Boosting of HIV protease inhibitors by ritonavir in the intestine: the relative role of Cyp and P-gp inhibition based on Caco-2 monolayers versus in situ intestinal perfusion in mice.

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List of nonstandard abbreviations: ABT, aminobenzotriazole; APV, amprenavir; AZV, atazanavir; DRV, darunavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; TPV, tipranavir; CYP, cytochrome P450; FaSSIF, Fasted State Simulated Intestinal Fluid; HBSS, Hanks’ Balanced Salt Solution; PBS, phosphate buffered saline; P_app, apparent permeability coefficient; P-gp, P-glycoprotein; PI, HIV protease inhibitor; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide.
Abstract

HIV protease inhibitors are essential components of most recommended treatment regimens for HIV infection. They are always co-administered with ritonavir as a pharmacokinetic booster. Their bioavailability may be impaired due to the fact that they are substrates of CYP3A4 and several transporters, including P-glycoprotein. The aim of this study was to explore the impact of ritonavir on the intestinal absorption of HIV protease inhibitors in two models: the Caco-2 system and the in situ intestinal perfusion model with mesenteric blood sampling in mice. Using the Caco-2 system, the effect of ritonavir on the permeability of the other HIV protease inhibitors was significant for saquinavir (2-fold increase) and indinavir (3-fold increase), negligible for darunavir and amprenavir, and non-existent for nelfinavir, lopinavir, tipranavir and atazanavir. However, performing the in situ intestinal perfusion technique in mice for 3 selected HIV protease inhibitors showed a significant increase in the intestinal permeability for all: indinavir (3.2-fold), lopinavir (2.3-fold) and darunavir (3-fold). The effect of aminobenzotriazole (a nonspecific Cyp inhibitor) on lopinavir permeability was comparable to using ritonavir, while there was no effect for indinavir and darunavir. We conclude that ritonavir can boost drug absorption by inhibiting P-glycoprotein and/or metabolism, in a compound-specific manner. The results of this study illustrate that a combination of absorption models needs to be considered to elucidate drug-drug interactions at the level of the intestinal mucosa.
Introduction

In order to eliminate new compounds with inadequate drug-like properties during drug discovery and development, various techniques are being used to assess the intestinal absorption potential. In early drug discovery, the absorption properties of new chemical entities are usually assessed by high throughput techniques, e.g. automated Caco-2 screening. In the later drug discovery stages, when drug properties are being optimized and/or when detailed insight into the mechanisms underlying oral drug disposition is required, Caco-2 cells or more advanced absorption models (Ussing Chambers or intestinal perfusion systems) are being used. Caco-2 cells have many advantages, including the fact that (1) they are well characterized, (2) they are from human origin and (3) the system can be adopted for high throughput screening. However, Caco-2 cells are endowed with several disadvantages including (1) a very low Cytochrome P450 (CYP) 3A4 expression (which is the most abundant phase I drug-metabolizing enzyme present in the human small intestine), (2) the absence of a mucus layer which protects the cells and forms an extra barrier for compounds to reach the cells and (3) interlaboratory differences in enzyme and drug transporter expression (Hayeshi e.a., 2008). Because of the absence of CYP3A4 expression in Caco-2 cells, the intestinal absorption of some drugs may be overestimated; in addition, the Caco-2 system might be insufficient to study drug-drug interactions, especially for compounds for which the interplay between transporters and drug-metabolizing enzymes is important. In order to address the low CYP3A4 expression, Caco-2 cells have been co-incubated with 1,25-dihydroxyvitamin-D3 (vitamin D3) or transfected with CYP3A4; however, both models have their drawbacks, being the high operating costs and the progressive loss of the expression vector (Cummins e.a., 2001), respectively.

