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P-gp activity and inhibition in the different regions of human intestine *ex vivo*

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ABSTRACT: Although intestinal P-glycoprotein (P-gp) has been extensively studied *in vitro* and in animals, its activity and the consequences of P-gp inhibition for drug disposition and toxicity in humans are still difficult to accurately extrapolate from these studies. Moreover, existing *in vitro* models do not take into consideration that the intestine is heterogeneous with respect to P-gp expression. Recently, we reported rat precision-cut intestinal slices (PCIS) as a physiological *ex vivo* model to study the regional gradient of P-gp activity and inhibition. Here we extended the application of PCIS to the human intestine. For this purpose rhodamine 123 (R123) accumulation in the presence or absence of the P-gp inhibitors verapamil, cyclosporine A, quinidine, ketoconazole, PSC833 and CP100356 was measured in PCIS of human duodenum, jejunum, ileum and colon. R123 accumulation in the presence of the P-gp inhibitors appeared to be most enhanced in the ileum compared to the other regions. Moreover, the regional differences in accumulation are in line with published differences in abundance of P-gp. The rank order of the potency of the P-gp inhibitors, reflected by their IC₅₀, was comparable to that in rat PCIS. However, the increase in accumulation of the P-gp substrate R123 by the inhibitors was larger in human ileum PCIS than in rat PCIS, indicating species difference in P-gp abundance. These data show that human PCIS are an appropriate *ex vivo* model to study the activity of intestinal P-gp and predict the inhibitory effect of drugs and of transporter-mediated drug-drug interactions in the human intestine. Copyright © 2016 John Wiley & Sons, Ltd.

Key words: P-gp; Intestinal regions; precision-cut intestinal slices; inhibitors; Rhodamine123

Introduction

In the last decades, the intestine has been more and more acknowledged to be actively involved not only in the absorption [1], but also in distribution, metabolism, excretion and toxicity (ADME-Tox) of orally administered drugs [2–4]. The drug transporters that are expressed along the intestine play an important role in these processes [5,6].

Among these transporters, P-glycoprotein (P-gp), a member of the ATP-binding cassette family, which is expressed on the apical membrane of the enterocytes, plays a key role, since it exhibits a high expression level, especially in the ileum, and has a broad substrate specificity [5,7]. By actively excreting its substrates back into the gut lumen, it forms a protective barrier in the intestine against xenobiotics, limiting the absorption specifically of P-gp substrates with low passive permeability. Besides this, P-gp also influences drug metabolism in the intestine, on the one hand by controlling the accessibility of parent drugs to the metabolizing enzymes [8] and on the other hand by preventing product inhibition of the

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metabolic enzymes by active excretion of the metabolites that are often also substrates of P-gp [9]. Thus, P-gp plays a role in intestinal first-pass metabolism, as well as in excretion of endogenous compounds. Consequently, P-gp controls the intracellular concentration of drugs and their metabolites in the intestine, determining the local exposure and thus the potential local toxicity of drugs and/or metabolites [10]. Furthermore, since many drugs are P-gp substrates and/or inhibitors, many drug-drug interactions (DDIs) are known that are related to P-gp activity [11,12]. Therefore, pre-clinical investigations on the influence of P-gp efflux and P-gp inhibition in the intestine as well as on the inhibitory potency of new molecular entities (NMEs) are highly important for safety assessment for those drugs that have a low therapeutic index such as digoxin or low permeability substrates [13].

Many different models, ranging from *in vitro* to *in vivo* models, have been developed to investigate the role of P-gp on the ADME-Tox processes in the intestine. The most common models are, in order of increasing physiological complexity, inside-out membrane vesicles, P-gp-expressing cell lines, the Ussing chamber, intestinal perfusion, and *in vivo* animal experiments [14–17]. However, it should be noted that the expression and activity of P-gp may be very different in cell lines compared to the intestine and vary considerably along the different parts of the intestine [5]. In addition, the number of tests that are possible in one single animal experiment becomes smaller when the complexity of the model increases. Therefore, none of these models can serve as a fast and efficient screening method, and at the same time, fully represent the properties of the intestine with respect to the *in vivo* gradients of expression along the length of the intestine. More importantly, since these models are often derived from laboratory animals, the accuracy of the prediction for humans is uncertain due to species differences, not only in P-gp expression but also in uptake and other efflux transporters as well as in metabolic enzymes.

