

University of Groningen

The consequence of regional gradients of P-gp and CYP3A4 for drug-drug interactions by P-gp inhibitors and the P-gp/CYP3A4 interplay in the human intestine ex vivo

Li, Ming; de Graaf, Inge A M; van de Steeg, Evita; de Jager, Marina H; Groothuis, Geny M M

Published in:
Toxicology in Vitro

DOI:
[10.1016/j.tiv.2016.12.002](https://doi.org/10.1016/j.tiv.2016.12.002)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Li, M., de Graaf, I. A. M., van de Steeg, E., de Jager, M. H., & Groothuis, G. M. M. (2017). The consequence of regional gradients of P-gp and CYP3A4 for drug-drug interactions by P-gp inhibitors and the P-gp/CYP3A4 interplay in the human intestine ex vivo. *Toxicology in Vitro*, 40, 26-33. <https://doi.org/10.1016/j.tiv.2016.12.002>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.



The consequence of regional gradients of P-gp and CYP3A4 for drug-drug interactions by P-gp inhibitors and the P-gp/CYP3A4 interplay in the human intestine *ex vivo*

Ming Li^{a,1}, Inge A.M. de Graaf^{a,*}, Evita van de Steeg^b, Marina H. de Jager^a, Geny M.M. Groothuis^a

^a Pharmacokinetics, Toxicology & Targeting, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

^b TNO (Netherlands Organization for Applied Scientific Research), Utrechtseweg 48, 3700 AJ Zeist, The Netherlands

ARTICLE INFO

Article history:

Received 29 August 2016

Received in revised form 28 September 2016

Accepted 2 December 2016

Available online 7 December 2016

Keywords:

P-glycoprotein

CYP3A4

Interplay

Selective P-gp inhibitor

Precision-cut intestinal slice

Ex vivo

ABSTRACT

Intestinal P-gp and CYP3A4 work coordinately to reduce the intracellular concentration of drugs, and drug-drug interactions (DDIs) based on this interplay are of clinical importance and require pre-clinical investigation. Using precision-cut intestinal slices (PCIS) of human jejunum, ileum and colon, we investigated the P-gp/CYP3A4 interplay and related DDIs with P-gp inhibitors at the different regions of the human intestine with quinidine (Qi), dual substrate of P-gp and CYP3A4, as probe. 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, in line with the different P-gp expression in these intestinal regions. The selective P-gp inhibitors (CP100356 and PSC833) enhanced 3-hydroxy-quinidine (3OH-Qi) in jejunum and ileum, while dual inhibitors of P-gp and CYP3A4 (verapamil and ketoconazole) decreased the 3OH-Qi production, despite of the increased intracellular Qi concentration, due to inhibition of CYP3A4. 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, varied among the intestinal regions, probably due to the different expression of P-gp and CYP3A4, and were different from those found in rat PCIS, which may have important implications for the disposition and toxicity of drugs and their metabolites.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

P-gp and CYP3A4 are important and extensively studied members of the families of efflux transporters and metabolizing enzymes (Bojczew et al., 2013; DeGorter et al., 2012; Zhen, 2013). 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 colocalization in the intestinal epithelium and the largely overlapping substrate specificities (von Richter et al., 2004; Wachter et al., 1995; Watkins, 1997). This P-gp/CYP3A4 interplay is the most well-known

transport-metabolism interplay (Benet, 2009). Because of the broad and overlapping spectrum of inhibitors for both proteins, drug-drug interactions (DDIs) based on this interplay may occur frequently (Shi and Li, 2015; Wachter et al., 1995; Wandel et al., 1999). The studies on the P-gp/CYP3A4 interplay and related DDIs are of clinical importance, as they may result in altered bioavailability and altered intestinal toxicity due to altered intracellular exposure and require investigation in the pre-clinical phase of drug development. For instance, Choi et al. demonstrated a significant interaction between verapamil and atorvastatin in healthy volunteers (Choi et al., 2008). 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 verapamil metabolism, probably due to inhibition of CYP3A4 (Srinivas, 2008). 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. Results of mRNA and Western blot analysis show that their expression patterns are very different along the intestinal tract, with colon \leq duodenum $<$ jejunum $<$ ileum for P-gp (MacLean et al., 2008; Takano et al., 2006) and colon \leq ileum $<$ jejunum $<$ duodenum for CYP3A4 (Mitschke et al., 2008). The most recent quantification of protein abundance by LC-MS/

Abbreviations: 3OH-Qi, 3-hydroxy-quinidine; ADME-Tox, absorption, distribution, metabolism, excretion and toxicity; CP100356, N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2[1H]-yl)-6,7-dimethoxyquinazolin-2-amine; CYP, cytochrome P450; DDI(s), drug-drug interaction(s); HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid; PCIS, precision-cut intestinal slices; P-gp, P-glycoprotein; PSC833, Valsopodar, 6-[(2S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A; Qi, quinidine.

* Corresponding author.