An absorption model which can be considered in late discovery and early development stages is the in situ intestinal perfusion with mesenteric blood collection, a model which is much
closer to the *in vivo* situation. It offers a better prediction of human absorption than cell-based assays (Salphati e.a., 2001) because of (1) an intact intestinal mucosa, nerve system and blood flow, (2) the presence of sink conditions, and (3) the expression of all enzymes and transporters. The rat is the standard animal used for this technique, but in 2009, Mols *et al.* (Mols e.a., 2009) introduced the use of mice in the *in situ* intestinal perfusion technique with mesenteric blood sampling. This offers the possibility to use knockout and knockin animals or perform *in situ* absorption studies when only small quantities of test compound are available. Using the *in situ* intestinal perfusion in wild-type and P-gp knockout mice, we have previously demonstrated that ritonavir (RTV) significantly increases the intestinal absorption of darunavir (DRV) by inhibition of P-glycoprotein (P-gp) (Holmstock e.a., 2010). P-gp is an ATP-binding cassette (ABC) efflux transporter present throughout the body, including the intestinal brush border membrane. RTV is usually being combined with HIV protease inhibitors (PIs) at a sub-therapeutic dose to enhance their bioavailability through irreversible inhibition of CYP3A4 (Sevrioukova en Poulos, 2010). The previous study clearly demonstrated that the P-gp modulatory effect of RTV can also contribute to the enhanced bioavailability of DRV. Since all PIs have been found to be a substrate of P-gp (Kis e.a., 2010), we wanted to explore whether RTV boosts the intestinal absorption of other PIs using the aforementioned absorption models. The results of this study illustrate that a combination of absorption models needs to be considered to elucidate drug-drug interactions at the level of the intestinal mucosa.
Materials & methods

Chemicals

Darunavir ethanolate (DRV), tipranavir (TPV), ritonavir (RTV), atazanavir sulfate (AZV) and amprenavir (APV) were provided by the NIH AIDS Research and Reference Reagent Program. Saquinavir mesylate (SQV), indinavir sulfate (IDV), nelfinavir mesylate (NFV) and lopinavir (LPV) were donated by Hetero Drugs Ltd (Hyderabad, India). GF120918 (elacridar) was provided by GSK (London, UK). Butyl-4-hydroxybenzoate, protease inhibitor cocktail for use with mammalian cell extracts, and ethyl acetate (puriss. p.a. ACS) were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium formate was from Acros Organics (Geel, Belgium). Ketamine (Anesketin) and xylazin (Xyl-M 2%) were from Eurovet (Heusden, Belgium) and VMD (Arendonk, Belgium), respectively. Sodium acetate trihydrate, methanol and glycerol were purchased from VWR International (Leuven, Belgium). Diethyl ether was from Lab-Scan (Gliwice, Poland). Phosphate buffered saline (PBS), 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 (Basel, Switzerland). All other reagents were used as supplied. 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 - 100 µg/ml streptomycin. Transport medium consisted of HBSS containing 25 mM glucose and was buffered with HEPES (10 mM) at pH 7.4. FaSSIF (Fasted State Simulated Intestinal Fluid) was made according to the composition reported by Vertzoni et al.
Caco-2 cells

Caco-2 cells were from ATCC (Manassas, Virginia) and were grown in culture medium at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity. Cells were passaged every 3–4 days (at 80 - 90% confluence) at a split ratio of 1 to 6. Transport experiments were performed according to a previously described method (Brouwers e.a., 2007). For transport experiments, cells were seeded at a density of 90,000 cells/cm² in Costar® Transwell membrane inserts (3 µm pore diameter, 12mm diameter; Corning Inc., USA) and were used for experiments 17-18 days after seeding. Transport medium containing 0.2% TPGS was added to the basolateral compartment to create sink conditions. The experiment was initiated by adding FaSSIF or transport medium + 0.2% DMSO containing the test compound to the apical compartment. Samples were taken from the basolateral compartment after 60 minutes and diluted 10 times in transport medium containing 0.2% TPGS to prevent adsorption to glass prior to analysis.

