Drug transport and transport-metabolism interplay in the human and rat intestine

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Chapter 5

The Consequence of Drug-Drug Interactions by P-gp inhibitors on the P-gp/CYP3A4 Interplay in the Human Intestine Ex Vivo


*Submitted*

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ABSTRACT

Due to the co-localization and overlapping substrate specificities, intestinal P-gp and CYP3A4 work coordinately to reduce the intracellular concentration of absorbed drugs, whereas drug-drug interactions (DDIs) based on this interplay occur frequently. These phenomena are of clinical importance and require pre-clinical investigation preferably with human tissue. Using precision-cut intestinal slices (PCIS), we investigated the P-gp/CYP3A4 interplay and related DDIs with P-gp inhibitors at the different regions, jejunum, ileum and colon, of human intestine ex vivo with quinidine (Qi), a dual substrate of P-gp and CYP3A4, as probe. The results showed that all the P-gp inhibitors increased the intracellular concentrations of Qi by 2.1 - 2.6 fold in jejunum, 2.6 - 3.8 fold in ileum but only 1.2 - 1.3 fold in colon, which is in line with the different P-gp expression in these intestinal regions. Consequently, the selective P-gp inhibitors (CP100356 and PSC833) enhanced Qi metabolism with profound increase of 3-hydroxy-quinidine (3OH-Qi) in jejunum and ileum, while dual inhibitors of both P-gp and CYP3A4 (verapamil and ketoconazole) decreased the 3OH-Qi production, despite of the increased intracellular Qi, due to inhibition of CYP3A4. The results indicate that the outcome of DDIs based on P-gp/CYP3A4 interplay shown as remarkable changes in the intracellular concentration of both the parent drug and the metabolite, varies among the intestinal regions, probably due to the different expression of P-gp and CYP3A4. Moreover, the effect depends on the specificity of the P-gp inhibitors, which may have important implications for the disposition and toxicity of drugs and their metabolites.

Keywords  P-glycoprotein; CYP3A4; interplay; selective P-gp inhibitor; precision-cut intestinal slice; ex vivo
INTRODUCTION

P-gp and CYP3A4 are important members of the families of efflux transporters and metabolizing enzymes and also the most studied [1-3]. In addition to their respective roles of excretion and metabolism of xenobiotics, they can work coordinately to reduce the intracellular concentration of xenobiotics and the absorption of orally taken drugs, due to their co-localization in the intestinal epithelium and the largely overlapping substrate specificities [4-6]. Their interdependent relationship is also known as P-gp/CYP3A4 interplay, which is the most well-known transport-metabolism interplay [7]. Furthermore, drug-drug interactions (DDIs) based on this interplay may occur frequently because of the broad and overlapping spectrum of inhibitors for both proteins [4, 8, 9]. The studies on the P-gp/CYP3A4 interplay and related DDIs are of clinical importance and require investigation in the pre-clinical phase. For instance, Choi et al. demonstrated a significant interaction between verapamil and atorvastatin in healthy volunteers. Atorvastatin, dual inhibitor of both P-gp and CYP3A4, enhanced the oral bioavailability of verapamil by inhibiting the P-gp efflux pump, and meanwhile decreased the verapamil metabolism, probably due to inhibition of CYP3A4 [10]. Thus, the studies on the P-gp/CYP3A4 interplay and related DDIs are of clinical importance and require investigation in the pre-clinical phase.

