a pivotal role as a biochemical barrier for compounds at concentrations relevant for the intraluminal environment, because of a possible saturation of the efflux system. In addition, it has been shown that compounds present in intestinal fluids may attenuate the effect of efflux carriers such as P-gp (Deferme et al., 2003). Also, RTV is a P-gp inhibitor, and its coadministration with darunavir may have an influence on the intestinal efflux system.

Darunavir was found to be a P-gp substrate (Lachau-Durand et al., 2006; Fujimoto et al., 2009), which implies that intestinal P-gp could modulate the uptake of darunavir from the intestinal lumen. However, the effect of P-gp toward darunavir absorption remains controversial: a recent article (Fujimoto et al., 2009) shows a clear role for P-gp in the cellular uptake of darunavir, but the darunavir concentration (10 μM) used in this study is less relevant for the intestinal luminal environment. Lachau-Durand et al. (2006) reported that there was only a limited impact of efflux transporters on the intestinal absorption of darunavir.

The purpose of this study was to investigate whether P-gp has a modulatory effect on the intestinal uptake of darunavir at a concentration of 100 μM, which is relevant for the in vivo situation. In addition, we explored the effect of fasted state simulated intestinal fluid (FaSSIF) and RTV coadministration (75 μM) on darunavir uptake using Caco-2 monolayers. We investigated intestinal absorption using the recently developed in situ intestinal perfusion technique in wild-type and mdr1a/1b(−/−) mice by measuring the appearance...
of darunavir in the mesenteric blood (Mols et al., 2009). To the best of our knowledge, this is the first time that intestinal absorption of DRV is explored in knockout mice.

**Materials and Methods**

**Chemicals.** DRV ethanolate was provided by Tibotec (Mechelen, Belgium). RTV was donated by Hetero Drugs Ltd (Hyderabad, India). PSC833 was provided by Novartis (Basel, Switzerland). GF120918 was provided by GSK (London, UK). Verapamil hydrochloride, MES sodium salt, and butyl-4-hydroxybenzoate were purchased from Sigma-Aldrich (St. Louis, MO). Sodium acetate trihydrate and methanol were purchased from VWR International (Leuven, Belgium). Diethyl ether was purchased from Lab-Scan (Gliwice, Poland). Hanks’ balanced salt solution (HBSS), Dulbecco’s modified Eagle’s medium, penicillin-streptomycin (10,000 IU/ml), nonessential amino acid medium (100X), and HEPES were provided by Lonza (Verviers, Belgium). Water was purified with a Maxima system (Elga Ltd., High Wycombe Bucks, UK). Stock solutions were prepared in dimethyl sulfoxide.

**Media.** Cell culture medium consisted of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acid, and 100 IU/ml penicillin-streptomycin. Transport medium consisted of HBSS containing 25 mM glucose and was buffered with MES (10 mM) to pH 6.5 or with HEPES (10 mM) to pH 7.4. FaSSIF and fed state simulated intestinal fluid (FeSSIF) were made according to the composition reported by Vertzoni et al. (2004) (revised standard FaSSIF and FeSSIF with practical grade taurocholate and soybean lecithin). Human intestinal fluid (HIF) was aspirated from one volunteer in the fasted and as the fed state. The pH of HIF in fasted state measured 5.7; in the fed state, the pH was measured 5.5. The methods used to aspirate HIF have been previously described by Brouwers et al. (2007).

**Caco-2 Cells.** Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in culture medium at 37°C in an atmosphere of 10% CO2, and 90% relative humidity. Cells were passaged every 3 to 4 days (at 80–90% confluence) at a split ratio of 1:6. For transport experiments, cells were seeded at a density of 90,000 cells/cm2 on Costar Transwell membrane inserts (3-μm pore diameter, 12-mm diameter; Corning Inc., Corning, NY) and were used for experiments 17 to 18 days after seeding. Only monolayers with transepithelial electrical resistance values higher than 400 Ω cm2 were used. Transport experiments were performed using a previously described method (Brouwers et al., 2007). Transport medium at pH 7.4 was added to the basolateral compartment. The incubation medium at the apical compartment was, depending on the experiment, transport medium (pH 6.5 or 7.4) or FaSSIF. The experiment was initiated by adding the incubation medium, containing DRV, to the donor compartment. Samples were taken from the acceptor compartment in function of time during 60 min and were diluted 10 times in HBSS containing 0.1% d-tocopheryl polyethylene glycol 1000 succinate to prevent adsorption to glass before analysis.

**In Situ Intestinal Perfusion.** Experiments were performed using male FVB and Ndr1/1b(−/−) FVB mice (the Netherlands Cancer Institute, Amsterdam, The Netherlands). The setup for the in situ perfusion experiments in mice has previously been described by Mols et al. (2009). The perfusion experiments were performed using an open-loop set-up of the perfusate. The perfusate consisted of FaSSIF containing DRV (100 μM) in the absence and presence of the P-gp inhibitor verapamil (150 μM) or RTV (75 μM). Blood samples were collected from the mesenteric vein for 60 min over 5-min time intervals.