In situ intestinal perfusion

Experiments were performed using male NMRI mice (Janvier, France). The setup for the in situ perfusion experiments in mice has previously been described by Mols et al. (Mols e.a., 2009). The perfusion experiments were performed using an open-loop set-up. The perfusate consisted of FaSSIF containing the test compound in the absence or presence of RTV (75 µM) and the nonspecific CYP inhibitor aminobenzotriazole (100 µM). Blood samples were collected from the mesenteric vein for 60 min over 5-min time intervals. Approval for the experiments with mice was granted by the Institutional Ethical Committee for Animal Experimentation of the KU Leuven.
Intestinal microsomes

Mouse intestinal microsomes were harvested according to a combination of the recommendations of Sigma Aldrich and the method previously described by Mohri et al. (Mohri et al., 2001). NMRI mice (n=6) were anesthetized with an intraperitoneal injection of ketamine (150 mg/kg) and xylazin (12.5 mg/kg). All solutions were used at 4 °C. Only the distal part of the small intestine was used for generating microsomes. The intestine was flushed with PBS and placed on a plate on ice. One end was clamped and the intestine was filled with solution A [PBS, pH 7.2, containing 5 mM EDTA, 0.5 mM dithiothreitol, 5 U/ml heparin and protease inhibitor cocktail (1%)]. The intestine was tapped gently several times during 10 minutes after which the solution was collected and kept on ice. This procedure was repeated four times. The collected cells were centrifuged at 800 x g for 10 minutes at 4°C and resuspended in solution B [pH 7.8, containing 10 mM HEPES, 250 mM sucrose, 25 mM KCl, 1 mM EDTA and protease inhibitor cocktail (1%)]. Next, the cells were homogenized using a Potter-Elvehjem homogenizer and the homogenate was centrifuged at 1000 x g for 10 minutes at 4 °C. The thin floating lipid layer was carefully removed by aspiration and the supernatant was transferred to a new recipient and centrifuged at 12,000 x g for 15 minutes at 4 °C. The supernatant was collected and CaCl2 was added to a final concentration of 10 mM. This solution was kept on ice for 15 minutes and was centrifuged at 8000 x g for 10 minutes at 4 °C. The pellet containing the microsomes was resuspended in 800 µl of solution C (pH 7.4, containing 100 mM Tris-HCl, 10 mM EDTA and 20% glycerol) and stored at -30 °C until use. The protein content was determined with the method of Lowry (Lowry et al., 1951) using BSA as standard and amounted to 1.63 mg/ml. Mouse microsomes were used as such.
Pooled human intestinal microsomes were purchased from BD Biosciences (Woburn, MA), and were diluted in solution C to a concentration of 0.5 mg/ml.

Metabolism studies

The metabolic stability of selected PIs (5 µM) was examined using 100 µl of the microsome-containing solution. In addition, the inhibitory effect of RTV (5 µM) was tested. NADPH was added to a final concentration of 1 mM. To the control conditions, no NADPH was added. PIs were incubated at 37 °C during 1 h when using the mouse microsomes and 30 minutes when using the human microsomes. Samples were diluted 1:2 in MeOH to arrest enzymatic activity. The samples were centrifuged at 20,817 × g for 10 minutes at 4 °C and the supernatant was analyzed by HPLC.

Analysis of the Caco-2 samples

Samples obtained from Caco-2 experiments were directly injected into the HPLC system. The HPLC system consisted of a Waters Alliance 2695 separations module and a Novapak C-18 column under radial compression (Waters, Milford, MA). Fluorescence was monitored by a Waters Fluorescence detector (W2475) (AZV, DRV and APV: excitation 268 nm, emission 347 nm), UV absorption was monitored by a Waters Absorbance detector (W2487) (LPV: 200 nm, IDV: 260 nm, RTV 240 nm, SQV 241 nm, NFV and TPV 252 nm). The mobile phase consisted of 25 mM sodium acetate (pH 5.5) and methanol (25:75 v/v) at a flow rate of 1.5 ml/min. The retention times for the PIs were: DRV, APV: 2.4 min, LPV: 8.7 min, AZV: 5.4 min, IDV: 3.9 min, NFV: 10.3 min, SQV: 8.4 min, RTV: 5.7 min and TPV: 6.5 min. The observed peaks were integrated using Empower Pro (Empower 2) software.

