The Consequence of Drug–Drug Interactions Influencing the Interplay between P-Glycoprotein and Cytochrome P450 3a: An Ex Vivo Study with Rat Precision-Cut Intestinal Slices

Ming Li, Inge A. M. de Graaf, Sanna Siissalo,† Marina H. de Jager, Annie van Dam, and Geny M. M. Groothuis

Groningen Research Institute of Pharmacy (M.L., I.A.M.G., S.S., M.H.J. G.M.M.G.), and Interfaculty Mass Spectrometry Center (A.D.), University of Groningen, Groningen, the Netherlands

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ABSTRACT

P-glycoprotein (P-gp) and cytochrome P450 3A (CYP3A) are differentially expressed along 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 preclinical investigation. We investigated the P-gp/Cyp3a interplay and related DDIs with different P-gp inhibitors in the various regions of the rat intestine ex vivo using precision-cut intestinal slices (PCIS) with quinidine (Qi), a dual substrate of P-gp and Cyp3a, as the 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 saturated at [Qi] > 50 µM. The selective P-gp inhibitors CP100356 [N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2[1H]-yl)-6,7-dimethoxyquinazolin-2-amine] and PSC833 [valspodar, 6-[[2S,4R,6E]-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A] 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 the jejunum and ileum. Dual inhibitors of both P-gp and Cyp3a (verapamil and ketoconazole) increased the concentration of Qi in the jejunum and ileum, but less 3-hydroxy-quinidine was produced due to inhibition of Cyp3a. The results indicate that the P-gp/Cyp3a interplay depends 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.

Introduction

Efflux transporters and metabolizing enzymes play important roles in the disposition and toxicity of orally administrated drugs. Among the efflux transporters, P-glycoprotein (P-gp) plays a central role due to its high expression and broad substrate specificity (Zhou, 2008). Also cytochrome P450 3A (CYP3A), a major CYP enzyme, is highly expressed and accounts for metabolism of approximately 50% of therapeutic drugs (Martignoni et al., 2006). The influence of P-gp and CYP3A on the pharmacokinetics and pharmacodynamics of drugs has been widely studied; drug–drug interactions (DDIs) based on their activities are responsible for many unexpected/altered therapeutic effects (Guengerich, 2001; Linardi and Natalini, 2006).

In the intestine, P-gp and CYP3A are believed to work coordinately as a barrier to reduce the intracellular concentrations of xenobiotics and thus lower the intestinal absorption of drugs (Coustein et al., 2007). 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 (Sun and Pang, 2008). 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. Therefore, DDIs based on the P-gp/CYP3A interplay are of clinical importance and require preclinical investigation.

The P-gp/CYP3A interplay relies on the relative activity of P-gp and CYP3A, which is determined by their expression. Therefore, we hypothesize that the P-gp/CYP3A interplay is different among the intestinal regions because the expression of P-gp and CYP3A is different and opposite along the intestine: ileum > jejunum > duodenum ≥ colon for P-gp, and duodenum > jejunum > colon ≥ ileum for CYP3A (Englund et al., 2006; Mitschke et al., 2008; Drozdzik et al., 2014). This differential intestinal regional expression of P-gp and CYP3A is quite similar in rats and humans, so the P-gp/Cyp3a interplay in the rat is expected to be qualitatively similar to that in humans. However, as the protein abundance may show

ABBREVIATIONS: ANOVA, analysis of variance; AUC, area under the curve; CP100356, N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2[1H]-yl)-6,7-dimethoxyquinazolin-2-amine; CYP3A, cytochrome P450 3A enzyme; DDI, drug–drug interaction; GG918, N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)-ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine; K77, N-methylpirperazine-urea-phenylalanine-homophenylalanine-vinylsulfone-benzene; 3OH-Qi, 3-hydroxy-quinidine; PCIS, precision-cut intestinal slices; P-gp, P-glycoprotein; PSC833, valspodar, 6-[[2S,4R,6E]-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A; Qi, quinidine; WME, William’s medium E with Glutamax-I.
species differences, the quantitative extrapolation from rats to humans may be uncertain.