Moreover, it is obvious that the effect of P-gp/CYP3A4 interplay and related DDIs highly correlates with the expression levels of P-gp and CYP3A4 in the intestine. However, as the results of mRNA and western blot show, their expression patterns are very different along the intestinal tract, which is colon ≤ duodenum < jejunum < ileum for P-gp [11, 12] while colon ≤ ileum < jejunum < duodenum for CYP3A4 [13]. The most recent quantification of protein abundancy by LC-MS/MS confirmed that the expression of P-gp differs among human intestinal regions [14] while the abundance of CYP3A4 in human intestine is not available currently. However, the expression of transporters and metabolizing enzymes in in vitro models of the human intestine, such as Caco-2 cell lines, is notably different from their physiological levels thus they cannot mimic the situation in each intestinal region [15]. Therefore, a proper model which could directly use the intestinal tissue from human and/or animal, and meanwhile provide efficient screening capacity, is preferable. The precision-cut intestinal slice (PCIS) model has been established to investigate drug metabolism, toxicity, and more recently transport in both human and animal [16-20], and was recently presented as an adequate model for the study on transport-metabolism
interplay in rat intestine [21]. The aim of this study is to investigate the P-gp/CYP3A4 interplay and the DDIs based on this interplay to P-gp inhibition in different regions of the human intestine. To the best of our knowledge, the consequences of the P-gp/CYP3A4 interplay is still under debate [7, 22] and have not been studied before in human tissue *ex vivo*.

Since several drug transporters and metabolizing enzymes are expressed in PCIS, selective P-gp inhibitors are necessary in order to avoid the influence of inhibition of other transporters than P-gp. CP100356 and PSC833 were chosen as selective P-gp inhibitors, due to their high selectivity for P-gp inhibition [8, 23], whereas verapamil and ketoconazole, two well-studied inhibitors of both P-gp and CYP3A4 [8], were employed as dual inhibitors to investigate the consequences of DDIs under non-selective P-gp inhibition, as most of P-gp inhibitive compounds are not specific. Quinidine (Qi), which has been used clinically for more than 200 years and is still the most frequently prescribed antiarrhythmic drug [24], was chosen as the probe drug for the P-gp/CYP3A4 interplay, because it is a well-known dual substrate of P-gp and CYP3A4 that does not significantly inhibits CYP3A4 [25]. It was reported to inhibit P-gp but at relative high concentration with IC$_{50}$ 14.1 μM in MDCKII cells [26], 23.6 μM in rat PCIS [20] and 35.7 μM in human PCIS [27]. Furthermore, Qi is extensively metabolized by CYP3A4 into 3-hydroxy-quinidine (3OH-Qi) [28], which is considered to be the specific marker reaction for CYP3A4 activity [29].

**MATERIALS AND METHODS**

**Chemicals**

Quinidine, verapamil hydrochloride, ketoconazole and agarose (low gelling temperature, type VII-A) were from Sigma-Aldrich (USA). PSC833 and CP100356 were obtained from Tocris Bioscience (UK). Amphotericin B (fungizone)-solution, gentamicin, and Williams medium E with glutamax-I (WME) were purchased from Invitrogen (UK). HEPES was from MP Biomedicals (Germany). Antipyrine was purchased from O. P. G. Pharma (the Netherlands). 3-Hydroxy-quinidine was purchased from Toronto research chemicals Inc. (Canada).

**Human intestinal tissue**

The use of human intestinal tissue, which was obtained from surgical resections, was approved by the Medical Ethical Committee of the University Medical Center Groningen. Human jejunum tissue was obtained from patients undergoing pylorus-preserving
pancreaticoduodenectomy (PPPD), whereas human ileum and colon tissue was from patients undergoing hemicolectomy. The characteristics of the donors are listed in Table 1. Due to the relative scarcity of human ileum and colon tissue and the considerable P-gp activity in human jejunum, all studies apart from the study on regional differences, were performed with human jejunum.

Table 1 Characteristics of the human intestine donors

<table>
<thead>
<tr>
<th>Intestine ID</th>
<th>Gender</th>
<th>Age</th>
<th>Region</th>
<th>Surgical Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>66</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>42</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>62</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>57</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>69</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>84</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>76</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>69</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>67</td>
<td>jejunum</td>
<td>PPPD</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>71</td>
<td>colon</td>
<td>hemicolecotomy</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>67</td>
<td>colon</td>
<td>hemicolecotomy</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>76</td>
<td>ileum &amp; colon</td>
<td>hemicolecotomy</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>64</td>
<td>ileum &amp; colon</td>
<td>hemicolecotomy</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>66</td>
<td>ileum</td>
<td>hemicolecotomy</td>
</tr>
</tbody>
</table>