E-mail addresses: liming@hec.cn (M. Li), i.a.m.de.graaf@rug.nl (I.A.M. de Graaf), evita.vandesteeg@tno.nl (E. van de Steeg), M.H.de.Jager@rug.nl (M.H. de Jager), G.M.M.Groothuis@rug.nl (G.M.M. Groothuis).

¹ Present address: Sunshine Lake Pharma Co. Ltd., 1 Northern Industry Road, Songshan Lake, Dongguan, Guangdong, PR China.

MS confirmed the different expression of P-gp in human intestinal regions (Drozdziak et al., 2014), while the absolute abundance of CYP3A4 protein in human intestine is not available currently. Notably, the expression of transporters and metabolizing enzymes in *in vitro* models of the human intestine, such as Caco-2 cell lines, is different from their physiological levels, thus they cannot mimic the physiological situation in different intestinal region (Artursson and Borchardt, 1997). Therefore, a proper model which could directly use the intestinal tissue from human and/or animals, and meanwhile provide efficient screening capacity, is preferable. The precision-cut intestinal slice (PCIS) model has been established to investigate intestinal drug metabolism, toxicity, and more recently transport in both human and animals (Khan et al., 2011; Li et al., 2015; Martignoni et al., 2006; van de Kerkhof et al., 2005; van de Kerkhof et al., 2006), and was recently presented as an adequate model to study transport-metabolism interplay in rat intestine (Li et al., 2016b). The aim of this study was 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. As species differences with respect to metabolizing enzymes and transporters functions between rats and human are prominent, results as obtained in the previous study with rat intestine (Li et al., 2016b) cannot be directly extrapolated to human. To the best of our knowledge, the consequences of the P-gp/CYP3A4 interplay is still under debate (Benet, 2009; Pang et al., 2009) and have not been studied before in human tissue *ex vivo*.

Since several drug transporters and metabolizing enzymes are present in PCIS, selective P-gp inhibitors are needed in order to avoid the influence of inhibition of metabolism, as many P-gp inhibitors are also cytochrome P450 inhibitors. CP100356 and PSC833 were chosen as selective P-gp inhibitors, due to their high selectivity for P-gp inhibition (Kalgutkar et al., 2009; Wandel et al., 1999), whereas verapamil and ketoconazole, two well-studied inhibitors of both P-gp and CYP3A4 (Wandel et al., 1999), were employed as dual inhibitors to investigate the consequences of DDIs under non-selective P-gp inhibition, as most of P-gp inhibitive compounds inhibit both P-gp and CYP3A4. Quinidine (Qi), which has been used clinically for >200 years as antiarrhythmic drug (Grace and Camm, 1998), 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 inhibit CYP3A4 (McLaughlin et al., 2005). It was reported to inhibit P-gp but at a relative high concentration with an IC₅₀ of 14.1 μM in MDCKII cells (Keogh and Kunta, 2006), 23.6 μM in rat PCIS (Li et al., 2015) and 35.7 μM in human PCIS (Li et al., 2016a). Furthermore, Qi is extensively metabolized by CYP3A4 into 3-hydroxy-quinidine (3OH-Qi) (Hu et al., 2004), which is considered to be the specific marker reaction for CYP3A4 activity (Nielsen et al., 1999).

2. Materials and methods

2.1. 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 William's 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).

2.2. 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

Table 1
Characteristics of the human intestine donors.

Intestine ID	Gender	Age	Region	Surgical procedure
1	M	66	Jejunum	PPPD
2	F	42	Jejunum	PPPD
3	F	62	Jejunum	PPPD
4	F	57	Jejunum	PPPD
5	M	69	Jejunum	PPPD
6	F	84	Jejunum	PPPD
7	M	76	Jejunum	PPPD
8	M	69	Jejunum	PPPD
9	F	67	Jejunum	PPPD
10	M	71	Colon	Hemicolectomy
11	F	67	Colon	Hemicolectomy
12	M	76	Ileum & colon	Hemicolectomy
13	M	64	Ileum & colon	Hemicolectomy
14	F	66	Ileum	Hemicolectomy

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.

2.3. 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 min. Precision-cut intestinal slices were prepared from the human jejunum, ileum and colon as previously described (de Graaf et al., 2010; van de Kerkhof et al., 2005). 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 (Li et al., 2015). Briefly, all the slices were first pre-incubated individually in a 12-well culture plate with 1.3 ml William's 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.

2.4. Viability of PCIS

Intracellular ATP levels in the PCIS were evaluated to monitor the overall viability of the slices during incubation in parallel groups (van de Kerkhof et al., 2006). To evaluate the toxicity of Qi and P-gp inhibitors, the ATP content in slices was measured after 3 h 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 (de Graaf et al., 2010) 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.

2.5. 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

Table 2

Multiple reaction monitoring (MRM) transitions of the P-gp and villin peptides and the corresponding internal standard (AQUA).