A suitable model to investigate the effect of DDIs on P-gp/CYP3A interplay in the intestine should express drug transporters and metabolizing enzymes at physiologic levels and at the same time reflect the different intestinal regions. Classic in vitro models, such as Caco-2, MDCKII, and transfected cell lines, do not meet these criteria because they have nonphysiologic expression of efflux transporters and/or metabolizing enzymes and do not represent the various intestinal regions (Artursson and Borchardt, 1997). In vivo studies on the P-gp/CYP3A interplay and the related DDIs are not practically suitable for large numbers of tests (Glaeser and Fromm, 2008). Precision-cut intestinal slices (PCIS) have been established as the ex vivo model to investigate drug metabolism, toxicity, and more recently transport in rat intestines (van de Kerkhof et al., 2006; Niu et al., 2013; Li et al., 2015). These PCIS contain the appropriate array and expression levels of metabolizing enzymes, transporters, and cofactors along the small and large intestines, hence representing a miniorgan model ex vivo and closely representing the segments of the intestine (van de Kerkhof et al., 2006). This makes PCIS highly suitable for studying DDIs based on the P-gp/CYP3A interplay.

In this study we investigated the P-gp/Cyp3a interplay in the rat intestine. We show the application of rat PCIS to study transport and metabolism simultaneously in rat intestines, characterize the intestinal P-gp/Cyp3a interplay and the DDI with P-gp inhibitors ex vivo, and investigate the consequences 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 the probe (Nielsen et al., 1999; Sziráki et al., 2011). Importantly, Qi is not an inhibitor of CYP3A/Cyp3a (McLaughlin et al., 2005), which allows selecting a concentration at which Qi only functions as a P-gp substrate (Li et al., 2015). Furthermore, the 3-hydroxylation of Qi can be used as a specific marker reaction for CYP3A4 (Nielsen et al., 1999).

To determine the effect of P-gp inhibition on the interplay, we employed CP100356 [N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroxyquinolin-2-[1H]-yl)-6,7-dimethoxyquinazolin-2-amine] and PSC833 [valspodar, 6-{2-[3,4,6(8H)]-4-methyl-2-(methylamino)-3-oxo-6-oxo octenoic acid}-7-t-valine-cyclosporin A] as selective P-gp inhibitors because of their high selectivity ratio for P-gp to CYP3A (Wandel et al., 1999; Kalugutkar et al., 2009). We included verapamil and ketoconazole, well-established inhibitors of both P-gp and CYP3A with different ratios of inhibitory potency (Wandel et al., 1999), 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 (St. Louis, MO). PSC833 and CP100356 were purchased from Tocris Bioscience (Bristol, United Kingdom). Gentamicin, William’s medium E with Glutamax-I (WME), and amphotericin B (Fungizone) solution were obtained from Invitrogen (Paisley, United Kingdom), and HEPES from MP Biomedicals (Eschwege, Germany). We obtained 3-hydroxy-quinidine (3OH-Qi) from Toronto Research Chemicals (Toronto, ON, Canada), and antipyrine was from OPG (Utrecht, the Netherlands). We purchased amphotericin B (Fungizone) solution from Invitrogen (Paisley, United Kingdom), and HEPES from MP Biomedicals (Eschwege, Germany). We obtained 3-hydroxy-quinidine (3OH-Qi) from Toronto Research Chemicals (Toronto, ON, Canada), and antipyrine was from OPG (Utrecht, the Netherlands).

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

Preparation and Incubation of Rat PCIS. After the rats had been anesthetized with isoflurane/O2, the intestine was excised and divided into four parts: duodenum, jejunum, ileum, and colon, where the 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 to 5 mg) were cut using a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL) in ice-cold oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM d-glucose, pH 7.4) as previously described elsewhere (van de Kerkhof et al., 2005; de Graaf et al., 2010). The slices from the same region were randomized and incubated individually in a 12-well culture plate (Greiner Bio-One GmbH, Kremsmunster, Austria) with 1.3 ml WME (with Glutamix-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 prewarmed 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, Burgwedel, Germany). All the slices were preincubated for 30 minutes in culture medium with or without inhibitor, and then incubated with substrate by adding its stock solution to reach the indicated final concentration, depending on the aim of the experiment.