Preparation and incubation of human PCIS

After resection, the intestine explant was quickly submerged and stored in ice-cold carbogenated Krebs-Henseleit buffer, and then delivered to the laboratory within 20 minutes. Precision-cut intestinal slices were prepared from the human jejunum, ileum and colon as previously described [17, 30]. Briefly, after arrival, the tissue was gently flushed with ice-cold, carbogenated Krebs-Henseleit buffer to remove blood and other remaining luminal content. After removing the muscle layer and cutting the mucosa layer into sheets of 10 × 20 mm, each sheet of tissue was embedded in 3 % (w/v) agarose solution (maintained at 37 °C) in a precooled embedding unit (Alabama R&D, USA). PCIS, approximately 350 - 450 µm of
thickness and 2 - 4 mg of wet weight, were made using a Krumdieck tissue slicer (Alabama R&D, USA) after the agarose solution had solidified. After randomization of the slices from the same regions, the slices were incubated as described previously [20]. Briefly, all the slices were first pre-incubated individually in a 12-well culture plate with 1.3 ml Williams medium E in a pre-warmed cabinet (37 °C) under humidified carbogen (95 % O₂ and 5 % CO₂) for 30 min without or with P-gp inhibitors. Then incubation was started by adding the required amount of Qi stock solution.

**Viability of PCIS**

Intracellular ATP levels in the PCIS were evaluated to monitor the overall viability of the slices during incubation in parallel groups [18]. To evaluate the toxicity of Qi and P-gp inhibitors, the ATP content in slices was measured after 3 hours of incubation with the highest concentrations used in the study, i.e. 200 µM Qi, 5 µM CP100356, 2 µM PSC833, 20 µM verapamil or 20 µM ketoconazole, and compared with the corresponding control group. The ATP content was determined using the ATP Bioluminescence Assay Kit as previously described [30] in the supernatant after homogenization of the slices in 70 % ethanol and 2 mM EDTA and centrifugation of the slice homogenate. The pellet after drying was used for protein determination.

**The P-gp/CYP3A4 interplay**

To study the time course of Qi uptake and metabolism in human PCIS, slices prepared from human jejunum were incubated with Qi (final concentration: 2 µM). Tissue and medium samples were harvested from parallel wells at 0, 15, 30, 60, and 120 min after addition of Qi and then stored at -20 °C until further analysis.

To investigate the concentration dependency of Qi uptake and metabolism, slices from human jejunum were incubated with various concentrations of Qi, ranging from 0 to 200 µM, for 120 min. At the end of the incubation, 1 ml of medium was collected and slices were rinsed in ice-cold PBS for 5 min and stored at -20 °C.

**Interplay-based DDIs with P-gp inhibitors**

PCIS prepared from human jejunum, ileum and colon, were pre-incubated for 30 min in the absence or presence of an inhibitor (CP100356, PSC833, verapamil and ketoconazole, respectively) and then incubated with 2 µM of Qi for 120 min. The addition of the inhibitor during the pre-incubation allowed sufficient uptake to ensure the presence of the inhibitor in
the enterocytes at the moment the substrate was added. Tissue and medium samples were harvested and stored as described above. Based on literature reports [8] and our preliminary studies, 0.5 µM CP100356 and 2 µM PSC833 were used to achieve sufficient P-gp inhibition without inhibition on CYP3A4, while verapamil (20 µM) and ketoconazole (20 µM) were used as dual inhibitors for P-gp and CYP3A4.

**LC-MS/MS of Qi and 3OH-Qi**
In order to quantify the amount of Qi and 3OH-Qi from the tissue and medium samples, the samples were pretreated as described earlier [21]. Briefly, by adding acetonitrile (containing 10 nM antipyrine as internal standard), Qi and 3OH-Qi were extracted and the protein was precipitated. After centrifugation, the supernatant was frozen at -80 °C and then lyophilized at -20 °C by freeze-drying (Martin Christ Gefriertrocknungsanlagen, Germany). After reconstitution with 200 µl of 40 % methanol (containing 0.1 % formic acid) and centrifugation, 150 µl supernatant was transferred into a 96-well plate with a piercable cover. The plate was centrifuged at 2000 rpm at 4 °C for 20 min (Beun de Ronde, the Netherlands). The LC-MS/MS analysis was performed as described earlier [21].