Protein	Peptide sequence	MW	Q1	Q3-1	Q3-2	Q3-3
P-gp	NTTGALTR	934.0	467.7	719.4	216.1	618.4
P-gp-AQUA	NTTGAL ¹³ C, ¹⁵ N]TR	950.0	471.2	726.5		
Villin	GDVFLDLGK	1076.3	538.8	805.5	658.4	545.4
Villin-AQUA	GDVFLDL ¹³ C, ¹⁵ N]GK	1083.3	542.3	812.5		

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 .

2.6. 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 erythrocytes at the moment the substrate was added. Tissue and medium samples were harvested and stored as described above. Based on literature reports (Wandel et al., 1999) and our preliminary studies, 0.5 μM CP100356 and 2 μM PSC833 were used to achieve sufficient P-gp inhibition without inhibition of CYP3A4, while verapamil (20 μM) and ketoconazole (20 μM) were used as dual inhibitors for P-gp and CYP3A4.

2.7. 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 (Li et al., 2016b). 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

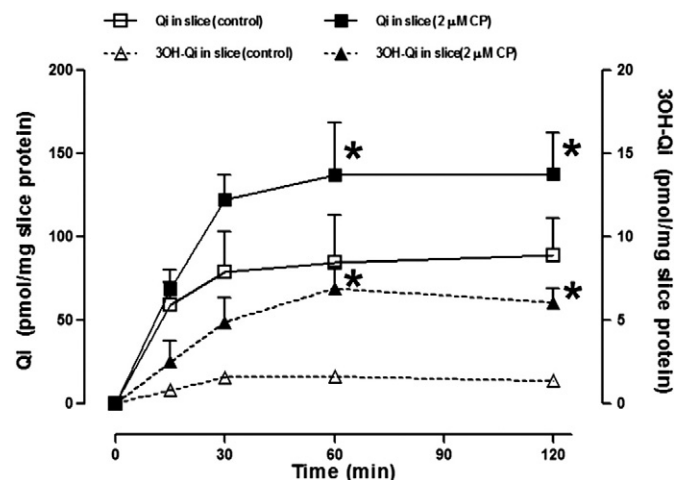


Fig. 1. The time course of Qi (left Y-axis) and 3OH-Qi (right Y-axis) content in the slice after incubation with 2 μM Qi in the absence or presence of the P-gp inhibitor CP100356 at 2 μM in PCIS from human jejunum ($n = 3$). *Significant increase of Qi and 3OH-Qi in the slice was found at 60 and 120 min by P-gp inhibition (one-way ANOVA and the Bonferroni test as post-hoc test).

centrifugation, 150 μl supernatant was transferred into a 96-well plate with a pierceable 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 (Li et al., 2016b).

2.8. 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 H_2O 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.

2.9. 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 (Bosgra et al., 2014; van de Steeg et al., 2013). 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). The peptide sequence was chosen according to the *in silico* peptide criteria defined by Kamiie et al. (2008) and is exclusively present in the target protein of interest (i.e. P-gp or villin). 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.

2.10. Statistical analysis

All the PCIS experiments were performed with tissue from 3 to 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 \pm 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.

3. Results

3.1. Viability of the human PCIS

After 3 h of incubation the PCIS from human jejunum, ileum and colon retained $>80\%$ ($p > 0.05$) of the level of intracellular ATP in fresh slices at 0 h, as shown earlier (Li et al., 2016a), indicating that the PCIS remain viable during incubation. Furthermore, the exposure to the P-gp substrate (Qi, 200 μM) or inhibitors at their highest concentration had no significant influence on the ATP content in PCIS (results not shown). These results indicate that Qi and the P-gp inhibitors did not influence the viability of PCIS during incubation.

3.2. Time course of uptake and metabolism of quinidine

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

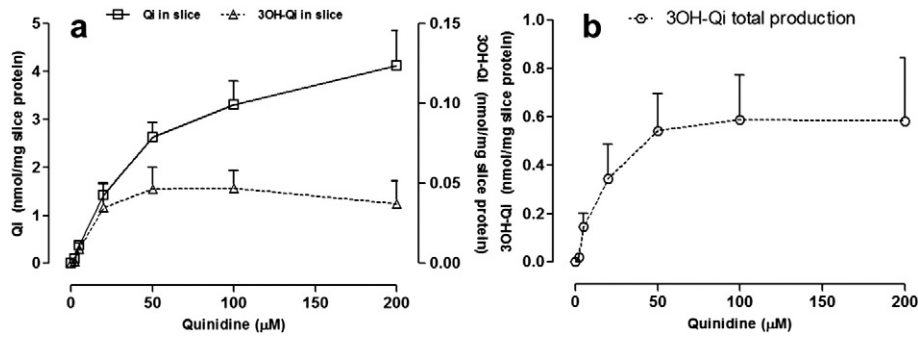


Fig. 2. The concentration dependency of Qi (left Y-axis) and 3OH-Qi (right Y-axis) content in PCIS (a) and the total 3OH-Qi production by PCIS (b) from human jejunum PCIS after 120 min of incubation with different concentrations of Qi ($n = 4$).

