Chapter 2

Rat Precision-Cut Intestinal Slices to Study P-gp Activity and the Potency of its Inhibitors Ex Vivo

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Abstract

Rat Precision-Cut Intestinal Slices (PCIS) were evaluated as \textit{ex vivo} model to study the regional gradient of P-gp activity, and to investigate whether the rank order of inhibitory potency of P-gp inhibitors can be correctly reproduced in this model with more accurate IC$_{50}$ values than with current \textit{in vitro} models. PCIS were prepared from small intestine (duodenum, jejunum, ileum) and colon. Rhodamine 123 (R123) was used as P-gp substrate, while verapamil, cyclosporine A, quinidine, ketoconazole, PSC833 and CP100356 were employed as P-gp inhibitors. Increase in tissue accumulation of R123 in the presence of the inhibitors was considered as an indication of the inhibitory effect. The P-gp inhibitors increased the tissue accumulation of R123 in a concentration dependent manner. Fluorescence microscopy elucidated that this increase occurred predominantly in the enterocytes. The rank order of the corresponding IC$_{50}$ values agreed well with reported values from cell lines expressing rat P-gp. The activity of and inhibitory effects on P-gp were significantly higher in ileum compared to the other regions. These data suggest that rat PCIS are a reliable \textit{ex vivo} model to study the activity of intestinal P-gp and the inhibitory effect of drugs. PCIS have potential as \textit{ex vivo} model for the prediction of transporter-mediated drug-drug interactions.

\textbf{Keywords} P-glycoprotein; IC$_{50}$; P-gp inhibitor; precision-cut intestinal slices; \textit{ex vivo}
1. Introduction

The intestine is the main location for drug absorption after oral administration. Its role in drug metabolism, excretion and toxicity has been intensively studied and is well acknowledged in the last decade [1-3]. Although many drugs are mainly absorbed by passive diffusion, intestinal transporters are also involved in this process. Located on the apical membrane of intestinal epithelial cells, influx transporters, such as members of the solute carrier (SLC) super-family, facilitate intestinal absorption. Efflux transporters on the apical membrane of the enterocytes, mainly belonging to the ATP-binding cassette (ABC) family, excrete their substrates from the cells into the intestinal lumen, thereby lowering the intracellular concentration and decreasing intestinal absorption [4-6]. Thus, this influence of drug transporters on the oral absorption must be taken into account in drug development. Particularly the apical efflux transporters with broad substrate specificity limit absorption thereby exerting a large impact on bioavailability [7, 8]. The three major types of ABC transporters expressed on the apical side of the epithelial cells, namely multidrug resistance protein (MDR1/P-gp), multidrug resistance- associated protein 2 (MRP2) and breast cancer resistance protein (BCRP), actively excrete their substrates, including drugs, back into the gut lumen and thus form a protective barrier in the intestine [5, 7]. Among these transporters, P-gp plays a key role due to its broad substrate specificity and high expression level. Furthermore, since many drugs are P-gp substrates and/or inhibitors, drug-drug interactions (DDIs) related to P-gp activity occur frequently [9, 10]. Since P-gp activity influences both the intracellular concentration of xenobiotics and its metabolites in the intestine but also determines the systemic exposure to these compounds, prediction of inhibitory potency of (new) drugs is highly important for the safety assessment of P-gp substrates. Furthermore, it is important to take into account the regional differences in P-gp expression and activity in the various parts of the intestine, because this is an important determinant of local exposure to xenobiotics.

To study the activity of intestinal transporters, several in vitro models have been developed, such as membrane vesicles, cell lines, everted gut sac, intestinal perfusion and Ussing chamber [4, 11-13]. However, none of these models can fully represent the properties of the intestine with respect to the in vivo gradient of expression along the length of the intestine, and at the same time, serve as a fast and efficient screening method. To meet these challenges, we investigated whether the rat precision-cut
intestinal slices (PCIS) model could be used to study the activity of intestinal transporters. Precisely sliced from the intestine immediately after harvesting the tissue, PCIS represent an *ex vivo* mini-organ model which contains all types of intestinal cells in their natural environment, i.e. *in vivo* 3D structure with intact intercellular and cell-matrix interactions. They have been successfully used in the investigation of drug metabolism and toxicity in the intestine [14-20]. The localization along the length of the intestine and the regulation of some of the transporters were studied on mRNA level before [21]. To date, studies on the functional activity of these transporters *ex vivo* is limited to the study of Possidente et al. who investigated the interactions of xenobiotics with Mrp2 and P-gp in rat PCIS with calcein-AM as a probe and concluded that rat PCIS are a reliable system to study interaction of xenobiotics with these transporters [22]. However in this paper only jejunum slices were studied and information on the activity in the different regions of the intestine was lacking. In addition the cells involved in the accumulation of the substrates were not identified.

