Chapter 3

The Consequence of Drug-Drug Interactions Influencing the Interplay between P-gp and Cyp3a: An Ex Vivo Study with Rat Precision-Cut Intestinal Slices

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ABSTRACT

P-gp and CYP3A are differentially expressed in the intestine, and work coordinately to reduce the intracellular concentration of xenobiotics and the absorption of orally taken drugs. Drug-drug interactions (DDIs) based on P-gp/CYP3A interplay are of clinical importance and require pre-clinical investigation. We investigated the P-gp/Cyp3a interplay and related DDIs with different P-gp inhibitors at the various regions of rat intestine *ex vivo* using Precision-Cut Intestinal Slices (PCIS) with Quinidine (Qi), dual substrate of P-gp and Cyp3a, as probe. The results showed that P-gp efflux was the main factor limiting the intracellular Qi content at concentrations below 5 µM, whereas both efflux and metabolism were non-linear and saturated at [Qi] > 50 µM. The selective P-gp inhibitors (CP100356 and PSC833) enhanced the Qi accumulation in slices in line with the different P-gp expression in the intestinal regions, and as a result also enhanced metabolism in jejunum and ileum. Dual inhibitors of both P-gp and Cyp3a (verapamil and ketoconazole) increased the concentration of Qi in jejunum and ileum, but less 3-OH-quinidine (3OH-Qi) was produced due to inhibition of Cyp3a. The results indicate that the P-gp/Cyp3a interplay is dependent on the concentration of the drug and on the intestinal region under study. Furthermore, due to the P-gp/Cyp3a interplay, DDIs can lead to remarkable changes in the intracellular concentration of both the parent drug and the metabolite, which varies among the intestinal regions and depends on the selectivity of the inhibitors, with potentially important implications for disposition and toxicity of drugs and their metabolites.

Keywords: P-gp, Cyp3a, P-gp inhibition, drug-drug interaction, transport-metabolism interplay, precision-cut intestinal slices
INTRODUCTION

Efflux transporters and metabolizing enzymes play important roles in disposition and toxicology of orally administrated therapeutic drugs. Among the efflux transporters, P-glycoprotein (P-gp), plays a central role due to its high expression and broad substrate specificity [1, 2]. Cytochrome P450 3A (CYP3A) is one of the major CYP-enzymes, accounting for metabolism of approximately 50% of therapeutic drugs [3]. Their respective influence on the pharmacokinetics and pharmacodynamics of drugs have been widely studied showing that their activities are responsible for many unexpected/changed therapeutic effects [1, 4].

In the intestine, P-gp and CYP3A are believed to work coordinately as a barrier to reduce the intracellular concentrations of xenobiotics and therefore lower the intestinal absorption of drugs [5]. In cells in vitro P-gp inhibitors increase the extent of metabolism of the parent drug by increasing its intracellular concentration, and thus its availability for CYP3A [6]. Drug-drug interactions (DDIs) based on the P-gp/CYP3A interplay therefore could influence not only the systemic and local exposure to the parent drug, but also the exposure to the metabolites of that drug. For this reason, DDIs based on the P-gp/CYP3A interplay are of clinical importance and require pre-clinical investigation.

As the P-gp/CYP3A interplay is dependent on the relative expression of the transporter and the enzyme, the consequence of DDIs on this interplay for local and systemic exposure to P-gp/CYP3A substrates and their metabolites, can only be predicted using models that express drug transporters and metabolizing enzymes at physiological levels. Since P-gp and CYP3A concentrations vary considerably among the regions of the intestine [7, 8], such a model should also reflect the different regions. In vivo studies on the P-gp/CYP3A interplay and the related DDIs are cost- and time-consuming [9]. On the other hand, in vitro models, such as Caco-2, MDCKII and transfected cell lines, are known to have non-physiological expression of efflux transporters and/or metabolizing enzymes and do not represent the various intestinal regions [10]. Precision-cut intestinal slices (PCIS) have been established as ex vivo model to investigate drug metabolism, toxicity, and more recently transport in rat intestine [11-13]. Their viability and metabolic activity can be maintained for at least 5 hours. These PCIS contain the appropriate array and expression levels of metabolizing enzymes, transporters and cofactors along the small and large intestine, and hence
represent a mini-organ model *ex vivo* and closely represent the segments of the intestine they were derived from [12], which makes them highly suitable for studying DDIs based on the P-gp/CYP3A interplay. In addition, this technique can be applied to both human and animal tissue which could facilitate cross-species comparisons and prediction of the human situation.