Viability of PCIS. Intracellular ATP levels in the PCIS were evaluated to monitor the overall viability of the tissue during incubation in parallel groups (Leist et al., 1997; van de Kerkhof et al., 2006). 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 the 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 an ATP bioluminescence assay kit, as previously described elsewhere (de Graaf et al., 2010), in the supernatant after homogenization in 70% ethanol and 2 mM EDTA and centrifugation of the slice samples. The pellet was used for protein determination.

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, 15, 30, 60, and 120 minutes 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 minutes. At the end of the incubation, 1 ml of medium was collected, and the slices were rinsed in ice-cold phosphate-buffered saline for 5 minutes and stored at −20°C.

Interplay-Based DDIs with P-gp Inhibitors. Intestinal slices prepared from duodenum, jejunum, ileum, and colon were preincubated for 30 minutes in the absence or presence of an inhibitor (CP100356, PSC833, verapamil, or ketoconazole, respectively) and then incubated with 2 μM Qi for 120 minutes. Tissue and medium samples were harvested and stored as described previously. The addition of inhibitor during preincubation allowed sufficient uptake to ensure the presence of the inhibitor in the enterocytes at the moment the substrate was added. Based on literature reports (Wandel et al., 1999) and our preliminary studies, 2 μM PSC833 was chosen, whereas 0.5, 2.0, or 5.0 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 with Tandem Mass Spectrometry. To extract Qi and 3OH-Qi from the medium samples, 100 μl medium samples were mixed with 400 μl of acetonitrile (containing 10 nM antipyrine as the internal standard) + 100 μl of blank WME + 100 μl of Milli-Q water. The slice tissue samples were added to 10 nM antipyrine (as the internal standard) + 200 μl of blank
High-pressure liquid chromatography was performed using an Acquity I-Class UPLC system (Waters, Milford, MA). Chromatographic separation was achieved at room temperature on an Alltima C18 column (2.1 × 15 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 was achieved at room temperature on an Alltima C18 column (2.1 × 15 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% formic acid. The elution was performed starting at 10% B, followed by a linear gradient to 65% B for 5 minutes, followed by a linear gradient to 75% B for 1 minute. Then the column was washed at 95% B for 3 minutes, after which it was returned to the starting conditions. The flow rate was 0.250 ml/min. The injection volume was 30 μl.

The high-pressure liquid chromatography system was coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters) 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.

After thorough mixture and centrifugation at 13,000 rpm for 15 minutes at 4°C, 400 μl of supernatant was transferred into clean tubes and frozen at −80°C. The samples were then lyophilized at −20°C overnight until dryness via freeze drying (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). After reconstitution with 200 μl of 40% methanol (containing 0.1% formic acid), the samples were centrifuged again at 13,000 rpm for 15 minutes at 4°C. We transferred 150 μl of supernatant into a 96-well plate with a pierceable cover. The whole plate was centrifuged at 2000 rpm at 4°C for 20 minutes (Beun De Ronde, Abcoude, the Netherlands) before injection.

Viability of PCIS

As shown in Fig. 1, after 5 hours of incubation with Qi at 50 μM, 100 μM, or 200 μM, no statistically significant difference in the intracellular ATP level was observed when compared with 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 coincubation with the P-gp inhibitors at their highest concentration used, the ATP content of the ileum slices was retained at a similar level as that of the control group (P > 0.05). This indicates that the P-gp inhibitors do not influence the viability of the intestinal slices.

Interplay between P-gp and Cyp3a with Qi as a Substrate

Time Course.

As shown in Fig. 2A, the Qi content of the slices increased rapidly in the first 30 minutes of incubation whereas equilibrium was reached after this period toward the end of incubation (120 minutes), indicating a balance among uptake, efflux, and metabolism. However, the steady-state level of Qi in the slice at this equilibrium was different in PCIS from different regions (duodenum > jejunum = colon > ileum). As uptake of quinidine is passive and metabolism is relatively low (results as seen in Fig. 2, B and D), this can be explained by the different expression of P-gp influencing the efflux rate, assuming that the efflux results from P-gp-mediated excretion and passive efflux. In ileum PCIS, where P-gp expression is high, exposure to Qi tended to be lower than in other regions, especially in the duodenum where P-gp expression is low, as indicated by the area under the curve (AUC) at 120 minutes calculated from the concentration–time curve (16.3 ± 5.6 mmol-min/mg protein in duodenum and 5.9 ± 3.6 mmol-min/mg protein in ileum in Fig. 2C). However, the difference was not statistically significant due to the large variation in the duodenal samples.