Recently, we reported the use of rat precision-cut intestinal slices (PCIS) as a possible screening method for P-gp inhibitory potential of drugs [18]. In PCIS, the intestinal organ structure is

maintained, and many slices can be prepared from a small piece of intestine, ensuring efficient use of animals and their organs [19]. It appeared that the inhibitory potencies of several model P-gp inhibitors could be appropriately predicted with PCIS. Furthermore, it was shown that the influence of P-gp inhibition on the tissue retention of its substrate varied greatly between the different segments of the intestine [18]. In the present study, we aimed to extend the use of this model for P-gp inhibition to human tissue. The influence of two strong and specific P-gp inhibitors, CP100356 and PSC833 [20], as well as several drugs known as P-gp inhibitors, namely quinidine, verapamil, cyclosporine A, and ketoconazole [21,22], on the retention of the P-gp substrate rhodamine 123 (R123) in the slices was investigated. The rank order of inhibitory potential of these compounds as well as the IC₅₀ values were determined and compared to data from previously reported studies. Furthermore, by using PCIS of the different segments of the human intestine (duodenum, jejunum, ileum and colon), we investigated the importance of regional differences in P-gp inhibition in the human intestine.

Materials and Methods

Chemicals

Rhodamine 123 (R123), ketoconazole, cyclosporine A, quinidine, verapamil hydrochloride, and agarose (low gelling temperature, type VII-A) were from Sigma-Aldrich (St. Louis, MO, USA). PSC833 (Valspodar, or 6-[(2S,4R,6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A) and CP100356 (N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydro-isoquinolin-2[1H]-yl)-6,7-dimethoxyquinazolin-2-amine) and CP100356 were purchased from Tocris Bioscience (Bristol, UK). Williams Medium E with glutamax-I, gentamicin, and amphotericin B (fungizone) solution were from Invitrogen (Paisley, UK). HEPES was obtained from MP Biomedicals (Heidelberg, Germany).

The stock solutions were prepared in ethanol (R123), methanol (quinidine) or DMSO (verapamil, cyclosporine A, ketoconazole, PSC 833 and CP100356).

Human intestinal tissue

Human intestinal tissue originated from surgical resections, which was approved by the Medical Ethical Committee of the University Medical Center Groningen. One piece of human duodenum tissue was obtained from a patient undergoing a pancreaticoduodenectomy by a Whipple procedure. Nine pieces of human jejunum tissue were obtained from patients undergoing pylorus-preserving pancreaticoduodenectomy (PPPD). Both ileum and colon tissue was obtained from five patients undergoing hemicolectomy. The characteristics of the donors are listed in Table 1. Due to the relative scarcity of human ileum tissue and the considerable P-gp activity in human jejunum, studies were performed with human jejunum except for those dedicated to compare the various intestinal regions. After resection, the tissue was immediately stored in ice-cold carbogenated Krebs-Henseleit buffer and transported to the laboratory within 20 minutes.

Preparation and incubation of human PCIS

Precision-cut intestinal slices were prepared from the different regions of the human intestine (duodenum, jejunum, ileum and colon) as previously described [19,23]. Briefly, after arrival, the human intestinal tissue was gently flushed with ice-cold, oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM D-glucose, pH 7.4) to remove the blood and luminal content. After the

muscle layer was removed, the mucosa layer was cut into sheets of 10×20 mm and then embedded in 3 % (*w/v*) agarose solution in 0.9 % NaCl (37 °C) in a precooled tissue embedding unit (Alabama R&D, USA). After the agarose solution had gelled, PCIS (thickness approximately 350–450 µm and wet weight about 2–4 mg) were made using a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA). The slices were incubated individually in a 12-well culture plate (Greiner Bio-One GmbH, Frickenhausen, Austria) containing 1.3 ml William's medium E in a pre-warmed cabinet (37 °C) under humidified carbogen (95 % O₂ and 5 % CO₂) as described previously [18]. All the slices were first pre-incubated for 30 min in culture medium with or without inhibitors. After the pre-incubation, incubation with the P-gp substrate was started by adding the required amount of its stock solution.