In this study we investigated the P-gp/Cyp3a interplay in rat intestine based on its similarity with P-gp/CYP3A interplay in human intestine. We aimed to (1) show the application of rat PCIS to study the transport and metabolism of a model compound simultaneously in rat intestine by measuring its uptake and metabolism; (2) to characterize the intestinal P-gp/Cyp3a interplay and the DDI with P-gp inhibitors *ex vivo*; and (3) to investigate the consequence of DDIs based on the P-gp/CYP3A interplay on the intracellular concentration of a model substrate and its metabolite in various intestinal regions.

For this purpose, quinidine (Qi), a well-known substrate of both P-gp and CYP3A/Cyp3a, was used as probe [14, 15]. Importantly, Qi is not an inhibitor of CYP3A/Cyp3a [16], whereas its inhibition on P-gp is quite weak, which allows to select a concentration at which Qi only functions as a P-gp substrate [13, 17]. Furthermore, the 3-hydroxylation of Qi can be used as a specific marker reaction for CYP3A4 [15].

To determine the effect of P-gp inhibition on the interplay between metabolism and transport of Qi, CP100356 and PSC833 were employed as selective P-gp inhibitors, due to their high selectivity ratio for P-gp to CYP3A [17, 18], whereas verapamil and ketoconazole, well-established inhibitors of both P-gp and CYP3A with different ratios of inhibitory potency to P-gp/CYP3A [17], were included as dual inhibitors to further explore the consequences of the interplay-based DDIs.

**MATERIALS AND METHODS**

**Chemicals**

Quinidine, verapamil, ketoconazole and low gelling temperature agarose (type VII-A) were purchased from Sigma-Aldrich (USA). PSC833 and CP100356 were from Tocris Bioscience (UK). Gentamicin, William’s Medium E with glutamax-I (WME), and amphotericin B (fungizone)-solution were obtained from Invitrogen (UK). HEPES was obtained from MP Biomedicals (Germany). 3-Hydroxy-quinidine was from Toronto research chemicals Inc. (Canada). Antipyrine was from O. P. G. Pharma (the Netherlands)
The stock solutions were prepared in methanol (quinidine) or DMSO (verapamil, ketoconazole, PSC833 and CP100356).

**Animals**

Male Wistar (HsdCpb: WU) rats with a body weight of ca. 300-350 g were purchased from Harlan (Horst, the Netherlands). They were housed in a temperature- and humidity-controlled room with a 12 hours light/dark cycle and had free access to food and tap water. The rats were allowed to acclimatize for at least 7 days after arrival in the animal facility before the experiments were performed. All the animal experiments were approved by the animal ethical committee of the University of Groningen.

**Preparation and incubation of rat PCIS**

After the rats were anesthetized with isoflurane/O₂, the intestine was excised and was divided into four parts: duodenum, jejunum, ileum and colon, where duodenum was considered to be the segment between 2 and 12 cm, jejunum the segment between 20 and 40 cm from the pylorus, ileum the segment of the last 20 cm before the ileocecal junction and colon the segment after the ileocecal junction. The segments were embedded in 3 % (w/v) low-gelling agarose solution in 0.9 % NaCl (37 °C) and precision-cut slices (thickness about 300 µm and wet weight about 3-5 mg) were cut using a Krumdieck tissue slicer (Alabama R&D, USA) in ice-cold oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM D-glucose, pH 7.4) as previously described [19, 20]. The slices were incubated individually in a 12-well culture plate (Greiner Bio-One GmbH, Austria) with 1.3 ml WME (with glutamax-I), supplemented with D-glucose, gentamicin and amphotericin B (final concentration: 25 mM, 50 µg/ml, and 2.5 µg/ml, respectively). The culture plates were placed in plastic boxes in a pre-warmed cabinet (37 °C) under humidified carbogen (95 % O₂ / 5 % CO₂) and shaken back and forth approximately 90 times per minute (Reciprocating Shaker 3018, Gesellschaft für Labortechnik GmbH, Germany). All the slices were pre-incubated for 30 min in culture medium with or without inhibitor, then incubated with substrate by adding its stock solution to reach the indicated final concentration, depending on the aim of the studies.