**Protein determination**
The remaining pellet left from the ATP assay and LC-MS/MS analysis was dried overnight at 37 °C and dissolved in 200 µl of 5 M NaOH for 30 min. After dilution with H2O to 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Germany) with a calibration curve prepared from bovine serum albumin. The protein content of each slice was used to normalize for the size variation of the intestinal slices.

**Quantification of P-gp in the intestine**
The absolute expression levels of P-gp and villin at the outer plasma membrane of human intestinal samples (jejunum, n = 4) were determined as previously described [31, 32]. Samples were processed in duplicate (except for one sample, which was processed in mono due to insufficient amounts of tissue), and approximately 350 mg human intestinal tissue was used for plasma membrane isolations per run. After trypsin digestion, the samples were analyzed by a UPLC coupled to a 6500 QTrap mass spectrometer (AB Sciex). For each peptide, three transitions (Q3–1, Q3–2, and Q3–3) were used for quantitation and confirmation, listed in Table 2. A peptide labeled with 15N and 13C (AQUA peptide) was synthesized (Sigma Aldrich,
Steinheim DE) and used as an internal standard for quantification (Table 2). Peak identification and quantification was performed using Masslynx software version 4.1.

**Table 2** Multiple reaction monitoring (MRM) transitions of the P-gp and villin peptides and the corresponding internal standard (AQUA). The peptide sequence is chosen according to the in silico peptide criteria defined by Kamiie et al. [33] and is exclusively present in the target protein of interest (i.e. P-gp or villin).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide sequence</th>
<th>MW</th>
<th>Q1</th>
<th>Q3-1</th>
<th>Q3-2</th>
<th>Q3-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>NTTGALTTR</td>
<td>934.0</td>
<td>467.7</td>
<td>719.4</td>
<td>216.1</td>
<td>618.4</td>
</tr>
<tr>
<td>Villin</td>
<td>GDVFLLDLGK</td>
<td>1076.3</td>
<td>538.8</td>
<td>805.5</td>
<td>658.4</td>
<td>545.4</td>
</tr>
</tbody>
</table>

**Statistical analysis**

All the experiments were performed with tissue from 3 - 5 different human donors, and within each experiment 3 parallel slice incubations were performed for each experimental condition. The average of the three individual slices of one human intestine was considered as one observation. The results are expressed as mean ± S.E.M of the values for the different human donors. One-way ANOVA and two-way ANOVA followed by the Bonferroni test as post-hoc test were used to compare multiple groups with one factor and two factors, respectively. In all cases, \( p < 0.05 \) was predetermined as the criterion for significance.

**RESULTS**

**Viability of the human PCIS**

After 3 h of incubation the PCIS from human jejunum, ileum and colon retained more than 80 % (\( p > 0.05 \)) of the level of intracellular ATP in fresh slices at 0 h, as shown earlier [27], indicating that the PCIS remain viable during incubation. Furthermore, the exposure to the P-gp substrate (quinidine, 200 µM) or inhibitors at their highest concentration had no significant influence on the ATP content in PCIS. These results indicate that quinidine and the P-gp inhibitors did not influence the viability of PCIS during incubation.