Viability of the intestinal slices

As in pilot studies a good correlation was found between morphological integrity and ATP content, intracellular ATP levels in the human PCIS were evaluated to judge the overall viability of the tissue during 3 h of incubation in parallel groups as described before [24]. Immediately after arrival of the tissue in the laboratory, three tiny pieces were cut and stored as controls of untreated tissue, which was used to evaluate the quality of the tissue, whereas three freshly prepared slices were taken as 0 h controls. Moreover, to evaluate the influence of R123 on the viability of the slices during incubation, the ATP content was measured after 3 h of incubation with or without R123 (highest concentration: 10 µM). In addition, to estimate the toxicity of the P-gp inhibitors, slices were incubated for 3 h with the P-gp inhibitors, thereafter the ATP levels were measured and compared to those in control groups. ATP samples were collected and analyzed as described before [24,25].

Kinetics of R123 uptake

After pre-incubation and the addition of R123 stock solution (final concentration 0.5 or 2 µM), the slices were harvested after 5, 15, 30, 60 and 120 minutes of incubation to study the time-course of R123 accumulation, which is the result

Table 1. Characteristics of the human intestine donors

Patient No.	Gender	Age	Region	Surgical Procedure
1	F	76	jejunum	PPPD
2	M	66	jejunum	PPPD
3	F	42	jejunum	PPPD
4	M	74	jejunum	PPPD
5	F	62	jejunum	PPPD
6	F	57	jejunum	PPPD
7	M	54	jejunum	PPPD
8	M	68	jejunum	PPPD
9	M	69	jejunum	PPPD
10	M	58	duodenum	Whipple
11	M	71	ileum & colon	hemicolectomy
12	F	67	colon	hemicolectomy
13	M	76	ileum & colon	hemicolectomy
14	M	64	ileum & colon	hemicolectomy
15	F	66	ileum & colon	hemicolectomy

Note: PPPD: pylorus-preserving pancreaticoduodenectomy

of passive influx and P-gp mediated efflux. At these relatively low concentrations, the accumulation of R123 was linear, indicating that there was no saturation of P-gp efflux. Subsequently, the concentration dependent R123 uptake was determined after 120 minutes of incubation by measuring accumulation in the PCIS after incubation with 0.5, 2 and 10 μM R123 that were chosen as a low, medium and high concentration. Slices were also incubated with these R123 concentrations in the absence or presence of CP100356 (2 μM) for 120 min. After incubation, slices were harvested and rinsed in blank PBS for 5 min and stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

Inhibition study

To determine the inhibitory effects of P-gp inhibitors, PCIS were incubated with a range of concentrations to be able to construct an inhibitory effect *vs* concentration curve, which was used to calculate the IC_{50} . The inhibitors were added during pre-incubation to ensure the presence of the inhibitors in the enterocytes at the moment the substrate was added. The incubation time of 120 minutes and R123 concentration of 2 μM were selected based on the results of the kinetics study mentioned above.

To study the differences in P-gp activity in the different intestinal regions, slices were prepared from human duodenum, jejunum, ileum and colon, whereas quinidine, CP100356 and verapamil were employed as P-gp inhibitors with final concentrations in the range of 0–200 μM , 0–5 μM , and 0–50 μM , respectively (7 concentrations were selected for each inhibitor). To compare the inhibitory potency of various P-gp inhibitors, slices were made from human jejunum and incubated with verapamil (0–50 μM), cyclosporine A (0–20 μM), quinidine (0–200 μM), ketoconazole (0–50 μM), PSC833 (0–2 μM) and CP100356 (0–5 μM), respectively (7 concentrations were selected for each inhibitor). The increase of tissue accumulation of R123 was considered to reflect P-gp inhibition.