**Solubility Measurement.** The methods used to determine the thermodynamic solubility of DRV were previously described by Bevernage et al. (2010). Experiments were done in triplicate.

**Degradation of DRV in Rat Intestinal and Liver Homogenate.** We investigated whether DRV is metabolized by intestinal and hepatic rat Cyp3a. Intestinal and liver homogenates were prepared using a method previously described by Van den Mooter et al. (1998), in which a segment of the ileum (±40 cm) and a part of the liver was used. The protein content of the homogenates was determined using the Lowry method (Lowry et al., 1951), whereas amounts to approximately 6 and 11 mg/ml for intestine and liver, respectively. NADPH was added to a final concentration of 1 mM. Degradation of DRV in the homogenates was determined by adding DRV at a final concentration of 10 μM to the homogenate. Verapamil was used as a positive control. Samples (200 μl) were taken at predetermined time points and added to 200 μl of methanol to arrest enzymatic activity.

**Analysis.** Samples obtained from Caco-2 experiments and solubility experiments were directly injected into the HPLC system. Before quantification of DRV in blood samples by HPLC, DRV was extracted from the blood. After diluting 100 μl of blood in 400 μl of KH2PO4 (0.1 M, pH 6.0), 100 μl of internal standard solution (butyl-4-hydroxybenzoate, 10 μg/ml) was added. After extraction with 5 ml of diethyl ether and centrifugation (2880g, 5 min), the organic layer was transferred to a clean test tube and evaporated to dryness under a gentle stream of air. The residue was dissolved in 150 μl of a solution of water and methanol (50:50 v/v), of which 25 μl was injected in the HPLC system. DRV and the internal standard were detected with a fluorescence detector. The HPLC system consisted of a Waters Alliance 2695 separations module and a Novapak C18 column under radial compression (Waters, Milford, MA). Fluorescence (excitation 268 nm, emission 347 nm) was monitored by a Waters fluorescence detector (W2475). The column was equilibrated with a mobile phase consisting of a 50 mM sodium acetate buffer (pH 3.5) and methanol (40:60 v/v). The retention times of DRV and the internal standard amounted to 5.50 and 10.90 min, respectively. After elution, the column was flushed with acetonitrile/water (80:20 v/v) for 3 min and re-equilibrated with mobile phase for 3 min. The flow was maintained at a rate of 1.3 ml/min. The observed peaks were integrated using Empower Pro (Empower 2) software.

The calibration curve was linear over the concentration range of 62.5 nM–2 μM. The assessment of intraday repeatability, determined at 0.2 and 1 μM, resulted in a relative S.D. (n = 6) of 0.3 and 1.4%, respectively. The deviation from the theoretical concentration amounted to 0.25 and 1.4%, respectively.

The apparent permeability coefficient ($P_{app}$) was calculated according to the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_{donor}}$$

where $Q$ is the cumulative amount of drug appearing in the mesenteric blood or acceptor compartment, $A$ is the surface area of the perfused cylindrical intestinal segment or Transwell membrane, and $C_{donor}$ is the drug concentration in the perfusate or donor compartment.

**Statistics.** Analysis of variance followed by Dunnett’s test was used to evaluate statistically differences (GraphPad Prism 5 for Windows; GraphPad Software Inc., San Diego, CA).

**Results and Discussion**

It has previously been shown that DRV, like all other HIV PI’s, is a substrate of P-gp (Lachau-Durand et al., 2006; Fujimoto et al., 2009). However, it is not clear whether P-gp has a modulatory effect on the uptake of DRV from the intestinal lumen in vivo: 1) if DRV is present at relatively high concentrations in the intestinal lumen, P-gp efflux may be saturated, resulting in only a limited impact of P-gp (Lachau-Durand et al., 2006); 2) there is evidence that compounds present in intestinal fluids may attenuate the effect of efflux carriers such as P-gp (Deferme et al., 2003); 3) darunavir is always coadministered with RTV, a P-gp substrate, which may interact with the efflux system. In a previous Caco-2 study (Fujimoto et al., 2009), DRV was tested at a relatively low concentration (10 μM), which makes the results less relevant for the in vivo situation. In the present study, 10 times higher concentrations were used to explore the transport characteristics of DRV using Caco-2 cells. In addition, by using the recently developed in situ intestinal perfusion technique (Mols et al., 2009), we investigated the influence of P-gp on the uptake of DRV from the intestinal lumen at concentrations that are more relevant for the in vivo situation, based on the solubility of DRV in intestinal fluids.