The aims of the present study were: (1) to verify the application of rat PCIS to the study the activity of rat intestinal P-gp using a P-gp-specific substrate; (2) to identify the cells involved (3) investigate the intestinal regional difference with respect to P-gp inhibition; and (4) to verify whether rat PCIS correctly reflect the inhibitory potency, i.e. rank order and IC$_{50}$ values, of different P-gp inhibitors in the rat intestine *ex vivo*;

In this study, P-gp was chosen as the targeted transporter for its key role in efflux transport in the intestine. R123 was selected as P-gp substrate, because it is only excreted by P-gp, but not metabolized by CYP-enzymes, as that might interfere with the transport results [23]. Moreover, it is easy to detect with high sensitivity due to its intensive fluorescence [24], which also makes it possible to identify by microscopy the increased accumulation of this substrate in the presence of a P-gp inhibitor. Two strong and specific third generation P-gp inhibitors, CP100356 and PSC833, were employed [25], as well as several drugs well known as classical P-gp inhibitors, namely verapamil, cyclosporine A, ketoconazole, and quinidine [26-28].

### 2. Materials and Methods

#### 2.1. Chemicals

Rhodamine 123, verapamil hydrochloride, cyclosporine A, ketoconazole, quinidine and low gelling temperature agarose (type VII-A) were purchased from Sigma-Aldrich (USA). PSC833 and CP100356 were from Tocris Bioscience (UK). Gentamicin, Williams Medium E
(WME) with glutamax-I, and amphotericin B (fungizone) solution were obtained from Invitrogen (UK). HEPES was obtained from MP Biomedicals (Germany). The stock solutions were prepared in ethanol (R123), methanol (quinidine) or DMSO (verapamil, cyclosporine A, ketoconazole, PSC833 and CP100356).

2.2. Animals
Male Wistar (HsdCpb:WU) rats weighing ca. 300 - 350 g were purchased from Harlan (Horst, the Netherlands). Rats were housed in a temperature- and humidity- controlled room on a 12/12 h light/dark cycle with free access to food and tap water, and acclimatized at least 7 days before use. All the animal experiments were approved by the animal ethical committee of the University of Groningen.

2.3. Preparation and incubation of rat precision-cut intestinal slices
Precision-cut intestinal slices were prepared from the three different regions of the rat small intestine and from the colon as previously described [15, 29]. Briefly, the rat was anesthetized by isofurane around 9 am. After the small intestine and colon were excised and put into ice-cold, oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM D-glucose, pH 7.4), the rat was sacrificed by bleeding by dissection of aorta. The whole intestine was divided into four parts: duodenum, jejunum, ileum and colon (duodenum was the segment between 2 and 12 cm and jejunum was between 20 and 40 cm from the pylorus, while ileum was the segment of the last 20 cm before the ileocecal junction and colon was the segment after the ileocecal junction). A 3-cm-segment was cut from the required part and flushed with ice-cold buffer. With one end tightly closed, it was filled with 3 % (w/v) agarose solution in 0.9 % NaCl (37 °C) and then cooled in ice-cold buffer, allowing the agarose solution to gel. Subsequently, the filled segment was embedded in 37 °C agarose solution in a precooled tissue embedding unit (Alabama R&D, USA). After the agarose solution had gelled, precision-cut slices (thickness about 300 µm and wet weight about 3-5 mg) were made using a Krumdieck tissue slicer (Alabama R&D, USA). The slices were incubated individually in a 12-well culture plate (Greiner Bio-One GmbH, Austria) with 1.3 ml WME (with glutamax-I), supplemented with D-glucose, gentamicin and amphotericin B (final concentration: 25 mM, 50 µg/ml, and 2.5 µg/ml, respectively). The culture plates were placed in plastic boxes in a pre-warmed cabinet (37 °C) under humidified carbogen (95 % O₂ and 5 % CO₂) and shaken back and forth approximately 90 times per
2.4. Viability of the intestinal slice