**Viability of PCIS**

Intracellular ATP levels in the PCIS were evaluated to monitor the overall viability of the
tissue during incubation in parallel groups [12, 21]. Also, the ATP content was measured in slices after 5 hours of incubation with or without Qi (50 µM, 100 µM, and 200 µM, respectively) to evaluate the toxicity of Qi. Furthermore, to estimate the influence of inhibitors on the viability during incubation, ileum slices were co-incubated with a P-gp inhibitor at the highest concentration used in the study (5 µM CP100356, 2 µM PSC833, 20 µM verapamil or 20 µM ketoconazole). The final concentration of the solvents in culture medium was always lower than 1 %, which did not influence the viability of the slice (evaluated by intracellular ATP, data not shown). The ATP content was determined using the ATP Bioluminescence Assay Kit as previously described [19] in the supernatant after homogenization in 70 % ethanol and 2 mM EDTA and centrifugation of the slice samples. The pellet is used for protein determination.

The interplay between P-gp and Cyp3a with Qi as a substrate
To study the time course of Qi transport and metabolism in rat PCIS, slices prepared from different regions of rat intestine were incubated with Qi (final concentration: 2 µM). Tissue and medium samples were harvested at 0 min, 15 min, 30 min, 60 min, and 120 min after addition of Qi and then stored at -20 °C until further analysis.

To evaluate the influence of Qi concentration on the interplay, slices from different regions were incubated with Qi (final concentration in the range of 0 - 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.

The interplay-based DDIs with P-gp inhibitors
Intestinal slices, prepared from duodenum, 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. Tissue and medium samples were harvested and stored as described above. The addition of inhibitor during pre-incubation allowed sufficient uptake to ensure the presence of the inhibitor in the enterocytes at the moment the substrate was added. Based on literature reports [17] and our preliminary studies, 2 µM PSC833 was chosen, whereas 0.5, 2 and 5 µM CP100356 was used to achieve sufficient P-gp inhibition without inhibition on Cyp3a. Verapamil (20 µM) and ketoconazole (20 µM) were used as dual inhibitors for P-gp and Cyp3a.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)
In order to extract Qi and 3OH-Qi from the medium samples, 100 µl medium samples were mixed with 400 µl acetonitrile (containing 10 nM antipyrine as internal standard) + 100 µl blank WME + 100 µl Milli-Q water. The slice tissue samples were added to 10 nM antipyrine (as internal standard) + 200 µl blank WME + 100 µl Milli-Q water (note: 100 µl extra blank WME was added to achieve the same final composition as the medium samples), and then they were homogenized using a Mini-BeadBeater-8 (BioSpec, USA). Calibration curves for Qi and 3OH-Qi were prepared separately with 100 µl in WME at each concentration plus the same solution mixture that was added into medium samples. After thorough mixture and centrifugation at 13000 rpm for 15 min at 4 °C, 400 µl supernatant was transferred into clean tubes and frozen at -80 °C. The samples were then lyophilized at -20 °C overnight till dryness by freeze-drying (Martin Christ Gefriertrocknungsanlagen, Germany). After reconstitution with 200 µl of 40 % methanol (containing 0.1 % formic acid), the samples were centrifuged again at 13000 rpm for 15 min at 4 °C. 150 µl supernatant was transferred into a 96-well plate with pierceable cover. The whole plate was centrifuged at 2000 rpm at 4 °C for 20 min (Beun de Ronde, the Netherlands) before injection.