**Solubility of DRV in Intestinal Fluids.** We determined the thermodynamic solubility of DRV in different media and compared the solubility in HIF with this in simulated intestinal fluid (FaSSIF and FeSSIF). We found that the solubility in fasted HIF (392 ± 16 μM)
was comparable with that in FaSSIF (397 ± 15 μM). The solubility was higher in fed HIF (494 ± 41 μM). This food effect on solubilizing capacity was expected because intestinal fluids collected in the fed state contain various compounds that enhance solubility (including bile salts phospholipids and lipid degradation products). This finding corresponds with a reported enhanced bioavailability when darunavir is administered in the fed state compared with the fasted state (Sekar et al., 2007). We were surprised to find that the solubility in FeSSIF (321 ± 18 μM) was lower than that in FaSSIF. In view of the recently reported high intra- and interindividual variability of the solubilizing capacity of HIF (Clarysse et al., 2009), the reported values of the solubility values in HIF should be considered as an estimate of the intraluminal concentration. The observed solubility values suggest that, after oral administration, DRV can be present at high concentrations in the intestinal lumen. Based on these observations, and to assure solubility in transport medium, we decided to use a DRV concentration of 100 μM for permeability experiments in different donor media.

The Effect of P-gp on the Transport of DRV in Caco-2 Cells. When studying the concentration-dependent transport characteristics of DRV, we found that, even at a concentration of DRV up to 100 μM, the efflux ratio (secretory \( P_{\text{app}} \) value/absorptive \( P_{\text{app}} \) value) is still 25, illustrating that the contribution of P-gp to the transport of DRV remains significant and that there is no saturation of P-gp. This conclusion is somehow contradictory to the conclusion of Lachaud-Durand et al., 2006, who also studied concentration-dependent transport of DRV in Caco-2 cells. They found a decreased efflux ratio for DRV at a concentration of 100 μM (efflux ratio was 3.7) and stated that there was only a limited impact of efflux transporters on the intestinal absorption of DRV. This difference in results may be due to the interlaboratory differences in P-gp expression in the Caco-2 cell line (Hayeshi et al., 2008).

The Effect of Intestinal Fluids on P-gp in Caco-2 Cells. We studied the effect of FaSSIF as apical solvent system on the function of P-gp in Caco-2 cells, along with different P-gp inhibitors on transport. Figure 1 illustrates the absorptive transport of DRV in HBSS and FaSSIF (both adjusted to pH 6.5), in the absence and presence of 150 μM verapamil, 4 μM PSC833, 75 μM RTV, and 4 μM GF120918 as P-gp inhibitor. The use of FaSSIF causes a significant increase in permeability for darunavir compared with HBSS. This result can be attributed to an inhibition of P-gp as previously described by Deferme et al., 2003. FaSSIF does not seem to completely inhibit P-gp because all tested P-gp inhibitors cause a further increase in the permeability of darunavir.

**Intestinal Absorption of DRV in Knockout Mice and the Role of P-gp.** We measured the intestinal absorption of DRV using the recently developed in situ intestinal perfusion technique in male FVB and \( mdr1a/1b(−/−) \) FVB mice. The intestine was perfused with FaSSIF containing 100 μM DRV in the absence and presence of 150 μM verapamil or 75 μM ritonavir. Coperfusion with ritonavir resulted in a similar apparent permeability coefficient to that observed using P-gp knockout mice, which was 2.7-fold higher than in control mice (Fig. 2). This effect shows that P-gp significantly limits the intestinal absorption of 100 μM DRV; however, when darunavir is codeadministered with ritonavir, the absorption of darunavir is significantly increased due to an inhibition of P-gp by ritonavir.

**Intestinal Metabolism of DRV.** Because metabolism may confound the results presented so far, we investigated whether DRV is metabolized by intestinal Cyp3a and compared this to its hepatic metabolism, by use of rat intestinal and hepatic homogenate. After incubation of DRV with intestinal homogenate for 1.5 h, no metabolism was observed. We also analyzed the blood samples from the in situ intestinal perfusion experiments in mice and did not observe any signs of metabolite formation. In contrast to the absence of intestinal metabolism, we did see significant metabolism when incubating DRV with hepatic homogenate. After 1.5 h of incubation, we observed a decrease of the DRV concentration by 73%. These data suggest that intestinal metabolism does not play a significant role in the first-pass elimination of DRV in mice and rats.

**Conclusion**

We conclude that, in mice, even at a relevant intraluminal concentration of darunavir, P-gp has a modulatory effect on the absorption of darunavir. However, this P-gp-mediated darunavir transport is inhibited when combined with ritonavir.

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**References**


FIG. 1. Apparent permeability coefficients of a Caco-2 monolayer for darunavir (100 μM) in HBSS buffer and FaSSIF (both pH 6.5) with or without the P-gp inhibitors verapamil (150 μM), PSC833 (4 μM), ritonavir (75 μM), and GF120918 (4 μM). Transport in apical-to-basolateral direction. Bars represent the mean ± S.D. (n = 3). *** significantly different from FaSSIF condition (p < 0.001).


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