Intracellular ATP levels in the intestinal slices were evaluated to judge the overall viability of the tissue during incubation in parallel groups [17, 30]. After the intestine was excised from the abdomen, three tiny pieces were cut and stored as control of untreated tissue, which was used to evaluate the quality of intestine as it is the source of intestinal slices. In addition, three freshly prepared slices were stored as controls of 0 h. ATP content was measured in slices after 5 hours of incubation with or without R123 (highest concentration: 10 µM). Furthermore, to estimate the viability of the slices during incubation with R123 and inhibitors, the ATP levels of slices co-incubated with a P-gp inhibitor and/or R123 were measured and compared with the levels in control groups. All the ATP samples were snap-frozen in 1 ml of preservation solution (70% ethanol containing 2 mM EDTA, pH 10.9) in liquid N₂ immediately after sampling and then stored at -80 °C until further analysis. ATP was analyzed by using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Sciences, Germany) and by measuring the luminescence in a Spectramax micro-plate reader (Molecular Devices, USA) as described before [17, 31].

2.5. Kinetics of R123 uptake

After slices were pre-incubated for 30 min, R123 was added to the incubation medium. To study the time-course of the uptake, slices were harvested at 15, 30, 60 and 120 minutes after the addition of the substrate, respectively. Furthermore, the effect of the medium concentration of R123 on its cellular accumulation was tested by using 0.5, 2 and 10 µM R123 that were chosen as low, medium and high concentration based on literature studies. To select a suitable concentration to show the P-gp inhibition effect, slices were also incubated with these R123 concentrations in the absence or presence of CP100356 (2 µM) for 120 min. After incubation, slices were collected and rinsed in blank PBS for 5 min and stored at -20 °C until further analysis.

2.6. Inhibition study

To study the inhibitory effects of P-gp inhibitors, a range of concentrations was used to be able to depict the inhibition vs concentration curve and calculate the IC₅₀ value. Therefore, several concentrations below and above effective concentration of inhibitors were employed for each inhibitor. The inhibitors were pre-incubated for 30 minutes before adding R123 to
allow sufficient uptake to ensure the presence of the inhibitors in the enterocytes at the moment the substrate was added. The incubation time of 2 hours and R123 concentration of 2 µM were selected based on the results of kinetics study above.

To study the differences in P-gp activity in the different intestinal regions, slices were prepared from duodenum, jejunum, ileum and colon and pre-incubated for 30 min in the absence or presence of inhibitor. Quinidine and CP100356 were employed as P-gp inhibitors with final concentrations in the range of 0 - 200 µM and 0 - 5 µM, respectively. Thereafter, R123 was added into each well, followed by 2-h incubation.

To compare the inhibitory potency of various P-gp inhibitors, slices from rat ileum were 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) for 30 min, whereafter R123 was added into each well, followed by 2-h incubation. Then, tissue samples were harvested and stored as described above. The increase of tissue accumulation of R123 was considered as an indication of P-gp inhibition.

The final concentration of the solvents in culture medium was always lower than 1 %, which did not influence the viability of the slice (evaluated by intracellular ATP; data not shown) and the transport activity (compared to the control group without any of these solvent, data not shown).