The final concentration of the solvents in culture medium was kept lower than 1 %, which did not influence the viability of the slice (evaluated by intracellular ATP, data not shown) and

the transport activity (compared to the control group without any of these solvent, data not shown).

R123 measurement

After incubation, the slices were collected in 1.5 ml Eppendorf tubes and homogenized together with 200 μl blank WME and 400 μl acetonitrile using a Mini-BeadBeater-8 (BioSpecProducts, Bartlesville OK, USA). After centrifugation for 5 min at 13000 rpm and $4\text{ }^{\circ}\text{C}$, 150 μl supernatant was transferred into a 96-well plate, and the fluorescence of R123 was measured with a fluorescence plate reader (Molecular Devices, Wokingham, UK) (excitation/emission wavelength: 485 nm/530 nm). The R123 content in PCIS was calculated using a calibration curve prepared with homogenate from blank intestinal slices.

Protein determination

The pellets remaining after the ATP assay and the R123 measurement were dried overnight at $37\text{ }^{\circ}\text{C}$ and then 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, Munich, 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 PCIS.

Statistical analysis

All the experiments were performed with tissues from at least three different donors and within each experiment all incubations were carried out in triplicate. The average of the three individual slices of one human intestine was considered as one observation. The average $\pm$ SEM of the observations from the different individual donors is shown in the figures, unless specifically indicated otherwise. Two-tailed paired Student's *t*-test was employed for a two-group comparison, whereas one-way ANOVA and two-way ANOVA were used to compare multiple groups with one factor and two factors, respectively. $P < 0.05$ was considered as the level of significance.

In the inhibition studies, data were normalized to their control groups (100 %) and fitted into a

response *vs.* log[inhibitor] curve. The IC_{50} value of each P-gp inhibitor and at each region was calculated from the fitted curve using GraphPad Prism 5 (GraphPad Inc., USA).

Results

Viability of the intestinal slices

As shown in Figure 1, the ATP level in untreated tissue (human jejunum) was on average 3.0 ± 0.6 nmol/mg protein. After incubation for 3 h without treatment, the intracellular ATP content had slightly decreased from 4.0 ± 0.6 (fresh slices, 0 h) to 3.3 ± 0.5 nmol/mg protein ($p > 0.05$, $n=6$). Similarly, the intracellular ATP in both human ileum and colon also decreased less than 20 % (from 6.4 ± 0.5 to 5.0 ± 0.3 nmol/mg protein for ileum ($n=4$) and from 5.6 ± 1.1 to 5.4 ± 0.5 nmol/mg protein for colon ($n=4$) and no statistically significant decrease was found between 0 h and 3 h, indicating that the human PCIS were viable during incubation. In addition, the ATP content of the slices that were exposed to R123 (2 or 10 μ M) was not significantly different from control slices, indicating that R123 did not influence the viability of the slice during incubation. Furthermore, after co-incubation for 3 h with the P-gp inhibitors at the highest concentration used in the inhibition study, no significant different

Figure 1. ATP content of human jejunum (tissue), fresh PCIS (fresh slice) at 0 h, and after 3 h of incubation in the absence (control) or presence of 2 and 10 μ M of R123 ($n=6$). One-way ANOVA with Tukey's comparison as post-hoc test was employed for the comparison between every two groups. No statistical significance was found.

decrease of the slice ATP content was found compared to that in control group (data not shown), indicating that the human PCIS were also viable during the incubation with the P-gp inhibitors.