**Time course of quinidine uptake and metabolism**
As shown in Fig. 1, after the first 30 min of incubation, the Qi content in the slice reached equilibrium, indicating a balance between uptake, efflux and metabolism, which was maintained till the end of the incubation (120 min). During exposure to a P-gp inhibitor, an equilibrium was also achieved but at a higher steady state level of Qi in the slice ($p < 0.05$ at 60 and 120 min), which could be the result of inhibition of P-gp efflux or inhibition of metabolism. As the total metabolism into 3OH-Qi, calculated using the sum of the amounts of metabolite found in the tissue and in the medium, was not decreased in the presence of the P-gp inhibitor (see Fig. 2 and 5), the increased Qi content in the slice was caused by inhibition of P-gp efflux. In line with the Qi content in the slice, also the 3OH-Qi in the slice reached an equilibrium level at approximately 1.8 % of the intracellular Qi content, and this steady state level was also enhanced by P-gp inhibition. The AUCs of Qi and 3OH-Qi in slice calculated after the indicated incubation times, which represent the intracellular exposure to Qi and 3OH-Qi, were enhanced from $9.1 \pm 0.3$ to $14.1 \pm 2.6$ nmol•min/mg protein and from $0.16 \pm 0.01$ to $0.64 \pm 0.13$ nmol•min/mg protein ($p < 0.05$), respectively. The total metabolic rate, calculated as the sum of the amounts of metabolite in tissue and medium, was constant during this equilibrium after 30 minutes. In addition, under P-gp inhibition, despite to the significant increased 3OH-Qi concentration in slice, the 3OH-Qi excreted into the medium decreased. After 120 min of incubation the amount of metabolite retained in the slices was only 5.6 % of the total amount of metabolites produced, whereas under P-gp inhibition, the percentage was enhanced to 26.2 %

Concentration dependency of quinidine uptake and metabolism

The influence of different Qi concentrations on the P-gp/CYP3A4 interplay was studied at 120 minutes, when equilibrium was reached. As shown in Fig. 2a, the Qi content in the slice increased concentration dependently. As can be seen in the insert, this increase is somewhat steeper in the range of 5 - 50 μM than in the range of 0 - 5 μM and 50 - 200 μM. This may indicate that at a concentration higher than 5 μM P-gp efflux becomes saturated. The lower accumulation at concentrations higher than 50 μM may be the result of P-gp inhibition by Qi itself as it is known that Qi is a P-gp inhibitor at high concentrations [20, 34]. The 3OH-Qi content in the slices and the total 3OH-Qi production were also accordingly increasing in a concentration-dependent manner until 50 μM Qi. At higher concentrations of Qi, the slice content of 3OH-Qi and the total 3OH-Qi production did not increase further, despite an increase in slice concentration of Qi, possibly due to saturation of CYP3A4 metabolism. The plateau in the curve indicates that the 3OH-Qi production in human jejunum PCIS reached its
maximum. The $V_{\text{max}}$ of human intestinal Qi metabolism, calculated as the average of the production rate at Qi 50 - 200 µM, was $4.7 \pm 0.2$ pmol/(mg slice protein)/min in the jejunum. However, as the rate of metabolism at low concentrations is influenced by the P-gp efflux, the calculation of an apparent $K_m$ is not reliable.

**Fig. 1** The time course of Qi (left Y-axis) and 3OH-Qi (right Y-axis) content in the slice after incubation with 2 µM Qi in the absence or presence of the P-gp inhibitor CP100356 at 2 µM in PCIS from human jejunum (n = 3).

* Significant increase of Qi and 3OH-Qi in the slice was found at 60 and 120 min by P-gp inhibition (one-way ANOVA and the Bonferroni test as post-hoc test).

**Fig. 2**
- **a** the concentration dependency of Qi (left Y-axis) and 3OH-Qi (right Y-axis) content in PCIS and **b**, the total 3OH-Qi production by PCIS from human jejunum PCIS after 120 min of incubation with different
concentrations of Qi (n = 4).

The influence of P-gp inhibitors on the P-gp/CYP3A4 interplay in jejunum, ileum and colon
The effect of both selective and non-selective P-gp inhibitors on the P-gp/CYP3A4 interplay was studied in jejunum, ileum and colon PCIS at a concentration of 2 µM Qi, at which P-gp efflux appeared to limit the intracellular concentration. In slices that were not exposed to the inhibitors, the Qi content in the slices at steady state was low in jejunum and ileum but significantly higher in colon, in line with the lower P-gp and CYP3A4 content in colon (Fig. 3 right panel). By co-incubation of Qi with various P-gp inhibitors, as shown in Fig. 3, the Qi content in the slices was significantly enhanced by approximately 2.1 - 2.6 fold in jejunum and 2.6 – 3.8 fold in ileum, while the increase was not significant in colon.