HPLC was performed using an Acquity I-Class UPLC system (Waters, Milford, MA, USA). Chromatographic separation was achieved at room temperature on an Alltima C18 column (2.1x150 mm, 5 µm particle size) from Grace Davison Discovery Sciences (Breda, the Netherlands). Eluent A was 100 % H2O and eluent B was 100 % methanol, both containing 0.1 % (v/v) formic acid. The elution was performed starting at 10 % B, followed by a linear gradient to 65 % B in 5 min, followed by a linear gradient to 75 % B in 1 min. Then the column was washed at 95 % B for 3 min after which was returned to the starting conditions. The flow rate was 0.250 ml/min. The injection volume was 30 µl. The HPLC system was coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization source in the positive mode. The vaporizer temperature was set at 450 °C, the Capillary Voltage at 3 kV and the source offset at 60 V. Nitrogen was used as desolvation gas at a flow of 1000 L/h, and as cone gas with a flow of 150 L/h. Argon was used as collision gas at a flow of 0.15 ml/min. Selected reaction monitoring (SRM) scans were acquired and SRM transitions were optimized individually for each molecular species (precursor / product ion [m/z]: antipyrine 189.1/104.1; Qi 325.2/184.1; 3OH-Qi 341.2/226.1). Data acquisition and processing was performed using MassLynx V4.1 software (Waters, Milford, MA, USA).

Protein determination
The remaining pellet left from the ATP assay and from the sample prepared for 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.

**Statistical analysis**

All the experiments were performed with 3-5 different rats and within each experiment all slice incubations were carried out in triplicate. The results are expressed as mean ± S.E.M of the values for the different rats. One-way ANOVA and two-way ANOVA followed with the bonferroni test as post-hoc test were used to compare multiple groups with one factor and two factors, respectively. A difference of \( p < 0.05 \) was considered as the level of significance.

**RESULTS**

**Viability of PCIS**

As shown in Fig. 1, after 5-h incubation with Qi at 50 µM, 100 µM or 200 µM, no significant difference in the intracellular ATP level was observed, compared to the corresponding control in each region (\( p > 0.05 \)), suggesting that Qi does not influence the viability of the slice during incubation. In addition, after co-incubation with the P-gp inhibitors at their highest concentration used, the ATP content of ileum slices was found to be retained at a similar level as that in control group (\( p > 0.05 \)). This indicates that the P-gp inhibitors do not influence the viability of the intestinal slices.

**The interplay between P-gp and Cyp3a with Qi as a substrate**

**The time course**

As shown in Fig. 2A, the Qi content of the slices increased rapidly in the first 30 min of incubation, whereas an equilibrium was reached after this period towards the end of incubation (120 min), indicating a balance between uptake, efflux and metabolism. However, the steady state level of Qi in the slice at this equilibrium was different in the PCIS of the different regions (duodenum > jejunum = colon > ileum). As uptake of quinidine is passive and metabolism is relatively low (see results below: Fig. 2B&C), this can be explained by the different expression of P-gp, influencing the efflux rate, assuming that the efflux is the resultant of P-gp mediated excretion and passive efflux. In ileum PCIS, where P-gp
expression is high, a lower exposure to Qi was observed than in duodenum PCIS, in which the P-gp expression is low, which is indicated by the significantly lower area under the curve (AUC) in the concentration-time curve (16300 ± 5600 pmol•min/mg protein in duodenum and 5900 ± 3600 pmol•min/mg protein in ileum in Fig. 2A).

Qi metabolism, represented by the 3OH-Qi production, showed a similar time course in the slices during 120 min of incubation, i.e. increasing in the first 30 minutes and achieving equilibrium till the end of incubation (Fig. 2B). At equilibrium level, in each region a steady state of 3OH-Qi content in the slices has been reached due to a constant Qi concentration in the slice, a constant metabolism rate and a constant efflux rate of the metabolite. Differences among regions were larger than those observed for the Qi concentration in the slice, with the same rank order. The AUC of 3OH-Qi in slices, which represents the intracellular exposure to 3OH-Qi, was calculated after the indicated incubation times. Significant differences of the exposure at 120 min between duodenum (51 ± 14 pmol•min/mg protein) and the other regions (jejunum: 16.5 ± 0.9; ileum 4.4 ± 0.4; colon 15.1 ± 3.0 pmol•min/mg protein) as well as between jejunum and ileum were found. Interestingly, these differences were larger than the regional difference of the Qi content (Fig. 2A), which is probably due to the combined effects of the differences in expression of P-gp and Cyp3a along the intestine.