Kinetics of R123 accumulation in the slices

As shown in Figure 2a, the tissue concentration of R123 (0.5 and 2 μ M) increased linearly between 5 min and 120 min. The R123 content extrapolated to $t = 0$ min was higher than zero which is probably reflecting non-specific binding of R123 to tissue proteins. It can be calculated that the amount of R123 which is non-specifically bound is low compared to the amount of R123 in the medium and therefore does not influence the substrate concentration in the medium.

Figure 2. The time-course (a) and the concentration dependency (b) of R123 accumulation in human jejunum PCIS and the effects of P-gp inhibition (b) by CP100356 (2 μ M) (a: $n = 3-5$; b: $n = 3$ for 0.5 and 10 μ M R123, and $n = 6$ for 2 μ M R123). Two-tailed paired Student's t -test was employed for comparison between the control and CP100356 co-incubation group at each R123 concentration. * indicates $p < 0.05$.

The concentration-dependent uptake of R123 and the effects of P-gp inhibition were investigated after 120 min (Figure 2b). The R123 accumulation in human PCIS increased with increasing medium concentration of R123 of 0.5, 2 and 10 μM (16 ± 2 , 46 ± 4 , and 220 ± 55 pmol/mg protein, respectively). When P-gp efflux was inhibited with CP100356 (2 μM), the R123 accumulation in human PCIS was enhanced to 19 ± 1 , 73 ± 3 , and 379 ± 41 pmol/mg protein, respectively, which was statistically significant when the concentration of R123 was 2 μM and 10 μM . Based on these results, we can conclude that P-gp significantly contributes to the efflux of R123, and that the efflux of R123 from the tissue to the medium was not saturated up to 10 μM R123.

Inhibition study

Regional difference of P-gp inhibition. The accumulation of R123 in the absence of inhibitors in the slices of the different regions is depicted in Figure 3. A high tissue concentration of R123 in the colon compared to the ileum and jejunum was observed, whereas there was no significant difference between jejunum and ileum. Duodenum also showed a high R123 accumulation;

Figure 3. R123 accumulation after 120 min of incubation with 2 μM R123 in human PCIS from the different regions along the intestine without P-gp inhibition. The results were expressed as mean $\pm$ SEM. $n = 1$ for duodenum (data represent average $\pm$ S.D.), $n = 5$ for jejunum, $n = 4$ for ileum, and $n = 4$ for colon. One-way ANOVA with bonferroni post-hoc test was employed for the comparison between every two groups. * indicates $p < 0.05$ and significant differences were found between jejunum and colon, and between ileum and colon. Note: There was only one human duodenum tissue available during the study; therefore it was excluded from bonferroni post-hoc test.

however, as this was only measured in tissue of one donor, the result in duodenum was excluded from statistical comparison. As shown in Figure 4 and Table 2, the maximum effect of P-gp inhibition by each of the three P-gp inhibitors was higher in the ileum (on average 3.6 fold increase) compared to the jejunum (on average 2.0 fold increase). In contrast, R123 accumulation was only slightly enhanced in duodenum and colon (on average 1.3 and 1.1 fold increase, respectively), in line with the lower P-gp expression in these two regions. A similar ranking of the various intestinal regions with respect to the effect of P-gp inhibition was found with rat PCIS [18] (summarized and listed in Table 3). However, compared to rat PCIS, R123 accumulation was relatively more enhanced by P-gp inhibition in human ileum. The IC_{50} values of quinidine, CP100356 and verapamil were similar in each of the intestinal regions as expected since the IC_{50} values are mostly determined by the interaction between the transporter and the inhibitor and as such independent of the transporter abundance.