![Fig. 3](image)

**Fig. 3** Left panel: the fold change of the amount of Qi in the slices in the presence of P-gp inhibitors in different regions of human intestine. * Significant increase of Qi in the slices compared to the corresponding control group (two-way ANOVA and the Bonferroni test as post-hoc test). Right panel: the absolute amount of Qi in the slices of the control groups. #, Significant different amount of Qi (one-way ANOVA and the Bonferroni test as post-hoc test). (n = 6 for jejunum, n = 3 for ileum, n = 4 for colon).

In addition, the selective P-gp inhibitors, CP100356 and PSC833, enhanced the 3OH-Qi metabolite content in the slices considerably in jejunum (approximately 10 fold) and ileum (approximately 20 fold) but not in colon (less than 1.2 fold) (Fig. 4), which is in line with the relative P-gp expression in these 3 regions. However, for verapamil this increased metabolite content was not obvious, while ketoconazole clearly reduced the CYP3A4 metabolism, in line
with its strong inhibition of CYP3A4. As the baseline production of metabolite is much higher in jejunum than in ileum (Fig. 4 right panel), the absolute amount of produced metabolite after P-gp inhibition was still higher in jejunum than in ileum (increase from 17.5 to approximately 40 pmol/mg protein by P-gp inhibition in jejunum whereas from 1.6 to approximately 11 pmol/mg protein in ileum), although the fold change was higher in ileum.

The difference between the intestinal regions with respect to the total 3OH-Qi production (the total amount in slice and medium) is given in Fig. 5. A 2.1 - 2.7 fold increase of total 3OH-Qi production by the selective P-gp inhibitors was found in jejunum and a 6.6 - 8.5 fold increase in ileum, whereas no increase was found in colon. In contrast, verapamil and ketoconazole did not increase the metabolism but inhibited it. The lack of response in colon reflects the lack of both P-gp and CYP3A4. Similar with the changes of 3OH-Qi in slice, the enhancement on absolute amount of metabolite was actually larger in jejunum due to the higher production in the control group of human jejunum. In addition, after co-incubation with P-gp inhibitors, the fold increase of 3OH-Qi in the slices was always higher than that in the medium, suggesting that 3OH-Qi is a substrate of P-gp.
Correlation between P-gp abundance and inhibition effect

The P-gp abundance was measured in the tissue of 3 human jejunum samples (MH13, MH14 and MH17 in Table 1). The absolute abundance of intestinal P-gp and the responses to P-gp inhibition by 0.5 µM CP100356, with respect to the tissue content of Qi, 3OH-Qi and total metabolism, are listed in Table 3. The results show that the fold change of Qi and 3OH-Qi content in the slices and of the total metabolism after P-gp inhibition is well in concordance with the P-gp abundance.

![Graph showing the correlation between P-gp abundance and inhibition effect](image)

**Table 3** The correlation between P-gp abundance and the responses to P-gp inhibition by 0.05 µM CP100356 in human PCIS

<table>
<thead>
<tr>
<th>P-gp abundance (pmol/g tissue)</th>
<th>Responses (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Qi in slice</td>
</tr>
<tr>
<td>6</td>
<td>0.68</td>
</tr>
<tr>
<td>7</td>
<td>1.66</td>
</tr>
<tr>
<td>9</td>
<td>1.56</td>
</tr>
</tbody>
</table>
DISCUSSION

The activity of P-gp and CYP3A4 in the intestine is one of the reasons of the low oral bioavailability for many drugs. In addition, many studies support that P-gp and CYP3A4 act synergistically to reinforce their limiting effect on intestinal absorption and enhancement of intestinal metabolism of their shared substrates, such as Qi [5, 35, 36]. Furthermore, DDIs based on this interplay are expected to occur frequently, because P-gp and CYP3A4 not only share substrates but they are also sensitive to a broad spectrum of inhibitors. In the intestine, P-gp/CYP3A4 interplay-based DDIs with P-gp inhibitors have major consequences on the disposition of the shared substrate and their metabolites [37, 38].