Analysis of the blood samples
Quantifying the PIs in the blood samples required extraction. **DRV blood extraction**: 100 µl of blood was diluted into 400 µl of a mixture of KH₂PO₄ (0.1 M, pH 6.0) and methanol (80:20 v/v); subsequently, 100 µl of internal standard solution (butyl-4-hydroxybenzoate, 10 µg/ml) was added. After adding 4 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 10 µl was injected into the HPLC system. The mobile phase consisted of 25 mM sodium acetate (pH 5.5) and methanol (40:60 v/v); the flow rate amounted to 1.3 ml/min. The retention times of DRV and the internal standard amounted to 6.3 and 13.4 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 calibration curve was linear over the concentration range of 0.63 - 20 µM. The assessment of interday repeatability, determined at 5 µM, resulted in a relative standard deviation of 2.0% (n = 5). The deviation from the theoretical concentration amounted to -4.6 %.

**LPV blood extraction**: 150 µl of blood was diluted in 850 µl of HBSS (pH 7.4); subsequently, 500 µl of NaOH (2 M) was added. After adding 4 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 100 µl was injected into the HPLC system. The mobile phase consisted of 25 mM sodium acetate (pH 5.5) and methanol (21:79 v/v); the flow rate amounted to 1.5 ml/min. The retention time of LPV was 5.7 min. After elution, the column was flushed with acetonitrile:water (80:20 v/v) for 4 min and re-equilibrated with mobile phase during 4 min. The calibration curve was linear over the concentration range of 0.78 - 12.5 µM. The assessment of interday repeatability, determined at 2 µM, resulted in a relative standard deviation of 3.2 % (n = 4). The deviation from the theoretical concentration amounted to -1.2%.
The intraday repeatability, determined at 2 µM, resulted in a relative standard deviation of 5 % (n = 9), with a deviation from the theoretical concentration of -5.8 %. **IDV blood extraction:** 150 µl of blood was diluted in 850 µl of HBSS (pH 7.4); subsequently, 500 µl of NaOH (2 M) was added. After adding 4 ml of ethyl acetate 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 100 µl was injected in the HPLC system. The mobile phase consisted of 10 mM ammonium formate (pH 4.5), methanol and acetonitrile (45:20:35 v/v/v); the flow rate amounted to 1.5 ml/min. The retention time of IDV was 7 min. After elution, the column was flushed with acetonitrile:water (90:10 v/v) for 2 min and re-equilibrated with mobile phase during 3 min. The calibration curve was linear over the concentration range of 0.16 - 5 µM. The assessment of interday repeatability, determined at 1 µM, resulted in a relative standard deviation of 3.3 % (n = 5). The deviation from the theoretical concentration amounted to 1.9 %. The intraday repeatability, determined at 1 µM, resulted in a relative standard deviation (n = 5) of 1.2 %, with a deviation from the theoretical concentration of -2.1 %.

Calculations

For each compound, the apparent permeability coefficient (P<sub>app</sub>) was calculated according to the following equation:

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

where Q is the cumulative amount of drug appearing in the mesenteric blood or basolateral compartment, A is the surface area of the perfused cylindrical intestinal segment or Transwell membrane, and C<sub>donor</sub> is the drug concentration in the perfusate or apical compartment.
Statistics

Statistical analysis was performed using an unpaired t test or one way ANOVA followed by Dunnett’s test, as specified in the legends of the figures. P-values of less than 0.05 are considered as statistically significant.
Results

The effect of P-gp on the permeability of Caco-2 cells for PIs using transport medium.

In a preliminary set of experiments, we investigated the effect of GF120918 (a specific P-gp inhibitor at 4 μM (Matsson e.a., 2009)) on the absorptive permeability of Caco-2 cells for four PIs (SQV, IDV, DRV and APV) using transport medium as solvent system (figure 1). GF120918 significantly increased the absorptive permeability for each PI (SQV 5-fold, IDV 11-fold, DRV 6-fold and APV 2-fold). These observations clearly confirm that these PIs are P-gp substrates (Kis e.a., 2010).

The effect of GF120918 and RTV on the permeability of Caco-2 cells for all PIs using FaSSIF.