The majority of 3OH-Qi was found in the incubation medium. After 120 minutes the amount of metabolite found in the slices is 2.6 – 4.9 % of the total amount of metabolites produced.
The total metabolism into 3OH-Qi, calculated as the sum of the amounts of metabolite in tissue and medium, showed a linear increase during 120 min incubation ($R^2$ for duodenum, jejunum, ileum, and colon is 0.9533, 0.9275, 0.8673, and 0.9941, respectively. Fig. 2C). This again indicates that at the achieved equilibrium after 30 min of incubation the rate of 3OH-Qi production and excretion (by passive diffusion and/or active transport) is constant. Significant differences between the slice content in duodenum and other regions were found with the rank order: duodenum > jejunum > colon > ileum.
**Fig. 2** The time course of Qi content (A), 3OH-Qi content (B) in PCIS and the total 3OH-Qi production by PCIS (C) from the different intestinal regions of the rats after incubation with 2 µM Qi. Data-points represent mean ± S.E.M (n = 3-4 rats). The AUC of intracellular Qi (A) was significantly different between duodenum and ileum slices at 120 min (p < 0.05 by two-way ANOVA and the Bonferroni test as post-hoc test), whereas the AUC of intracellular 3OH-Qi (B) was found to be: significantly different with duodenum* and significantly different with jejunum# by two-way ANOVA followed with Bonferroni test. For the total 3OH-Qi production (C), trend lines were made by linear regression. Two-way ANOVA and the Bonferroni test as post-hoc test were performed to compare the total 3OH-Qi production by each region after the indicated incubation time: *, significantly different with duodenum.

**Concentration dependency**

Based on the results above, 120 min of incubation could be considered as the steady state and thus was employed to study the influence of Qi concentration on the P-gp/Cyp3a interplay. As shown in Fig. 3A, when the Qi concentration in medium was below 5 µM, the concentration dependent increase of the Qi content in the slice was lower in jejunum and ileum than in duodenum and colon, in line with a more active efflux by P-gp in jejunum and ileum than duodenum and colon. Consequently, the 3OH-Qi content in the slices was also low in each region at that Qi concentration. Between 5 µM and 50 µM Qi, the slice Qi content increased more than proportionally with the increasing extracellular concentration of Qi, indicating that the active efflux became saturated and probably inhibited by Qi. At the same concentration range, the slice content of 3OH-Qi increased even more than the slice content of Qi, which indicates active Cyp3a metabolism and/or retention of the metabolite in the slice due to saturation or inhibition of P-gp (shown in Fig. 3B). In addition, when the Qi concentration was above 50 µM, the production of 3OH-Qi reached a plateau, possibly due to saturation of Cyp3a metabolism as shown in Fig. 3B&C. As the result of saturation of both P-gp and Cyp3a,
the Qi content in slices then increased proportionally with the extracellular concentration, while the total production of 3OH-Qi remained unchanged. Furthermore, comparison of the different intestinal regions revealed that the Qi content was significantly higher in ileum slices compared to colon slices at Qi 200 µM. In addition, significantly more 3OH-Qi was produced and retained in duodenum slices compared to other regions, whereas less was retained in ileum slices (compared to duodenum and jejunum slices at Qi 50 µM and compared to duodenum slices at Qi 100 µM). In addition, significantly more 3OH-Qi was produced in total in duodenum at Qi 20 - 100 µM and in jejunum at Qi 50 µM, compared to that in ileum and colon (Fig 3B). The plateau in the curve reflects the maximum total 3OH-Qi production of intestinal slices from different regions. The apparent $V_{\text{max}}$ of rat intestinal Qi metabolism, calculated as the average of the production rate at Qi 50 - 200 µM, is $1.53 \pm 0.43$, $1.53 \pm 0.24$, $0.42 \pm 0.07$, and $0.46 \pm 0.05$ pmol/(mg slice protein)/min in duodenum, jejunum, ileum and colon, respectively. However, due to the influence of P-gp efflux at low concentration of Qi on the Michaelis-Menten curve, the calculation of an apparent $K_m$ is not reliable.
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Fig. 3 The Qi content (A), the 3OH-Qi content (B) in PCIS, and the total 3OH-Qi production by PCIS (C) from different intestinal regions of the rat after 120 min of incubation with different concentrations of Qi. Data points represent mean ± S.E.M. (3-4 rats for 0.5-50 µM and 3 other rats were used for 0 and 50-200 µM). Two-way ANOVA and the Bonferroni test as post-hoc test were performed: *, significant (p<0.05) difference between ileum and colon (A); *, significantly different from duodenum and #, significantly different with jejunum (B&C).