Inhibitory potency of various P-gp inhibitors. As expected, all six P-gp inhibitors enhanced the accumulation of R123 in the PCIS from jejunum and the increase was concentration-dependent, as shown in Figure 5. At maximum inhibition, R123 tissue concentration increased approximately 2.0 fold, for all six inhibitors. The potency of P-gp inhibition, estimated by the IC_{50} , varied greatly among the inhibitors as indicated by the inhibitory effect *vs* concentration curves. For CP100356 and PSC833, two potent P-gp inhibitors, an IC_{50} of 0.42 μM and 0.93 μM (Table 3), was observed respectively. The higher IC_{50} of the other classical P-gp inhibitors, quinidine (35.65 μM), cyclosporine A (1.79 μM), and verapamil (2.66 μM), indicated a lower potency of P-gp inhibition. However, the IC_{50} of ketoconazole could not be accurately calculated since no plateau in the inhibitory effect *vs* concentration curve was reached within the concentration range studied. However, assuming that the same maximum inhibition would be reached as with the other two inhibitors studied with PCIS of the same tissue (PSC833 and cyclosporine A), the IC_{50} value probably is estimated to exceed 20 μM . In Table 3 the IC_{50} values of the P-gp inhibitors are compared

Figure 4. Effects of P-gp inhibition by quinidine (a), CP100356 (b), and verapamil (c) on R123 accumulation (medium concentration of R123 at 2 μM) in human PCIS from the different regions along the intestine. The results are expressed relative to the control group (no inhibitor). $n = 1$ for duodenum (only data for quinidine and CP100356 were obtained, data represent average $\pm$ S.D.), $n = 3$ for jejunum, $n = 3$ for ileum, and $n = 4$ for colon. Note: 1: there was only one human duodenum tissue available during the study; 2: the increase by P-gp inhibitors was very low in human colon, the inhibition effect *vs* concentration curve could not be fitted to calculate the IC₅₀ value.

Table 2. The average increase in accumulation by the P-gp inhibitors in PCIS from rat and human PCIS

Regions	R123 accumulation by P-gp inhibitors (fold change)	
	Human PCIS	Rat PCIS
Duodenum	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1#
Jejunum	2.0 $\pm$ 0.1#	1.6 $\pm$ 0.1
Ileum	3.6 $\pm$ 0.6*	2.3 $\pm$ 0.2
Colon	1.1 $\pm$ 0.1#	1.2 $\pm$ 0.2#

The results are expressed as mean $\pm$ SEM. $n = 1$ for human duodenum (data represent average $\pm$ S.D.), $n = 5$ for human jejunum, $n = 4$ for human ileum, and $n = 5$ for human colon, $n = 6$ for rat. Two-way ANOVA with bonferroni post-hoc test was employed for the comparison between species and regions. * indicates $p < 0.05$ and significant difference between human and rat when comparing the same region; # indicates $p < 0.05$ and significant difference with that in ileum in the same species; \$, significant difference between jejunum and colon in the same species. Note: There was only one human duodenum tissue available during the study; therefore it was excluded from bonferroni post-hoc test.

Table 3. Comparison of IC₅₀ values of the P-gp inhibitors.

Inhibitors	IC ₅₀ (μM)	
	Human PCIS current study	Rat PCIS from [18]
CP100356	0.42	0.64
PSC833	0.93	0.60
Cyclosporine A	1.79	2.34
Verapamil	2.66	5.21
Ketoconazole	>20 ^a	8.22
Quinidine	35.65	23.57

^ano plateau could be accurately calculated for ketoconazole

with those from rat PCIS studied earlier [18]. It appears that the IC₅₀ values from human PCIS are comparable with those from rat PCIS. As expected, the rank order of P-gp inhibitors was largely the same for rat and human PCIS.

Discussion

Intestinal P-gp is an important factor in the pharmacokinetics and safety profile of drugs. The International Transporter Consortium (ITC) recommended that new drugs are screened as possible substrates or inhibitors of P-gp during drug development [13,26], which has been adopted in the guidelines of FDA (US), EMA (European), and PMDA (Japan).

Figure 5. Effect of 6 different P-gp inhibitors on R123 accumulation in human jejunum PCIS ($n = 3$). (a) quinidine, (b) cyclosporine A, (c) CP100356 (d) verapamil (e) ketoconazole (f) PSC833. The results are depicted as relative to the control group (no inhibitor). Note: Quinidine, CP100356 and verapamil were tested on the slices from the same three donors, whereas cyclosporine A, ketoconazole and PSC833 from three other donors.