In order to increase the biorelevance of the solvent system and to enhance the solubility of RTV, we determined the absorptive permeability for 9 PIs using FaSSIF (Fasted State Simulated Intestinal Fluid), a more biorelevant medium containing sodium taurocholate and phospholipids. The permeability values obtained from Caco-2 experiments using FaSSIF as apical medium are shown in figure 2 and illustrate that a wide range in values were obtained, with the apparent permeability for APV being 35 times higher than for SQV. When using FaSSIF, permeability of PIs appeared to be higher compared to using standard transport medium as apical medium, except for SQV. GF120918 further increased the absorptive transport, although its effect was limited compared to the effect observed in transport medium. The effect of RTV (50 μM) on absorptive transport was very variable, ranging from a significant increase (SQV: 2-fold, IDV: 3-fold) over negligible (DRV and APV) to non-existent (NFV, LPV, TPV and AZV). These results match with our previously published Caco-2 data on DRV (Holmstock e.a., 2010). Although the expression levels of CYP3A4 are very low in Caco-2 cells, they could potentially still limit the transport of PIs over a Caco-2
monolayer. To explore this possibility, we determined whether the non-specific CYP-inhibitor aminobenzotriazole (ABT, 100 µM) affects the permeability for SQV and IDV in Caco-2 cells, 2 PIs for which a large increase in absorptive permeability was observed in presence of RTV; in addition, one PI (DRV) for which a small effect of RTV had been observed was also included. In the presence of ABT, we observed no increase in the permeability values for SQV, IDV or DRV (data not shown), indicating that PIs are not metabolized by CYP-isozymes in Caco-2 cells.

The effect of RTV on the intestinal absorption of IDV, LPV and DRV in NMRI mice.

Based on the results obtained in the Caco-2 model, we selected 3 PIs which were tested with the \textit{in situ} intestinal perfusion technique; one for which RTV caused a large (IDV), negligible (DRV) and no (LPV) increase in permeability. The permeability values obtained were comparable to those obtained in the Caco-2 system, and the same rank order was maintained (figure 3). Since RTV has recently been shown to enhance the intestinal absorption of DRV through inhibition of P-gp, resulting in the same apparent permeability as observed for P-gp knockout mice (Holmstock et al., 2010), we focused on the effect of co-administration with RTV. Co-perfusion of the PIs together with RTV (75 µM) resulted in a significant increase in their permeability: IDV (3.2-fold), LPV (2.3-fold) and DRV (3-fold). The effect of RTV on IDV absorption was similar as observed in the Caco-2 system; however the inclusion of RTV resulted in a very significant increase in LPV and DRV absorption in the mouse model, while (almost) no effect was observed in the Caco-2 model. Since the presence of metabolic enzymes in the mouse intestine may be at the origin of this discrepancy between the two models, we investigated whether IDV, LPV and DRV are metabolized in the distal part of the small intestine, the segment which was also used for the intestinal perfusion experiments.
The intestinal metabolism of IDV, LPV and DRV.

We investigated the metabolic stability of IDV, LPV and DRV in the presence of microsomes prepared from the distal part of the mouse small intestine (n=6). Incubation of these PIs with intestinal microsomes revealed significant metabolism for LPV (fraction metabolized 37.2 % after 1 hr), while metabolism of IDV and DRV was negligible, being 0.6% and 1.2 %, respectively. We subsequently investigated the effect of the intestinal metabolism on the absorption of the selected PIs by co-perfusion of the intestinal segment together with the nonspecific CYP inhibitor aminobenzotriazole (100 µM). In line with the results obtained using the intestinal microsomes, we observed that for IDV and DRV, there was no effect of aminobenzotriazole on their intestinal absorption, while for LPV, aminobenzotriazole increased the absorption to the same level as when co-perfused with RTV (figure 3). Because of species differences in CYP-enzymes, the metabolism data in mouse intestine should be verified to be predictive for metabolism in the human intestine. Therefore, we examined the metabolism of IDV, LPV and DRV using human intestinal microsomes; in addition, the inhibitory potency of RTV was explored. The results are shown in figure 4; we observed a comparable ranking of PI metabolism between human microsomes (LPV>>DRV≈IDV) and mice microsomes, where especially lopinavir was prone to enzymatic degradation. The extensive metabolism of LPV was inhibited by RTV.
Discussion