Qi metabolism, represented by the 3OH-Qi production, showed a similar time course in the slices during 120 minutes of incubation: increasing in the first 30 minutes and reaching equilibrium until the end of incubation (Fig. 2B). At equilibrium, in each region a steady state of 3OH-Qi content in the slices was reached due to a constant Qi concentration in the slice, a constant metabolism rate, and a constant efflux rate of the metabolite. The AUC of 3OH-Qi in slices, which represents the intracellular exposure to 3OH-Qi, was calculated after the indicated incubation times. The exposure at 120 minutes to...
3OH-Qi in duodenum (51.3 ± 14.1 pmol•min/mg protein) was significantly different from that in the other regions (jejunum: 16.5 ± 0.9; ileum 4.4 ± 0.4; colon 15.1 ± 3.0 pmol•min/mg protein) (Fig. 2C). Interestingly, although the rank order of the different regions was the same, 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 was 2.6% to 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 minutes of incubation. (The R² for duodenum, jejunum, ileum, and colon was 0.95, 0.93, 0.87, and 0.99, respectively. See Fig. 2D.). This again indicates that at the achieved equilibrium after 30 minutes of incubation the rate of 3OH-Qi production and excretion (by passive diffusion and/or active transport) is constant.

Concentration Dependency. Based on the previous results, 120 minutes 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 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 the jejunum and ileum than the duodenum and colon. 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 and 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 with colon slices at Qi 200 μM. In addition, significantly more 3OH-Qi was produced and retained in duodenum slices compared with other regions, whereas less was retained in ileum slices (compared with duodenum and jejunum slices at Qi 50 μM and compared with 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 with the 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 Vₘₐₓ 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.

**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
Fig. 4. The Qi content (A), the 3OH-Qi content (B) in PCIS, and the total 3OH-Qi production (C) by PCIS after coincubation with P-gp inhibitors (expressed as the fold change compared with the control group of each rat intestinal region). The absolute Qi content or 3OH-Qi content in the control group of each region is indicated in the insert. The Qi and 3OH-Qi content in PCIS reflects the amounts in tissue whereas the total 3OH-Qi production by PCIS is the sum of the amount in tissue and medium. Data points represent the mean ± S.E.M. (n = 3 to 4 rats). Two-way ANOVA (two factors: inhibitor treatment and region) followed by the Bonferroni test as post-hoc test were performed for comparison among regions and between control and effects of P-gp inhibitors: *statistically significantly different from control group. One-way ANOVA with single factor (region) and the Bonferroni multiple comparison test as post-hoc test were performed to compare control groups: #statistically significant difference.
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 coincubation of Qi with various selective or non-selective P-gp inhibitors, the effect of the P-gp inhibition on the P-gp/Cyp3a interplay was revealed.

As shown in Fig. 4A, coincubation with P-gp inhibitors significantly enhanced the Qi content in the slices by approximately 2-fold in jejunum and 2.5-fold to 3-fold in ileum, but the increase was not significant in the 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 (Drozdzik et al., 2014).

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 <10-fold in colon) is in line with the increasing P-gp expression from the duodenum to ileum (Drozdzik et al., 2014).

Figure 4C shows the total 3OH-Qi production (slice content and medium content added up). We observed an approximately 2- to 3-fold increase of total 3OH-Qi production by P-gp inhibitors with a slight regional difference. This increase was much smaller compared with the increase of intracellular 3OH-Qi content (Fig. 4B). In other words, when coincubated with P-gp inhibitors, the fold increase of 3OH-Qi in a slice was always higher than that in the medium, suggesting that 3OH-Qi is probably also a substrate of P-gp. Therefore, in a situation where P-gp is inhibited, more 3OH-Qi is retained in the slice, leading to a larger increase than that in the 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 results from 3OH-Qi production and its P-gp-mediated efflux, which was decreased due to P-gp inhibition.

Discussion

Because P-gp and CYP3A work coordinately in the intestine to keep the intracellular concentration of xenobiotics low and thus suppress their systemic uptake, it can be anticipated that influencing this interplay has major consequences on disposition of these substrates and their metabolites. In our present study, we used rat PCIS prepared from fresh tissue, which are expected to express both metabolic enzymes and transporters at physiologic 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.