($p < 0.05$ 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 Figs. 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 the slice calculated after the indicated incubation times, which represent the intracellular exposure to Qi and 3OH-Qi, were enhanced from 9.1 ± 0.3 to 14.1 ± 2.6 nmol·min/mg protein and from 0.16 ± 0.01 to 0.64 ± 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 min. In addition, under P-gp inhibition, despite of the significantly 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%

3.3. Concentration dependent uptake and metabolism of quinidine

The influence of different Qi concentrations on the P-gp/CYP3A4 interplay was studied at 120 min, when equilibrium was reached. As shown in Fig. 2a, the Qi content in the slice increased in a concentration dependent manner. The 3OH-Qi content in the slices and the total 3OH-Qi production were also accordingly increasing in a concentration-dependent manner until $50 \mu\text{M}$ 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_{max} of human intestinal Qi metabolism, calculated as the average of the production rate at Qi 50–200 μM , was 4.7 ± 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

3.4. 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 $2 \mu\text{M}$ 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.

In addition, the selective P-gp inhibitors, CP100356 and PSC833, considerably enhanced the 3OH-Qi metabolite content in the slices of the jejunum (approximately 10-fold) and ileum (approximately 20-fold) but not in the slices of the colon (<1.2-fold) (Fig. 4), which is in line with the relative P-gp expression in these regions. However, for verapamil no change in metabolite content was observed, while ketoconazole clearly reduced the CYP3A4 metabolism, in line with its strong

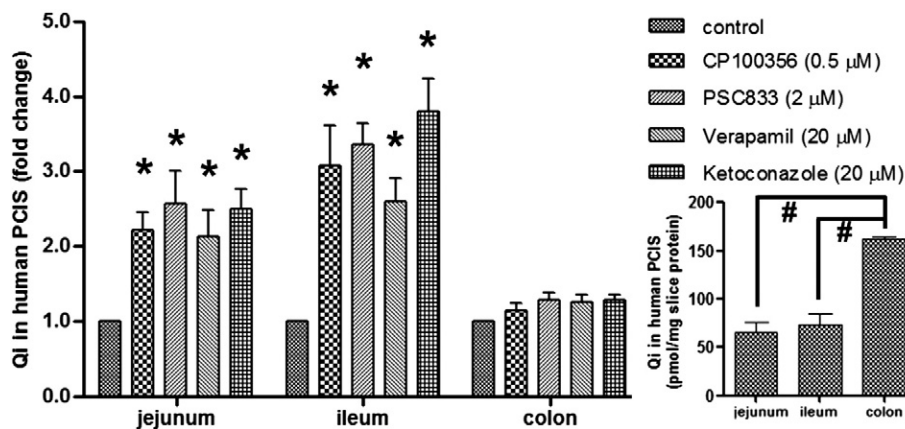


Fig. 3. Left panel: the fold change of the amount of Qi in the slices in the presence of P-gp inhibitors in different regions of human intestine. *Significant increase of Qi in the slices compared to the corresponding control group (two-way ANOVA and the Bonferroni test as post-hoc test). Right panel: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

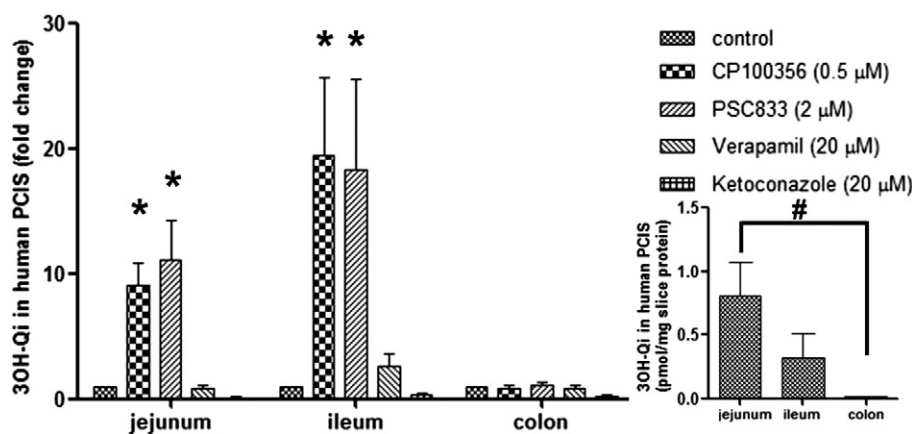


Fig. 4. Left panel: fold change in the amount of 3OH-Qi in the slices of the different regions of human intestine in the presence of P-gp inhibitors. *Significant increase of 3OH-Qi in the slices compared to the corresponding control group ($p < 0.05$ two-way ANOVA and the Bonferroni test as post-hoc test). Right panel: the absolute amount of 3OH-Qi in the slices of the control groups without P-gp inhibitors. #Significantly ($p < 0.05$) different amount of 3OH-Qi in the slices (one-way ANOVA and the Bonferroni test as post-hoc test). ($n = 6$ for jejunum, $n = 3$ for ileum, $n = 4$ for colon).

inhibition of CYP3A4. As the baseline production of metabolites is much higher in jejunum than in ileum (Fig. 4 right panel), the absolute amount of produced metabolites upon 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. We observed a 2.1–2.7-fold increase of total 3OH-Qi production by the selective P-gp inhibitors 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 to P-gp inhibition in colon is probably due to the low expression of both P-gp and CYP3A4. Similar to the changes of intracellular 3OH-Qi, the enhancement of 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 intracellular accumulated 3OH-Qi was always higher than that in the medium, indicating that 3OH-Qi is a substrate of P-gp.