When opting for a PI-based regimen during HIV treatment, PIs are always co-administered with a subtherapeutic dose of RTV in order to boost their plasma concentrations (Hull en Montaner, 2011). It is commonly accepted that RTV exerts its function by inhibition of intestinal and hepatic CYP3A4. Inhibition of intestinal efflux transporters has also been suggested to contribute to the ritonavir boosting effect, however the exact role of this mechanism remains somewhat elusive (Zeldin en Petruschke, 2004). Since RTV has been described to be an inhibitor of P-gp (Kis e.a., 2010), its co-administration is also expected to result in an enhanced intestinal absorption of P-gp substrates. By performing in situ intestinal perfusion experiments using wild-type and P-gp knockout mice, we previously confirmed that RTV does increase the permeability of the ileum for DRV 2.7-fold through inhibition of P-gp. Since all PIs have been found to be P-gp and CYP3A4 substrates, we investigated the effect of RTV on the intestinal permeability for other PIs. In order to exclude a confounding effect of CYP, we used Caco-2 cells as their CYP3A4 expression is very low, thereby allowing us to specifically explore the P-gp inhibitory effect of RTV.

Figure 1 shows that GF120918 significantly increases the absorptive permeability for PIs. However, the use of plain aqueous buffers may be questioned as it does not represent the intraluminal environment in which a multitude of compounds, including bile salts, are present. Previously, it has been shown that bile salts present in intestinal fluids may inhibit P-gp (Deferme e.a., 2003), meaning that using plain aqueous buffers may overestimate the contribution of P-gp. In addition, the effect of RTV on the absorptive permeability of PIs could not be explored in plain aqueous buffers because of the limited solubility of RTV. In order to increase the biorelevance of the solvent system and to enhance the solubility of RTV, we determined the absorptive permeability for 9 PIs using FaSSIF, a more biorelevant medium containing sodium taurocholate and phospholipids. The effect of using FaSSIF
consists in inhibition of P-gp on the one hand (resulting in an enhanced transport), and micellar encapsulation on the other hand (resulting in a decreased transport) (Ingels et al., 2004). Judging from figure 2, for PIs, the P-gp inhibitory effect appeared to be more important than micellar encapsulation. To explore whether P-gp was completely inhibited by compounds present in FaSSIF, we did an additional set of experiments in which GF120918 (4 µM) was included in the apical medium. GF120918 further increased the absorptive transport, although its effect was limited, probably due to the fact that the functionality of P-gp was already partially inhibited by taurocholate present in FaSSIF. Subsequently, we explored the effect of co-incubation with RTV, which has previously been shown as one of the most potent P-gp inhibitors among the PIs (Storch et al., 2007). The effect of RTV on absorptive transport was very variable, ranging from a significant increase (SQV and IDV) over negligible (DRV and APV) to non-existent (NFV, LPV, TPV and AZV). To further explore the dual inhibitory effect of RTV on CYP and P-gp, we used the in situ intestinal perfusion technique in mice. As this advanced absorption tool is a relatively labor intensive technique, we made a selection of 3 PIs based on the results obtained in the Caco-2 model; one for which RTV caused a large (IDV), negligible (DRV) and no (LPV) increase in permeability. The data obtained in the in situ intestinal perfusion of the mouse (figure 3) clearly illustrate that RTV increases the absorption of other PIs by inhibition of either metabolism (LPV) or P-gp mediated efflux (IDV and DRV). The observed differences in the effect of RTV between the Caco-2 and the mouse model can be explained in part by the presence of drug-metabolizing enzymes in the mouse intestine. However, other factors may be the underlying cause of the different effect of RTV observed in both models, including (1) species differences between human and murine P-gp resulting in differences in affinity for the protease inhibitors, (2) a differential inhibitory effect of sodium taurocholate (present in
FaSSIF) on human versus murine P-gp, and (3) the presence of mucus in the mouse model which is absent in the Caco-2 system.