We found that inhibition of P-gp increased the tissue concentrations of the P-gp substrate Qi (2- to 3.5-fold) and, more profoundly (10- to 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 DDIs based on P-gp/CYP3A interplay, despite the fact that this model does not allow the study of vectorial transport across the intestinal wall. Although in this model the basolateral side of the enterocytes is exposed to the substrate, we assume that for many substrates the basolateral uptake is much lower than the apical uptake due to the much smaller surface area and the diffusion barrier of the submucosa and muscle layer.

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 intestinal and systemic exposure to metabolites is under debate (Pang et al., 2009). Watkins (1997) hypothesized that P-gp may prolong the residence time of its substrates, thus increasing the possibility for intestinal metabolism. Furthermore, P-gp removes 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 absorption in the proximal small intestine, the absorption is shifted to more distal, less catalytically efficient segments that contain lower amounts of CYP3A (Watkins, 1997; Dudef et al., 2013), thus P-gp inhibition will increase metabolism. In addition, Pang et al. (2009) 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. Thus, they concluded that P-gp inhibition will increase metabolism because the intracellular substrate available to CYP3A will be increased.

In our present study, P-gp inhibition markedly increased (1.5- to 3-fold, depending on the region) the tissue concentration of Qi under nonsaturating 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 also is a substrate for P-gp. These results seem to be in contrast with results of Benet’s group, using K77 [N-methylpiperazine-urea-phenylalanine-homophenylalanine-vinylsulfonof-benzene] as a dual substrate of P-gp and CYP3A and GG918 [N-[4-[2-(3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine] 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 (Cummins et al., 2002; Cummins et al., 2003). However, it should be noticed that the extraction ratio in these studies reflects the relative change of produced metabolite over the absorbed parent drug but not the absolute production of the metabolite. The amount of the absorbed parent drug was increased under P-gp inhibition in Benet’s study. Consequently, although the extraction ratio was decreased, the total amount of the metabolite was increased, as we found in our study. This is in line with the findings of Pang et al. (2009), who recommended use of the fraction of the dose metabolized to represent the extent of metabolism in vitro. 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 nonsaturating 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 P-gp/CYP3A interplay 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 a concentration at which the interplay between P-gp and CYP3A was not limited by saturation.
The clinical relevance of DDI related to the P-gp/CYP3A interplay is dependent on the local concentration of the drug and 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 to the drug and its metabolites.

Regional difference is a 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 (Bojesey et al., 2013). In our present study, P-gp inhibition increased the tissue concentration of Qi and 3OH-Qi more dramatically in the ileum than in other intestinal regions. This can be explained by the different expression levels of P-gp and CYP3A in these regions (Englund et al., 2006; Mischke et al., 2008). Whereas the efflux of Qi and 3OH-Qi is very efficient in the ileum in the 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 the 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 topics of investigation and that PCIS is a good model for their study. As these regional expression gradients of P-gp and Cyp3a/CYP3A are qualitatively similar in rat and human, the consequences of the DDI on the interplay are expected to be qualitatively similar. However, the protein abundance might be different in human and rat intestines, leading to a different extent of DDIs. A study of human intestine using human PCIS is ongoing.

Many P-gp inhibitors are also inhibitors of CYP3A (Wacher et al., 1995). 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 P-gp/CYP3A substrate in the intestine. Although both selective and nonselective 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 and C). PSC833, a selective P-gp inhibitor, enhanced the total production of 3OH-Qi by 2- to 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 by 2 μM 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. When 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 the amount 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 what extent the 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 DDIs based on P-gp/CYP3A4 interplay and possibly also for other enzyme/transporter interplay such as between glucuronolucerase transferase and multidrug resistance-associated protein 2.

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Authorship Contributions
Participated in research design: Li, de Graaf, Siissasso, Groothuis. Conducted experiments: Li, de Jager, van Dam. Contributed new reagents or analytic tools: van Dam. Performed data analysis: Li, de Jager, van Dam. Wrote or contributed to the writing of the manuscript: Li, de Graaf, Groothuis, van Dam.

References


Address correspondence to: Dr. Inge A. M. de Graaf, Pharmacokinetics, Toxicology and Targeting, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands. E-mail: i.a.m.de.graaf@rug.nl