In situ intestinal perfusion in knockout mice demonstrates inhibition of intestinal P-glycoprotein by ritonavir causing increased darunavir absorption.

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List of nonstandard abbreviations: CYP, cytochrome P450; DRV, darunavir; HBSS, Hanks’ Balanced Salt Solution; FaSSIF, Fasted State Simulated Intestinal Fluid; FeSSIF, Fed State Simulated Intestinal Fluid; HIF, Human Intestinal Fluid; P-gp, P-glycoprotein; P_{app}, apparent permeability coefficient; PI, protease inhibitor; RTV, ritonavir.
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 co-administered with a sub-therapeutic 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 (FaSSIF) partially inhibited P-gp functionality, which was further inhibited by adding the P-gp inhibitors verapamil, PSC833, GF120918 or ritonavir. Using the in situ intestinal perfusion technique, it was demonstrated that coperfusion with ritonavir resulted in a similar apparent permeability coefficient as 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 combined with ritonavir.
Introduction

The combination of multiple antiretroviral drugs with different modes of action (HAART, 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 (NRTIs) combined with either a protease inhibitor (boosted with ritonavir) or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Darunavir (DRV, Prezista®, Tibotec Pharmaceuticals) is a second-generation protease inhibitor (PI) designed to have antiviral efficacy against HIV-1 with multiple resistance mutations to protease inhibitors (Tremblay, 2008; Busse and Penzak, 2007). Ritonavir is always co-administered in a sub-therapeutic 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, 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 if P-gp plays a pivotal role as 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 like P-gp (Deferme et al., 2003). Also, ritonavir is a P-gp inhibitor, and its co-administration with darunavir may have an influence on the intestinal efflux system.

Recently, darunavir was found to be a P-gp substrate (Fujimoto et al., 2009; Lachau-Durand et al., 2006), which implicates that intestinal P-gp could modulate the uptake of darunavir from the intestinal lumen. However, the effect of P-gp towards darunavir absorption remains controversial: a recent paper (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. reported that there was only a limited impact of efflux transporters on the intestinal absorption of darunavir (Lachau-Durand et al., 2006)

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. Also, we explored the effect of simulated intestinal fluid (FaSSIF) and ritonavir co-administration (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
Darunavir ethanolate (DRV) was provided by Tibotec (Mechelen, Belgium). Ritonavir 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 Medium (DMEM), penicillin-streptomycin (10,000 IU/ml), nonessential amino acid (NEAA) medium (100x) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (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 DMSO.

Media
Cell culture medium consisted of DMEM supplemented with 10% FBS, 1% NEAA 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 (Fasted State Simulated Intestinal Fluid) and FeSSIF (Fed State Simulated Intestinal Fluid) were made according to the composition reported by Vertzoni et al. (revised standard FaSSIF and FeSSIF with practical grade taurocholate and soybean lecithin) (Vertzoni et al., 2004).

HIF (human intestinal fluid) was aspirated from one volunteer in the fasted as well as in the fed state. The pH of HIF in fasted state measured 5.7; in fed state the pH measured 5.5. The
methods used to aspirate HIF have been previously described by Brouwers et al. (Brouwers et al., 2007)

Caco-2 cells
Caco-2 cells were from ATCC (Manassas, Virginia) and were grown in culture medium at 37 °C in an atmosphere of 10% CO₂ and 90% relative humidity. Cells were passaged every 3–4 days (at 80 - 90% confluence) at a split ratio of 1 to 6.

For transport experiments, cells were seeded at a density of 90,000 cells/cm² on Costar® Transwell membrane inserts (3 μm pore diameter, 12mm diameter; Corning Inc., USA) and were used for experiments 17-18 days after seeding. Only monolayers with transepithelial electrical resistance (TEER) values higher than 400 Ω × cm² were used. Transport experiments were performed following 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 minutes and were diluted 10 times in HBSS containing 0.1% TPGS to prevent adsorption to glass prior to analysis.

In situ intestinal perfusion
Experiments were performed using male FVB and mdr1a/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. (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 ritonavir (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. (Bevernage et al., 2010). Experiments were done in triplicate.