3.5. Correlation between P-gp abundance and inhibition effect

The P-gp abundance was measured in the tissue of 3 human jejunum samples (No. 6, No. 7 and No. 9 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 changes of Qi and 3OH-Qi content in the individual slices and of the total metabolism upon P-gp inhibition were well in concordance with the absolute P-gp abundance.

4. Discussion

The activity of P-gp and CYP3A4 in the intestine is one of the reasons of the low oral bioavailability of 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 (Dufek et al., 2013; Peng et al., 2006; Watkins, 1997). 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 for the disposition of the shared substrates and their metabolites (Audus et al., 1996; Cummins et al., 2002).

In the present study, we demonstrated that the Qi concentration in intestinal slices was considerably influenced by P-gp efflux and that consequently the concentration of 3OH-Qi in the slices and the total 3OH-Qi production correlated with the intracellular Qi concentration (Figs. 1 and 2). Furthermore, upon applying a selective P-gp inhibition,

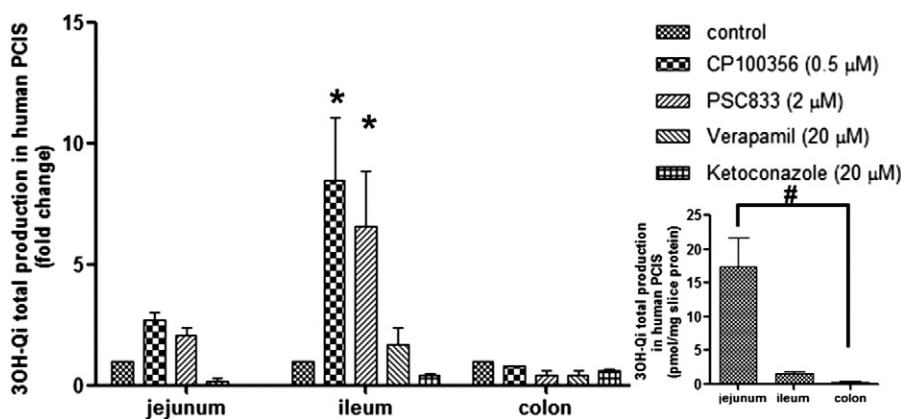


Fig. 5. Left panel: the fold change in total 3OH-Qi production in the presence of P-gp inhibitors by slices of the different regions of the human intestine. *Significant increase compared to the corresponding control group (two-way ANOVA and the Bonferroni test as post-hoc test). Right panel: the absolute amount of the total 3OH-Qi production of the control groups without P-gp inhibitors. #Significant difference in total 3OH-Qi production (one-way ANOVA and the Bonferroni test as post-hoc test). ($n = 6$ for jejunum, $n = 3$ for ileum, $n = 4$ for colon).

Table 3

The correlation between P-gp abundance and the responses to P-gp inhibition by 0.05 μ M CP100356 in human PCIS.

Intestine ID	P-gp abundance (pmol/g tissue)	Responses (fold change)		
		Qi in slice	3OH-Qi in slice	3OH-Qi total production
6	0.68	1.4	4.0	2.0
7	1.66	2.4	9.6	3.0
9	1.56	2.5	9.2	2.6

the tissue concentration of Qi increased 2.1–3.8 fold (Fig. 3) and the tissue concentration of 3OH-Qi, the metabolite of Qi, increased more remarkably 9.1–19.6 fold (Fig. 4). Moreover, the total 3OH-Qi production was enhanced by approximately 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 (The International Transporter Consortium, 2010). Furthermore, also the local tissue concentration of the metabolite can be remarkably accumulated, increasing the potential risk for intestinal toxicity. The total metabolite production also increased, which *in vivo* results in a higher metabolite formation. The net effect on the bioavailability will be the result of the increased net uptake and the increased metabolism, which depends on the affinity and maximal capacity of both P-gp and CYP3A4 for the parent drug and the metabolites. However, the concomitant inhibition of CYP3A4 activity of a non-P-gp-selective inhibitor may counteract the enhancement of intestinal CYP3A4 metabolism by P-gp inhibition.