2.7. Intestinal localization of R123

Fluorescence microscopy was applied to check the localization of R123 in the intestinal epithelial cells and to visualize the increase of its accumulation by the action of P-gp inhibitors. Based on the inhibition study, quinidine and CP100356 were selected to represent a weak and a strong P-gp inhibitor, respectively. The concentrations of inhibitor were selected as such, that at these concentrations >50% inhibition occurred, i.e. quinidine (50 µM) and CP100356 (2 µM). After pre-incubation for 30 min with or without P-gp inhibitor, intestinal slices made from each region were incubated with R123 (final concentration: 2 µM) for 2 h. Then the slices were washed in ice-cold PBS, embedded in Tissue-tek (3 slices in one core) and snap-frozen in isopentane placed on dry ice within 30 s. They were stored at -80 °C until sections of 8 µm were cut perpendicular to the surface of the slice in a Cryostat (Cryostat™ NX70, Thermo Scientific, USA) at -20°C. The sections were attached and dried on a glass slide, and examined unmounted under a fluorescence microscope (Leica DM4000 B, Leica Microsystems, Germany) with a +L5 filter (excitation 480/40 nm, emission 527/30 nm) by
using a DC350FX digital camera with QWin software (Leica) at a fixed exposure time (1.2 s). Thereafter, they were stained with hematoxylin and eosin as described previously [32]. Pictures were taken with light microscope (Olympus BX41, Olympus America Inc., USA) at approximately the same areas where pictures of the fluorescence were made. A comparison between these two groups of images was made to confirm the localization of R123 in the intestine in the absence or presence of P-gp inhibitors.

2.8. R123 measurement

The slices were homogenized using a Mini-BeadBeater-8 (BioSpec, USA) with 200 µl blank WME and 400 µl acetonitrile. After centrifugation for 5 min at 13000 rpm and 4 °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, USA) (excitation/emission wavelength: 485 nm / 530 nm). The intracellular content of R123 was calculated using a calibration curve prepared in a homogenate of blank intestinal slices.

2.9. Protein determination

The pellet remaining after the ATP assay and the R123 measurement was dried overnight at 37 °C and dissolved in 200 µl of 5 M NaOH for 30 min. After dilution with H₂O to 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Germany) with a calibration curve prepared from bovine serum albumin. The protein content of each slice was used to normalize for the size variation of the intestinal slices.

2.10. Statistical analysis

All the experiments were performed with at least three different rats and within each experiment all incubations were carried out in triplicate and the results were expressed as mean ± SEM. Two-tailed paired Student’s t-test was employed for a two-group comparison, while one way ANOVA and two way ANOVA were used to compare multiple groups with one factor and two factors, respectively. A difference of \( p < 0.05 \) was considered as the level of significance.

In the inhibition study with various concentrations of inhibitors, data were normalized to their control groups (100 %) and fitted into a log [inhibitor] vs. response curve. The IC₅₀ value of each P-gp inhibitor was calculated using GraphPadPrism 5 (GraphPad Inc., USA).
3. Results

3.1. Viability of the intestinal slices

As shown in Fig. 1, the ATP level in the control of untreated tissue was 2.7 nmol/mg protein whereas the slices directly after preparation had an ATP content of 4.0 nmol/mg protein. After incubation for 5 h, the ATP content decreased slightly, but not significantly by 12.5% compared to that in the fresh slices (0 h), until 3.5 nmol/mg protein. This level is comparable with that in the untreated tissue and also with that in the fresh slices (P > 0.05), indicating that the intestinal slices are viable during a 5-hour incubation. In addition, no significant difference in ATP content of the slice was observed when R123 (2 or 10 µM) was added, suggesting that R123 did not influence the viability of the slice during incubation. Furthermore, the slice ATP content after co-incubation for 3 h or 5 h with the P-gp inhibitors at the highest concentration used for the inhibition study with or without R123 was found to be retained at a similar level as that in control group (data not shown). This indicates that the intestinal slices are also viable during the incubation with P-gp inhibitors.

![Fig. 1 ATP content of the intestine (tissue untreated), fresh PCIS at the start of the incubation, and PCIS after 5 h of incubation in the absence or presence of 2 and 10 µM of R123 (n=7 to 9). Data are presented as Mean ± SEM of each group. 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.]

3.2. Intestinal localization of R123

In jejunum and ileum, the R123 content in the epithelial cells was very low, resulting in a faint,
nearly invisible line, contrary to the bottom of villi, crypt cells and muscle layer where the staining was more intense (Fig. 2 c&d). This is probably caused by the active efflux of R123 by P-gp which is mainly expressed in the mature epithelial cells on the villi, resulting in a gradient of P-gp expression along the villus axis. In line with this, when P-gp was inhibited by quinidine or CP100356, the fluorescence intensity of the epithelium lining clearly increased. As shown in Fig. 2, the increase of fluorescence intensity of R123 in the jejunum and ileum due to the presence of the P-gp inhibitor occurred predominantly in the villi and not in the other intestinal structures. In contrast in duodenum and colon, the effect of P-gp inhibition was less noticeable, as there was only a small increase of the fluorescence intensity, which probably indicates that much less P-gp is expressed in these segments.