Degradation of DRV in rat intestinal and liver homogenate
We investigated if DRV is metabolized by intestinal and hepatic rat Cyp3a. Intestinal and liver homogenate was prepared by a method previously described by Van den Mooter et al. (Van den Mooter et al., 1998) for which a segment of the ileum (± 40 cm) and a part of the liver was used. The protein content of the homogenates was determined with the method of Lowry, and amounted to about 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 (2880 × g, 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 C-18 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 during 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 standard deviation (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

ANOVA followed by Dunnett’s test was used to evaluate statistical differences (GraphPad Prism 5 for Windows).
Results and discussion

It has previously been shown that DRV, like all other HIV PI, is a substrate of P-gp (Fujimoto et al., 2009; Lachau-Durand et al., 2006). However, it is not clear if 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 like P-gp (Deferme et al., 2003); (3) darunavir is always co-administered with ritonavir, 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, ten times higher concentrations were used to explore the transport characteristics of DRV using Caco-2 cells. Also, by use of 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 which 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 human intestinal fluid (HIF) with this in simulated intestinal fluid (FaSSIF and FeSSIF). We found that the solubility in fasted HIF (392 µM ± 16 µM) was comparable to this in FaSSIF (397 µM ± 15 µM). The solubility was higher in fed HIF (494 µM ± 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 and lipid degradation products). This also corresponds with a reported enhanced bioavailability when
Darunavir is administered in the fed state compared to the fasted state (Sekar et al., 2007). Surprisingly, the solubility in FeSSIF (321 µM ± 18 µM) was lower than this 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 dependant transport characteristics of DRV, we found that, even at a concentration of DRV up to 100 µM, the efflux ratio (secretory $P_{app}$ value / absorptive $P_{app}$ 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 Lachau-Durand et al. 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 inter-laboratory 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 simulated intestinal fluid (FaSSIF) as apical solvent system on the function of P-gp in Caco-2 cells, along with different P-gp inhibitors on DRV 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 ritonavir and 4 µM GF120918 as P-gp inhibitor. The use of FaSSIF causes a significant increase in permeability for darunavir compared with HBSS. This can be attributed to an inhibition of P-gp as previously described by Deferme et al. FaSSIF does not seem to completely inhibit P-gp because all tested P-gp inhibitors cause a further increase in the permeability for 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 *mdrla/1b(-/-) FVB* mice. The intestine was perfused with FaSSIF containing 100 µM of DRV in the absence and presence of 150 µM of verapamil or 75 µM ritonavir. Coperfusion with ritonavir resulted in a similar apparent permeability coefficient as observed using P-gp knockout mice, which was 2.7-fold higher than in control mice (*figure 2*). This shows that P-gp significantly limits the intestinal absorption of 100 µM of DRV; however, when darunavir is co-administered 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 hours, 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 hours of incubation,
we observed a decrease of the DRV concentration by 73%. These data suggest that intestinal metabolism plays no 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

Drug supersaturation in simulated and human intestinal fluids representing different

Brouwers J, Tack J, and Augustijns P (2007) In vitro behavior of a phosphate ester prodrug of
amprenavir in human intestinal fluids and in the Caco-2 system: illustration of intraluminal

Health Syst Pharm* 64: 1593-1602.

Postprandial evolution in composition and characteristics of human duodenal fluids in


Fujimoto H, Higuchi M, Watanabe H, Koh Y, Ghosh AK, Mitsuya H, Tanoue N, Hamada A,
and Saito H (2009) P-glycoprotein mediates efflux transport of darunavir in human
intestinal Caco-2 and ABCB1 gene-transfected renal LLC-PK1 cell lines. *Biol Pharm Bull*
32: 1588-1593.

MA, Gatell JM, Hirsch MS, Jacobsen DM, Montaner JSG, Richman DD, Yeni PG, and
recommendations of the International AIDS Society-USA panel. *JAMA* 300: 555-570.

Fossati L, Hovenkamp E, Korjamo T, Masungi C, Maubon N, Mols R, Müller Z, A,
Mönkkönen J, O'Driscoll C, Oppers-Tiemissen HM, Ragnarsson EGE, Rooseboom M, and


Vertzoni M, Fotaki N, Kostewicz E, Stippler E, Leuner C, Nicolaides E, Dressman J, and
Legends for figures

**Figure 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).

**Figure 2**: Apparent permeability coefficients of intestinal tissue for darunavir (100 µM) in FaSSIF in male wild-type FVB mice, *mdrla/1b(-/-)* FVB mice and FVB mice coperfused with verapamil (150 µM) or ritonavir (75 µM). Bars represent the mean ± S.D. (n = 3). Significantly different from FVB control mice; *p < 0.05, **p < 0.01.
Fig. 2

The figure shows the effect of different conditions on the permeability coefficient ($P_{app}$) expressed as $P_{app} \times 10^6$ (cm/sec).

- **Control** shows a significantly lower $P_{app}$ compared to other conditions.
- **P-gp KO** has a significantly higher $P_{app}$ compared to Control.
- Adding **Verapamil** increases $P_{app}$ further, showing a significant difference from P-gp KO.
- Adding **Ritonavir** results in a $P_{app}$ similar to Verapamil, indicating no significant difference.

Significance levels: **p < 0.01**, *p < 0.05*. All data points are indicated with error bars.