In the present study, we have observed that the concentration of 3OH-Qi in slices and the total 3OH-Qi production correlated with the Qi concentration in the slice, which was considerably influenced by P-gp efflux (Fig. 1 and 2). Furthermore, the tissue concentrations of Qi, the shared substrate, increased 2.1 - 3.8 fold (Fig. 3) and 3OH-Qi, the metabolite of Qi, increased more remarkably 9.1 - 19.6 fold (Fig. 4) under P-gp inhibition. Moreover, the total 3OH-Qi production was enhanced by approximate 2.5 fold in jejunum and 7.5 fold in ileum (Fig. 5). These results indicate that selective inhibition of P-gp can have a significant effect on the local tissue concentration of a drug, because (at low drug concentrations) P-gp activity (and not CYP3A4 activity) is the rate-limiting step in the intestinal disposition of the drug [39]. Furthermore, also the local tissue concentration of the metabolite can be remarkably increased, increasing the risk for intestinal toxicity. The total metabolite production also increased which in vivo will result in a lower bioavailability. However, the non-P-gp-selective inhibition may counteract the enhancement of intestinal CYP3A4 metabolism by P-gp inhibition, because of the concomitant inhibition of CYP3A4 activity.

Benet et al. stated that in vivo the P-gp efflux would increase the intracellular residence time by cycling the drug several times between enterocytes and gut lumen [7], thus increasing the extent of intestinal CYP3A4 metabolism [38, 40]. In other words, P-gp inhibition will lead to a decrease of intestinal CYP3A4 metabolism. Our findings here and those earlier in rat PCIS [21], do not support this assumption, at least when the concentration of the substrate is low. In line with our findings, Dufek et al. found that P-gp inhibition, mimicked in P-gp-deficient mice, decreases intestinal absorption of loperamide in mouse by increasing the intestinal metabolism [36]. In addition, Pang et al. found that P-gp efflux limits intestinal metabolism due to the competition between P-gp and CYP3A4 within the cell and that their interplay is independent of the mean residence time of drug in the system [22]. Therefore, they also
concluded that P-gp inhibition will increase metabolism, since the intracellular substrate concentration, available to CYP3A4, will be increased. Anyway, there is no doubt that P-gp/CYP3A4 interplay-based DDIs by P-gp inhibitors change the disposition of dual P-gp/CYP3A4 substrates.

Most of the reported studies on P-gp/CYP3A4 interplay were performed in only one segment of the intestine [40], or in vivo including the whole intestine [36]. In vitro experiments made use of cell lines [38, 41], such as Caco-2, which overexpress P-gp but have low CYP expression or CYP3A4-transfected Caco-2, in which CYP3A4 expression might be unstable and its expression relative to P-gp is unknown. To be able to predict the consequences of DDI related to the P-gp/CYP3A4 interplay in vivo, however, the predictive model should express P-gp and CYP3A4 in the same ratio as in the intestine in vivo. This is complicated by the fact that in vivo the ratio of expression differs considerably in the different regions of the intestine. P-gp and CYP3A4 are expressed heterogeneously, actually in an opposite pattern along the intestine. The expression of P-gp increases from proximal intestine to the distal region of the small intestine (ileum) however it is low in colon [11, 14]. The expression level of CYP3A is highest in duodenum and jejunum but decreases towards the ileum and is even less expressed in colon [13]. As a result, several combinations of P-gp and CYP3A4 levels exist, i.e. duodenum (low P-gp, highest CYP3A4), jejunum (medium P-gp, medium CYP3A4), ileum (highest P-gp, low CYP3A4) and colon (low P-gp, low CYP3A4). Therefore, in the present study with human PCIS the full scope of the interplay has been shown in human intestine. It appeared that the P-gp inhibitors had a greater effect on the Qi concentration in the tissue in the ileum than in the jejunum (Fig. 3). Consequently the total production of the metabolite was more increased in ileum than in jejunum. The absolute amount of the metabolite formed, which depends on both the intracellular concentration of the substrate and the expression level of CYP3A4, increased more in the jejunum (Fig. 4 and 5), reflecting the differences in P-gp and CYP3A4 expression. In contrast, almost no response was observed in the colon, reflecting its low expression of both P-gp and CYP3A4.