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 (Benet, 2009), thus increasing the extent of intestinal CYP3A4 metabolism (Cummins et al., 2002; Cummins et al., 2003). In other words, P-gp inhibition will lead to a decrease of intestinal CYP3A4 metabolism. Our findings here and those earlier in rat PCIS (Li et al., 2016b), 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, increases the intestinal metabolism of loperamide when its intestinal concentration is near the apparent K_m for Cyp3a (Dufek et al., 2013). In addition, Pang et al., found that P-gp efflux limits intestinal metabolism due to the competition between P-gp and CYP3A4 for the substrate within the cell and that their interplay is independent of the mean residence time of the drug in the system (Pang et al., 2009). 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 (Cummins et al., 2003), or *in vivo* including the whole intestine (Dufek et al., 2013). Several reported *in vitro* experiments made use of cell lines (Cummins et al., 2002; Hochman et al., 2000), such as Caco-2, which overexpresses 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 (Drozdziak et al., 2014; MacLean et al., 2008). The

expression level of CYP3A is highest in duodenum and jejunum but decreases towards the ileum and is even less expressed in colon (Mitschke et al., 2008). 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 in human intestine has been studied. 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 further increased in ileum than in jejunum. The absolute amount of the metabolites formed, which depends on both the intracellular concentration of the substrate and the expression level of CYP3A4, was more increased in the jejunum (Figs. 4 and 5) compared to that in ileum and colon reflecting the differences in P-gp and CYP3A4 expression in the different intestinal regions. Almost no response to P-gp inhibition was observed in the colon, reflecting its low expression of both P-gp and CYP3A4.

The relevance of differences in P-gp and CYP3A4 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, similar to the regional differences, higher P-gp expression in the different individuals resulted in proportionally higher Qi accumulation and metabolite formation. This indicates that the intracellular Qi concentrations (even after P-gp inhibition) were probably below the K_m of CYP3A4 in these individual intestinal samples.

Human *in vivo* data on this interplay is scarce due to ethical limitations for *in vivo* experiments. Therefore, pre-clinical investigations are performed in animals. However, species differences can be a major problem in the translation of ADME results for drug candidates from animal models to human *in vivo*. The direct use of human tissue *ex vivo* to test ADME-tox properties of drug candidates could therefore offer a well-needed solution. To our best knowledge, the present study is the first P-gp/CYP3A4 interplay study on human intestinal tissue *ex vivo*. With human PCIS, we aim to get more relevant data to predict DDI in human intestine. When comparing to our earlier DDI study in rat PCIS (Li et al., 2016b), many species differences are found. The V_{max} of intestinal Qi metabolism was 4.76 ± 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 than the rat P-gp. The results support the use of human instead of animal PCIS for more adequate representation of the human situation. The limitation of the use of human PCIS lies in the limited availability of fresh human tissue, although when tissue is available, many slices can be prepared and many experiments can be performed in one tissue sample. A second limitation lies in the fact that although both basolateral and apical efflux takes place, vectorial transport cannot be measured, but this is of less importance when the effect of DDI's on local cellular concentrations of drugs and metabolites is studied.

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 a model probe 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 Qi after a therapeutic 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.

5. 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 in human intestine. We show that human PCIS are an adequate and efficient *ex vivo* model to study these effects. The results indicate that the outcome of DDIs based on P-gp/CYP3A4 interplay varies among the different intestinal regions (jejunum, ileum, and colon), probably because of the different abundance of P-gp and CYP3A4. Moreover, the DDI was shown to be dependent on the selectivity of the applied inhibitors for P-gp and CYP3A4. This may have important clinical 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 identify the risk of DDIs and inadequate pharmacotherapeutic effects of newly developed compounds.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

Ming Li is very grateful to be granted by the China Scholarship Council (CSC) (2011616035) for his PhD scholarship.

The authors thank Annie van Dam (Interfaculty Mass Spectrometry Center, University of Groningen) for the LC-MS/MS measurement of Qj and 3OH-Qj.

The authors thank Wouter Tobias van Haften, Prof. Dr. Peter Olinga (Pharmaceutical Technology and Biopharmacy, Department of Pharmacy, University of Groningen), Dr. Koert P. de Jong, Dr. Sijbrand Hofker and their colleagues (Department of Abdominal Surgery, University Medical Center Groningen) for the arrangements for obtaining human intestinal tissue.