Clinical data on RTV-boosted LPV are characterized by a remarkable 10-fold increase in LPV $C_{\text{max}}$, which has been associated with an important role of inhibition of intestinal (rather than hepatic) first pass elimination in the boosting effect (Zeldin en Petruschke, 2004; Hill e.a., 2009). Combined with our data obtained in the present study, these clinical findings support inhibition of presystemic LPV metabolism in human small intestine as a major mechanism underlying the pharmacokinetic boosting of LPV by RTV. In contrast to the 10-fold increase in LPV $C_{\text{max}}$, RTV-boosting resulted in a $C_{\text{max}}$ increase of only 49-77% for IDV (Hill e.a., 2009) and 88% for DRV (Vermeir e.a., 2009). This increase is lower as would be expected based on our results obtained in the in situ perfusion experiments, suggesting that data obtained in mice regarding the effect of P-gp inhibition might somewhat over-predict the situation in humans. This might be explained by (1) higher expression levels of P-gp in rodents compared to humans (Uchida e.a., 2011) or (2) the fact that high intraluminal concentrations may saturate P-gp functionality.

**Conclusion.**

Our results indicate that the boosting effect of RTV on the intestinal permeability for other PIs in the mouse is compound-specific: RTV significantly increases the intestinal permeability for IDV and DRV by P-gp inhibition, while the increase in the permeability for LPV is mainly achieved through inhibition of its metabolism. Since the dual effect of RTV on metabolism and transporter inhibition could only be observed in a system co-expressing both, the Caco-2 model might be insufficient when studying drug-drug interactions at the level of the intestinal mucosa.
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Authorship Contributions

Participated in research design: Holmstock, Annaert and Augustijns

Conducted experiments: Holmstock

Contributed new reagents or analytic tools: not applicable.

Performed data analysis: Holmstock

Wrote or contributed to the writing of the manuscript: Holmstock, Annaert and Augustijns
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Footnotes

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Legends for figures

**Figure 1:** Apparent absorptive permeability values of a Caco-2 monolayer for SQV (25 µM), IDV, DRV and APV (50 µM) in HBSS in absence (open bars) or presence (gray bars) of the P-gp inhibitor GF120918 (4 µM). Bars represent the mean ± SD. (n = 3). Statistical significance between the control and inhibitor condition was evaluated using an unpaired t test. ***, significantly different from control condition (p < 0.001).

**Figure 2:** Apparent absorptive permeability values of a Caco-2 monolayer for SQV, IDV, NFV, TPV, LPV, RTV, AZV, DRV, APV in FaSSIF (50 µM) in absence (open bars) or presence of the P-gp inhibitor GF120918 (4 µM) (light gray bars) and RTV (50 µM) (dark gray bars). Bars represent the mean ± SD. (n = 3). Statistical significance between the different conditions was evaluated using one way ANOVA followed by Dunnett’s test. Significantly different from control condition; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Figure 3:** Apparent permeability values of the ileum of NMRI mice for IDV (100 µM), LPV (25 µM) and DRV (100 µM) using FaSSIF in the absence (open bars) or presence of the P-gp inhibitor RTV (75 µM) (light gray bars) and the nonspecific Cyp inhibitor aminobenzotriazole (100 µM) (dark gray bars). Bars represent the mean ± SD. (n = 3). Statistical significance between the different conditions was evaluated using one way ANOVA followed by Dunnett’s test. Significantly different from control condition; **, p < 0.01; ***, p < 0.001.

**Figure 4:** The percentage of PI (5 µM) remaining after 30 minutes of incubation with human intestinal microsomes (0.5 mg/ml) in the absence (open bars) or presence (gray bars) of RTV (5 µM). Bars represent the mean ± SD. (n = 2).
Figure 4

The diagram shows the remaining compound percentages for DRV, IDV, and LPV. DRV has a lower remaining percentage compared to IDV and LPV. LPV has the highest remaining percentage among the three.