Effect of P-gp inhibitors on Qi disposition

For determining the effect of P-gp inhibition on the interplay between P-gp and Cyp3a, a concentration of 2 µM Qi, at which P-gp efflux appeared to limit the intracellular concentration, was chosen. In slices that were not exposed to the inhibitors, the Qi steady state content tended to decrease from duodenum to ileum although not significantly, but the content in the ileum slices was significantly lower than in the colon slices. By co-incubation of Qi with various selective or non-selective P-gp inhibitors, the effect of the P-gp inhibition on the interplay between Cyp3a and P-gp was revealed. As shown in Fig. 4A, co-incubation with P-gp inhibitors significantly enhanced the Qi content in the slices by approximately 2 fold in jejunum and 2.5 - 3 fold in ileum, while the increase was not significant in duodenum and colon. This indicates that the compounds functioned well as P-gp inhibitors at the indicated concentration. The Qi steady state concentration in the control groups and its enhancement by P-gp inhibitors were consistent with the P-gp expression difference along the intestine.

Almost all P-gp inhibitors enhanced the 3OH-Qi content in the slice remarkably, except ketoconazole at 20 µM, in line with its strong inhibition of Cyp3a. The increase in 3OH-Qi content by CP100356 at 2 µM and PSC833 at 2 µM in PCIS of the different regions (approximately 10 fold in duodenum, 20 fold in jejunum 30 fold in ileum, and less than 10 fold in colon) was in line with the increasing P-gp expression from duodenum to ileum.
In Fig. 4C, the total 3OH-Qi production (slice content and medium content added up) was shown. An approximately 2 - 3 fold increase of total 3OH-Qi production by P-gp inhibitors with a slight regional difference was observed. This increase was much smaller compared to the increase of intracellular 3OH-Qi content (Fig. 4B). In other words, when co-incubated with P-gp inhibitors, the fold increase of 3OH-Qi in slice was always higher than that in medium, suggesting that 3OH-Qi is probably also a substrate of P-gp. Therefore, in the situation where P-gp is inhibited, more 3OH-Qi is retained in the slice leading to a larger increase than that in medium.

In all regions, the largest increase in total 3OH-Qi formation was associated with the lowest CP100356 concentration. The higher concentration of CP100356 of 5 µM and verapamil at 20 µM caused an increase in 3OH-Qi in the slices, whereas they decreased the total 3OH-Qi production. This indicates that the Cyp3a activity reflected by the total 3OH-Qi production was decreased by these compounds at these concentrations, whereas the intracellular concentration of 3OH-Qi is the resultant of 3OH-Qi production and its P-gp mediated efflux, which was decreased due to P-gp inhibition.