References

- Artursson, P., Borchartd, R., 1997. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharm. Res.* 14, 1655–1658.
- Audus, K., Ng, L., Wang, W., Borchartd, R., Borchartd, R., 1996. Brain microvessel endothelial cell culture systems. In: Smith, P., Wilson, G. (Eds.), *Models for Assessing Drug Absorption and Metabolism*. Springer, US, pp. 239–258.
- Benet, L.Z., 2009. The drug transporter-metabolism alliance: uncovering and defining the interplay. *Mol. Pharm.* 6, 1631–1643.
- Bojcev, S., Almási, A., Simon, H., Perjési, P., Fischer, E., 2013. Investigation of drug metabolism in various segments of small intestine in the rat. *Acta Physiol. Hung.* 100, 115–123.
- Bosgra, S., van de Steeg, E., Vlaming, M.L., Verhoeckx, K.C., Huisman, M.T., Verwei, M., Wortelboer, H.M., 2014. Predicting carrier-mediated hepatic disposition of rosuvastatin in man by scaling from individual transfected cell-lines in vitro using absolute transporter protein quantification and PBPK modeling. *Eur. J. Pharm. Sci.* 65, 156–166.
- Choi, D.-H., Shin, W.-G., Choi, J.-S., 2008. Drug interaction between oral atorvastatin and verapamil in healthy subjects: effects of atorvastatin on the pharmacokinetics of verapamil and norverapamil. *Eur. J. Clin. Pharmacol.* 64, 445–449.
- Cummins, C.L., Jacobsen, W., Benet, L.Z., 2002. Unmasking the dynamic interplay between intestinal P-glycoprotein and CYP3A4. *J. Pharmacol. Exp. Ther.* 300, 1036–1045.
- Cummins, C.L., Salphati, L., Reid, M.J., Benet, L.Z., 2003. In vivo modulation of intestinal CYP3A metabolism by P-glycoprotein: studies using the rat single-pass intestinal perfusion model. *J. Pharmacol. Exp. Ther.* 305, 306–314.
- de Graaf, I.A.M., Olinga, P., de Jager, M.H., Merema, M.T., de Kanter, R., van de Kerkhof, E.G., Groothuis, G.M.M., 2010. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nat. Protoc.* 5, 1540–1551.
- DeGorter, M.K., Xia, C.Q., Yang, J.J., Kim, R.B., 2012. Drug transporters in drug efficacy and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 52, 249–273.
- Drozdzik, M., Gröer, C., Penski, J., Lapczuk, J., Ostrowski, M., Lai, Y., Prasad, B., Unadkat, J.D., Siegmund, W., Oswald, S., 2014. Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine. *Mol. Pharm.* 11, 3547–3555.
- Dufek, M.B., Knight, B.M., Bridges, A.S., Thakker, D.R., 2013. P-glycoprotein increases portal bioavailability of loperamide in mouse by reducing first-pass intestinal metabolism. *Drug Metab. Dispos.* 41, 642–650.
- Grace, A.A., Camm, A.J., 1998. Quinidine. *N. Engl. J. Med.* 338, 35–45.
- Hochman, J.H., Chiba, M., Nishime, J., Yamazaki, M., Lin, J.H., 2000. Influence of P-glycoprotein on the transport and metabolism of Indinavir in Caco-2 cells expressing cytochrome P-450 3A4. *J. Pharmacol. Exp. Ther.* 292, 310–318.
- Hu, M., Ling, J., Lin, H., Chen, J., Yan, Z., 2004. Use of Caco-2 cell monolayers to study drug absorption and metabolism. In: Caldwell, G. (Ed.), *Optimization in Drug Discovery*. Humana Press, pp. 19–35.
- Kalgutkar, A.S., Frederick, K.S., Chupka, J., Feng, B., Kempshall, S., Mireles, R.J., Fenner, K.S., Troutman, M.D., 2009. N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2[1H]-yl)-6,7-dimethoxyquinazolin-2-amine (CP-100,356) as a “chemical knock-out equivalent” to assess the impact of efflux transporters on oral drug absorption in the rat. *J. Pharm. Sci.* 98, 4914–4927.
- Kamiie, J., Ohtsuki, S., Iwase, R., Ohmine, K., Katsukura, Y., Yanai, K., Sekine, Y., Uchida, Y., Ito, S., Terasaki, T., 2008. Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm. Res.* 25, 1469–1483.
- Keogh, J.P., Kunta, J.R., 2006. Development, validation and utility of an in vitro technique for assessment of potential clinical drug–drug interactions involving P-glycoprotein. *Eur. J. Pharm. Sci.* 27, 543–554.
- Khan, A.A., Chow, E.C.Y., Porte, R.J., Pang, K.S., Groothuis, G.M.M., 2011. The role of lithocholic acid in the regulation of bile acid detoxication, synthesis, and transport proteins in rat and human intestine and liver slices. *Toxicol. in Vitro* 25, 80–90.
- Li, M., de Graaf, I.A.M., de Jager, M.H., Groothuis, G.M.M., 2015. Rat precision-cut intestinal slices to study P-gp activity and the potency of its inhibitors *ex vivo*. *Toxicol. in Vitro* 29, 1070–1078.
- Li, M., de Graaf, I.A.M., de Jager, M.H., Groothuis, G.M.M., 2016a. P-gp activity and inhibition in the different regions of human intestine *ex vivo*. *Biopharm. Drug Dispos.* <http://dx.doi.org/10.1002/bdd.2047>.
- Li, M., de Graaf, I.A.M., Siissalo, S., de Jager, M.H., van Dam, A., Groothuis, G.M.M., 2016b. The consequence of drug–drug interactions influencing the interplay between P-gp and Cyp3a: an *ex vivo* study with rat precision-cut intestinal slices. *Drug Metab. Dispos.* 44, 683–691.
- MacLean, C., Moenning, U., Reichel, A., Fricker, G., 2008. Closing the gaps: a full scan of the intestinal expression of P-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated protein 2 in male and female rats. *Drug Metab. Dispos.* 36, 1249–1254.
- Martignoni, M., Groothuis, G., de Kanter, R., 2006. Comparison of mouse and rat cytochrome P450-mediated metabolism in liver and intestine. *Drug Metab. Dispos.* 34, 1047–1054.
- McLaughlin, L.A., Paine, M.J.I., Kemp, C.A., Maréchal, J.-D., Flanagan, J.U., Ward, C.J., Sutcliffe, M.J., Roberts, G.C.K., Wolf, C.R., 2005. Why is quinidine an inhibitor of cytochrome P450 2D6? the role of key active-site residues in quinidine binding. *J. Biol. Chem.* 280, 38617–38624.
- Mitschke, D., Reichel, A., Fricker, G., Moenning, U., 2008. Characterization of cytochrome P450 protein expression along the entire length of the intestine of male and female rats. *Drug Metab. Dispos.* 36, 1039–1045.
- Nielsen, T.L., Rasmussen, B.B., Flinois, J.-P., Beaune, P., Brösen, K., 1999. In vitro metabolism of quinidine: the (3S)-3-hydroxylation of quinidine is a specific marker reaction for cytochrome P-4503A4 activity in human liver microsomes. *J. Pharmacol. Exp. Ther.* 289, 31–37.
- Pang, K.S., Maeng, H.-J., Fan, J., 2009. Interplay of transporters and enzymes in drug and metabolite processing. *Mol. Pharm.* 6, 1734–1755.
- Peng, S.X., Ritchie, D.M., Cousineau, M., Danser, E., DeWire, R., Floden, J., 2006. Altered oral bioavailability and pharmacokinetics of P-glycoprotein substrates by coadministration of biochanin A. *J. Pharm. Sci.* 95, 1984–1993.
- Shi, S., Li, Y., 2015. Interplay of drug-metabolizing enzymes and transporters in drug absorption and disposition. *Curr. Drug Metab.* 15, 915–941.
- Srinivas, N., 2008. Dual drug interactions via P-glycoprotein (P-gp)/cytochrome P450 (CYP3A4) interplay: recent case study of oral atorvastatin and verapamil. *Eur. J. Clin. Pharmacol.* 64, 1135–1136.
- Takano, M., Yumoto, R., Murakami, T., 2006. Expression and function of efflux drug transporters in the intestine. *Pharmacol. Ther.* 109, 137–161.
- The International Transporter Consortium, 2010. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* 9, 215–236.
- van de Kerkhof, E.G., de Graaf, I.A.M., de Jager, M.H., Meijer, D.K.F., Groothuis, G.M.M., 2005. Characterization of rat small intestinal and colon precision-cut slices as an