Recently, we reported on the rat PCIS model as a possible model to study P-gp-mediated transport [18]. In the present study we extended this to human tissue, aiming to overcome difficulties in translation of animal data to the situation in humans. It was found that selected model inhibitors of P-gp could significantly enhance the intracellular concentration of R123, indicating the involvement of P-gp. The inhibition potency of the used inhibitors was found to be approximately the same for rat and human PCIS. This is in agreement with the data of Sugimoto et al., who compared the inhibition potency of cyclosporine A, verapamil, ketoconazole and quinidine in LLC-PK1 cells either expressing human or rat P-gp, and found similar results for P-gp of both species [22]. This indicates that only minor differences in P-gp inhibitor specificity exist between the two species.

Since the slices are prepared from fresh tissue, and only incubated for a short time for these transport studies, we believe that the abundance of transporter proteins is close to *in vivo*, in contrast to the expression in cell lines and P-gp expression systems which lack this similarity to the intestine *in vivo* [27,28]. This makes it possible to not only determine the P-gp inhibitory potency of compounds, but also to estimate the extent of the effect of P-gp inhibition on the retention of P-gp substrates in the enterocytes. We found that the extent to which the intracellular concentration of R123 could be enhanced due to P-gp inhibition, was quite different between rats and humans. In ileum, where the expression of P-gp is highest in both rats [29] and humans [30], the R123 concentration was enhanced approximately 2.3–2.5 times in rats [18] and 3.5–4.5 times in humans (present study). This observation is of clinical importance, since the extent to which the intracellular

concentration of a P-gp substrate is increased, is likely to have a direct effect on the increase of the systemic concentration of the substrate due to P-gp inhibition. Also local intestinal toxicity, being dependent on the exposure of the cellular targets, is related to the increase of intracellular substrate concentration, and as such to the extent of inhibition. This difference between rats and humans may be due to the different abundances of the P-gp protein in the two species. Unfortunately, no data on P-gp abundance in rats is available to compare with abundance in human intestine and to support this hypothesis.

According to the decision tree that was proposed by the ITC, the necessity of an *in vivo* DDI study should be assessed by determining whether the ratio of the theoretical maximum intestinal concentration $[I]_{\max, \text{gut}}$ of a NME to its inhibitory potency on P-gp efflux is higher than 10 ($[I]_{\max, \text{gut}} / (K_i \text{ or } IC_{50}) \geq 10$) [31]. Based on this assessment, a further drug interaction study can be waived or recommended. Because the absolute IC_{50} or K_i value determines the cutoff value, it is essential that the applied models not only rank the potencies of potential P-gp inhibitors correctly, but also give reliable and reproducible IC_{50} or K_i values. For the six inhibitors used in our study, both the ranking of their inhibitory potency and the "absolute" IC_{50} values differed markedly from previous reports of studies with cell lines [20,32]. This could at least partly be explained by the fact that large variations in expression of transporters and metabolizing enzymes exist between and even within the different cell lines [33,34]. Since metabolism, transport and protein binding of the inhibitor can determine the concentration of the inhibitors intracellularly at the target (P-gp), it is very likely that these apparent IC_{50} values are influenced by the different expression of protein binding, metabolizing enzymes and uptake transporters in these different models. Moreover Bentz et al. showed that the IC_{50} values found for P-gp are not only highly dependent on the cell models used (due to differences in the amount of P-gp and the rate of passive transport), but also show large inter-laboratory variations due to differences in procedures and calculation methods [35]. We noticed that particularly the ranking of ketoconazole was highly different between the different systems, as it was ranked as relatively