Discussion

Since P-gp and CYP3A work coordinately in the intestine to keep the intracellular concentration of xenobiotics low and therefore suppress their systemic uptake, it can be anticipated that influencing this interplay has major consequences on disposition of these substrates and their metabolites. In the present study, we have used rat PCIS, prepared from fresh tissue, which are expected to express both metabolic enzymes and transporters at physiological levels, to shed light on the possible consequences of inhibition of P-gp and Cyp3a on the exposure of intestinal tissue to a dual substrate of both proteins. We have found that inhibition of P-gp increased the tissue concentrations of the P-gp substrate (2 - 3.5 fold) and, more profoundly (10 - 30 fold), of its metabolites. Moreover, we have shown that the influence of P-gp inhibition on the interplay between P-gp and Cyp3a differs greatly in the different parts of the intestine, which can be explained by the differences in expression levels of these proteins. These results indicate that PCIS are an adequate model to predict DDI’s based on P-gp/CYP3A interplay in human intestine when human PCIS are used.
Fig. 4 The Qi content (A), the 3OH-Qi content (B) in PCIS, and the total 3OH-Qi production (C) by PCIS after co-incubation with P-gp inhibitors (expressed as the fold change compared to the control group of each rat intestinal region). Absolute amount of Qi content or 3OH-Qi content in the control group of each region is indicated in the insert. Data-points represent mean ± S.E.M. (n = 3 – 4 rats). Two-way ANOVA and the
Bonferroni test as post-hoc test were performed for comparison among regions and between control and effects of P-gp inhibitors: *, significantly different from control group; One-way ANOVA and the Bonferroni multiple comparison test as post-hoc test were performed to compare control groups: #, significant difference.

Although it is generally accepted that P-gp inhibition in the intestine leads to a higher systemic exposure to its substrate, the effect of P-gp inhibition on the intestinal and systemic exposure to the metabolites is under debate [22]. Watkins et al. hypothesized that P-gp may prolong the residence time of its substrates in the intestine, therefore increasing the possibility for intestinal metabolism [23]. Furthermore, P-gp removes drug CYP3A metabolites, which are often P-gp substrates, from the enterocytes, thereby reducing the chance of product inhibition. Inhibition of P-gp would therefore decrease the total intestinal metabolism. On the other hand, when P-gp limits the absorption in the proximal small intestine, the absorption is shifted to more distal, less catalytically efficient segments that contain lower amounts of CYP3A [23, 24], thus P-gp inhibition will increase metabolism. In addition, Pang et al. stated that efflux by P-gp limits metabolism due to the competition between CYP3A and P-gp within the cell and that their interplay is independent of the mean residence time of drug in the system [22]. Thus, they conclude that P-gp inhibition will increase metabolism, since the intracellular substrate available to CYP3A will be increased.

In the present study, P-gp inhibition markedly increased (1.5 - 3 fold, dependent on the region) the tissue concentration of Qi under non-saturating conditions for both P-gp and Cyp3a. Moreover, we found an even greater increase of the metabolite concentration in the tissue, which can be explained by increased metabolism due to increased intracellular availability of Qi and reduced efflux of the metabolite, which very likely also is a substrate for P-gp. These results seem to be in contrast with results of Benet’s group, who used K77 as a dual substrate of P-gp and CYP3A and GG918 as a selective P-gp inhibitor. They found a reduced extraction ratio for K77 in CYP3A4-transfected Caco-2 cells and in perfused rat intestine, when P-gp was inhibited with GG918 [25, 26]. However, it should be noticed that the extraction ratio in these studies reflect the relative change of produced metabolite over the absorbed parent drug but not the absolute production of the metabolite. Nevertheless, the amount of the absorbed parent drug was increased under P-gp inhibition in Benet’s study. Consequently, although the calculated extraction ratio was decreased, the total amount of the produced metabolite was increased like in our study. This is in line with the
findings of Pang et al., who recommended to use the fraction of the dose metabolized to represent the extent of metabolism in vitro [22]. This would better reflect the change in systemic exposure that is induced by P-gp inhibition, indicating the chance of systemic toxicity of the metabolites.