The relevance of differences in P-gp expression for the outcome of DDI was also reflected by the correlation that we found between P-gp abundance in the different samples of jejunum and the increase of intracellular Qi and (produced) metabolite (Table 3). It was shown that, similarly to the regional differences, higher P-gp expression in the different individuals led to relatively higher Qi accumulation and metabolite formation. The individual expression of CYP3A4 was not measured but since the intracellular Qi concentrations (even after P-gp
inhibition) were probably well lower than $V_{\text{max}}$ in these intestinal samples, the increase of intracellular concentration by P-gp inhibition led to a proportional increase in metabolism. Human $in\ vivo$ data is scarce due to ethical limitations. Therefore, pre-clinical investigations are performed in animals. However, species differences can be a major problem that hinders the translation of ADME results for drug candidates from animal models to human $in\ vivo$. Thus the direct use of human tissue to test ADME-tox properties of drug candidates $ex\ vivo$ could offer a solution. To our best knowledge, the present study is the first P-gp/CYP3A4 interplay study on human intestine $ex\ vivo$. With human PCIS, we aim to get more relevant data. Compared to our earlier DDI study in rat PCIS [21], many species differences were found. The $V_{\text{max}}$ of intestinal Qi metabolism was $4.76 \pm 0.20$ pmol/(mg slice protein)/min in human jejunum, which was approximately 3 fold of that in rat jejunum, indicating the higher activity or expression of CYP3A in human jejunum. In addition, the increase of Qi in the slice by P-gp inhibition was higher in human intestine, suggesting that the activity or expression of P-gp in the human intestine may also be higher than in the rat intestine. In line with the difference of P-gp and CYP3A, the total 3OH-Qi production increased more in human by P-gp inhibition but the regional differences are smaller in human. In addition, the accumulation of 3OH-Qi was less increased by the P-gp inhibitor in human than in rat PCIS, indicating that this metabolite may have a different affinity for the human and the rat P-gp. The results support the use of human instead of animal PCIS for more adequate representation of the human situation.

All the above-mentioned regional differences and species differences give us indications about the effect of P-gp inhibitors on the systemic bioavailability and local exposure of the drug and its metabolite, which can have critical clinical implications. In the present study, Qi was used as probe drug to illustrate the P-gp/CYP3A4 interplay via Qi uptake and metabolism and the consequence of interplay-based DDI to P-gp inhibitors. However, the intestinal concentration of quinidine after a dose of 200 - 400 mg could be up to 2.5 - 5.0 mM, which is sufficient to inhibit intestinal P-gp efflux and saturate intestinal CYP3A4 metabolism. In this situation, the influence of P-gp/CYP3A4 interplay and P-gp inhibition would not be obvious. However, for drugs of which the luminal concentrations are lower than the respective $K_m$'s of CYP3A4 and P-gp, P-gp inhibition may have considerable effects on the absorption of the drug and the local concentration of the metabolite. When CYP3A4 mediated metabolism gives rise to formation of reactive metabolites, the human ileum and jejunum seem to be most prone to a toxic insult since P-gp inhibition may dramatically increase tissue concentrations of the CYP3A4 product, as demonstrated in our
study.

CONCLUSIONS
To the best of our knowledge, the present study is the first *ex vivo* study on P-gp/CYP3A4 interplay and its related DDIs on human intestine. It shows that the human PCIS are a reliable and efficient *ex vivo* model for these purposes and the results indicate that the outcome of DDIs based on P-gp/CYP3A4 interplay varies among the intestinal regions probably because of the different abundance of P-gp and CYP3A4 and depends on the selectivity of the inhibitors. This may have important implications for the disposition and toxicity of drugs and their metabolites, since remarkable changes in the intracellular concentration of both the parent drug and the metabolite were observed. In future studies, human PCIS can be used to predict the interplay between other intestinal transporters and metabolizing enzymes, which may help to decrease the risk of DDIs and inadequate pharmacotherapeutic effects of newly developed compounds.

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