potent in MDCKII cells [32,36], but much less potent in Caco-2, LLC-PK1 and NIH-G185 cells [20,22,37]. This might correlate with the relative expression of P-gp and CYP3A4 in the different cells, as ketoconazole is substrate and inhibitor of both proteins [38,39]. Similarly, in the present study we found an appreciable difference in IC_{50} of ketoconazole between human and rat PCIS, which could be explained by a different metabolic rate of ketoconazole in rat and human intestine. We may speculate that prediction of IC_{50}/K_i is more accurate in the PCIS, since metabolism and transport is expected to be more similar to *in vivo*. Moreover, in PCIS, similar to the situation *in vivo*, inhibitors may bind to extracellular matrix proteins and other cells, affecting the apparent IC_{50}/K_i . Unfortunately, no apparent IC_{50} or K_i values of P-gp inhibitors are available from *in vivo* studies to compare and validate this assumption.

The intestine is a highly differentiated and heterogeneous organ, in which the structure and function are considerably different among the regions. For instance, P-gp expression is low in the proximal intestine and increases gradually to the distal end of small intestine, and is again low in the colon [5,29]. Thus, the intestine is usually divided into several compartments in physiological-based pharmacokinetics models to simulate the *in vivo* situation. However, the regional difference of the intestine is practically impossible to mimic in cell lines and thus is often ignored. The use of PCIS made from different intestinal regions can reflect these regional differences.

In the present study, we used PCIS from the different parts of the human intestine to map regional differences in P-gp activity and inhibition. R123 intracellular concentration (which is dependent on P-gp activity) was enhanced by P-gp inhibition with an increasing gradient from duodenum (1.3 fold increase on average) to jejunum (2.0 fold increase) and ileum (3.6 fold increase), while the smallest effect was induced in colon (1.1 fold increase) (Table 2). This regional difference was also found in rat PCIS (Table 2). Previously it was reported that P-gp abundance in isolated membrane fractions of the human intestine, quantified with LC-MS/MS was approximately 0.30, 0.45, 0.95, and 0.25 pmol/(mg

membrane protein) in duodenum, jejunum, ileum and colon [30]. Thus the relative P-gp abundance in the different regions normalized to the abundance in duodenum (respectively 1, 1.5, 3.2, 0.8) correlates very well with the extent of P-gp inhibition in the present study (1, 1.6, 2.8, 0.9, from duodenum to colon). In addition Harwood et al. published a meta-analysis of the relative abundance of P-gp in 8 sections of the human intestine based on mRNA and protein data showing a similar relative abundance (1, 2, 3, 1 from duodenum to colon) and they stressed the importance of this gradient in abundance for a proper prediction of oral absorption [40]. It has been shown that R123 is also a substrate for OATP1A2 [41] and OCT1 [42], as OATP1A2 is not expressed in the human intestine [30], interference with this transporter is unlikely. OCT1 is expressed in the human intestine, but as the maximal inhibitory effect of the P-gp inhibitors was similar for all inhibitors and only a few of them (quinidine and verapamil) are known to be OCT1 inhibitors, we may speculate that the influence of OCT1 inhibition is limited.

Our data also indicate that considerable effects of P-gp mediated DDIs can be particularly expected between drugs and inhibitors that are absorbed in jejunum or ileum, whereas for drugs with a good permeability which are readily absorbed in duodenum, the influence of P-gp might be low.

In conclusion, the present study shows that human PCIS are an efficient and reliable *ex vivo* model to study the activity and inhibition of intestinal P-gp in the different regions of the human intestine. The IC₅₀ and rank order of P-gp inhibitors appeared similar in rat and human PCIS. However, the extent of the inhibitory effect on intestinal P-gp was larger in human ileum, suggesting that a larger effect of P-gp inhibition on AUC (area under the curve) and local tissue concentration of P-gp substrates can be expected in human ileum than in rat ileum, which could be of clinical importance. Furthermore, with PCIS we were able to map the regional activity of P-gp in the human intestine. Since the effect of P-gp inhibition was significant in jejunum and ileum, but relatively minor in the other regions, DDIs affecting AUC and local tissue of P-gp substrates can be expected for drugs that are absorbed in these regions.

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