The consequences of P-gp inhibition on disposition and toxicity of a dual P-gp/CYP substrate depend on the experimental conditions (saturating or non-saturating substrate concentration conditions). When the substrate concentration is above the saturation level of P-gp, inhibition of P-gp efflux will have relatively less influence on the intracellular concentration. Furthermore, if the intracellular substrate concentration becomes higher than the saturating level of CYP3A, higher cellular substrate concentrations due to P-gp inhibition will not lead to more metabolism. Therefore, for understanding the interplay between intestinal P-gp/CYP3A and the effect of P-gp inhibition, it is essential to take into account whether the proteins are saturated at the applied substrate concentrations. For this reason we performed a concentration dependency study from which we selected 2 µM Qi as an experimental concentration at which the interplay between P-gp and CYP3A was not limited by saturation of either protein. From a clinical point of view, the relevance of DDI related to the interplay between P-gp and CYP3A is dependent on the local concentration of the drug, determined by the given dose and the solubility of the drug and by the ability of the drug to enter the enterocytes. Particularly for drugs that are P-gp and CYP3A substrates and are given at low doses and/or that are poorly soluble or permeable to cells, P-gp efflux and CYP metabolism are major determinants of intracellular and systemic uptake, indicating that inhibition of P-gp would greatly induce local and systemic exposure of the drug and its metabolites.

Regional difference is a very unique feature of the intestine because of its heterogeneity of structure and expression profile of transporters and metabolic enzymes along the intestine, leading to considerable differences in drug absorption and metabolism in various segments [27]. In the present study, we have shown that P-gp inhibition increased the tissue concentration of Qi and 3OH-Qi more dramatically in ileum than in other intestinal regions. This can be explained by the different expression levels of P-gp and CYP3A in these regions, i.e. P-gp: ileum > jejunum > duodenum ≥ colon and CYP3A: duodenum > jejunum > colon ≥ ileum [8, 28]. Whereas the efflux of Qi and 3OH-Qi is very efficient in the ileum in absence of P-gp inhibitors due to the
high P-gp expression, in the presence of P-gp inhibitors tissue concentrations of both Qi and 3OH-Qi are strongly enhanced. On the other hand, in duodenum and colon, where P-gp expression is low, P-gp inhibition has less effect on the exposure. This observation confirms that regional differences induced by DDI on the P-gp/CYP3A in the intestine are relevant to investigate and that PCIS are a good model to study this.

Many P-gp inhibitors are also inhibitors of CYP3A [29]. For this reason in the present study we also included mixed inhibitors of P-gp and CYP3A to show their effect on disposition of a dual CYP3A substrate in the intestine. Although either selective or non-selective inhibitors could increase the intracellular content of Qi (Fig. 4A), the effect on metabolism by Cyp3a was more complex and largely dependent on the selectivity of P-gp inhibitors (Fig 4 B&C). PSC833, the well-known selective P-gp inhibitor, enhanced the total production of 3OH-Qi by 2 - 4 fold by increasing the intracellular availability of Qi to Cyp3a. The other selective P-gp inhibitor, CP100356, increased total metabolism in a similar way at the lowest concentration used, whereas at higher concentrations Cyp3a inhibition became more prominent and the total metabolism decreased. Similar results were obtained with the mixed inhibitors verapamil and ketoconazole, which apart from their potency to inhibit P-gp (as was reflected by the increased cellular Qi concentrations), also had a strong inhibitory potency on CYPs at the used concentrations. Interestingly, despite of the inhibition of total metabolism that was induced by 2 and 5 µM CP100356 and 20 µM verapamil, the intracellular metabolite concentrations were still increased, due to inhibition of P-gp efflux of the metabolite by these mixed inhibitors. This was not the case with ketoconazole, which inhibited metabolism almost completely.

In conclusion, our findings indicate that rat PCIS could help to determine the effect of P-gp inhibition for pairs of substrates and inhibitors in the various parts of the intestine. If the drug concentration is below the saturation level of P-gp efflux, P-gp inhibition not only enhances the amount of the parent compound available for systemic uptake, but also of metabolites that are P-gp substrates. Moreover, the tissue concentration of the parent compound, and even more prominently of the metabolite is markedly enhanced, which may increase the risk for intestinal toxicity. To which extent intracellular concentrations are enhanced by P-gp inhibition depends on expression of both P-gp and Cyp3a and as such on the region of the intestine as well as the selectivity of P-gp inhibitors. This PCIS model, when applied to human tissue from the different regions of the intestine, is a promising ex vivo model to predict in vivo DDI’s based on P-gp/CYP3A4 interplay and possibly also for other
enzyme/transporter interplay such as between glucuronyltransferase and MRP2.

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References