- in vitro system for drug metabolism and induction studies. *Drug Metab. Dispos.* 33, 1613–1620.
- van de Kerkhof, E.G., Ungell, A.-L.B., Sjöberg, Å.K., de Jager, M.H., Hilgendorf, C., de Graaf, I.A.M., Groothuis, G.M.M., 2006. Innovative methods to study human intestinal drug metabolism in vitro: precision-cut slices compared with Ussing chamber preparations. *Drug Metab. Dispos.* 34, 1893–1902.
- van de Steeg, E., Greupink, R., Schreurs, M., Nooijen, I.H.G., Verhoeckx, K.C.M., Hanemaaijer, R., Ripken, D., Monshouwer, M., Vlaming, M.L.H., DeGroot, J., Verwei, M., Russel, F.G.M., Huisman, M.T., Wortelboer, H.M., 2013. Drug-drug interactions between rosuvastatin and oral antidiabetic drugs occurring at the level of OATP1B1. *Drug Metab. Dispos.* 41, 592–601.
- von Richter, O., Burk, O., Fromm, M.F., Thon, K.P., Eichelbaum, M., Kivistö, K.T., 2004. Cytochrome P450 3A4 and P-glycoprotein expression in human small intestinal enterocytes and hepatocytes: a comparative analysis in paired tissue specimens. *Clin. Pharmacol. Ther.* 75, 172–183.
- Wacher, V.J., Wu, C.-Y., Benet, L.Z., 1995. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol. Carcinog.* 13, 129–134.
- Wandel, C., Kim, R.B., Kajiji, S., Guengerich, P., Wilkinson, G.R., Wood, A.J., 1999. P-glycoprotein and cytochrome P-450 3A inhibition: dissociation of inhibitory potencies. *Cancer Res.* 59, 3944–3948.
- Watkins, P.B., 1997. The barrier function of CYP3A4 and P-glycoprotein in the small bowel. *Adv. Drug Deliv. Rev.* 27, 161–170.
- Zhen, Y., 2013. The roles of membrane transporters on the oral drug absorption. *J. Mol. Pharm. Org. Process Res.* 1, e102.