3.3. R123 uptake assays

3.3.1. Time-dependent uptake
To determine the time-course of R123 uptake during incubation, intestinal slices from ileum were incubated with 0.5 or 2 µM R123 for 15, 30, 60 or 120 min. A relatively low R123 concentration was chosen in order to avoid saturation of P-gp efflux. As shown in Fig. 3a, the R123 uptake in both concentrations was linear between 15 min and 120 min. The extrapolated tissue concentration at t=0 min is probably due to the non-specific tissue binding of R123.

3.3.2. Concentration-dependent uptake and the effect of P-gp inhibition
Based on the results above, we investigated the effects of P-gp inhibition on accumulation of R123 at different concentrations during 120 min of incubation (shown in Fig. 3b). When incubated with R123 (0.5, 2 or 10 µM), the tissue accumulation of R123 increased linearly with the R123 concentration (38.7 ± 10.3, 154.0 ± 33.9, and 797.4 ± 272.7 pmol/mg protein, respectively). When P-gp efflux was blocked with CP100356, the R123 accumulation in the tissue was enhanced to 77.9 ± 14.4, 330.0 ± 51.4, and 1556.8 ± 189.5 pmol/mg protein, respectively, which was significant when the concentration of R123 was 2 µM. These data indicate that the R123 uptake in PCIS is concentration dependent and its efflux by P-gp is not saturated up to 10 µM R123.
Rat Precision-Cut Intestinal Slices to Study P-gp Activity and the Potency of its Inhibitors Ex Vivo

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<th>R123 (2 μM)</th>
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**Duodenum**

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**Jejunum**

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**Ileum**

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Fig. 2 The localization of R123 in the intestine (A, duodenum; B, jejunum; C, ileum; D, colon) (left: no inhibitor; middle: quinidine 50 µM; right: CP100356 2 µM). Pictures were taken at approximately the same areas of the sections using the fluorescent microscope (upper panels) and the light microscope (lower panels). Scale bar = 100 µm.

Fig. 3a (left panel) The time course of R123 uptake in PCIS at 0.5 and 2 µM R123 (n=3). Results were expressed as mean ± SEM. The fluorescence value of blank slice, which was very low and negligible, was included by the calibration curves using an homogenate of untreated slices.

Fig. 3b (right panel) Concentration-dependent uptake of R123 and the effect of P-gp inhibition by CP100356 (2 µM) (n=3). Results were expressed as mean ± SEM. Two-tailed paired Student’s t-test was employed for comparison between the control and CP100356 group at each R123 concentration. * indicates p<0.05.

3.4. Inhibition study

3.4.1. Regional difference of P-gp inhibition
The regional difference of P-gp inhibition was studied with intestinal slices made from duodenum, jejunum, ileum and colon co-incubated with R123 and at a range of concentrations of quinidine or CP100356. As shown in **Fig. 4a&b**, the maximum effect of P-gp inhibition by quinidine was highest in the ileum (approximately 2.5-fold increase) compared to the duodenum, and jejunum (approximately 1.5-fold increase). No effect (CP100356) or highly variable (quinidine) P-gp inhibition was shown for colon. However, the IC$_{50}$ values of quinidine and CP100356 were similar for each of the intestinal regions.

When the absolute concentrations of R123 (without inhibitor) in the slices of the different regions are depicted it becomes clear that P-gp is differently expressed in the different regions resulting in a lower tissue concentration of R123 in the ileum (high efflux activity) and a high tissue concentration in the colon (low efflux activity) (**Fig. 5**). After P-gp efflux was inhibited by either CP100356 or quinidine, R123 accumulation in ileum slices was significantly enhanced, eliminating the regional differences in accumulation of the substrate caused by the efflux pump.

![Fig. 4 a&b](https://example.com/figure4.png) Effects of P-gp inhibition by quinidine and CP 100356 on R123 accumulation in PCIS of the different regions along the intestine. The results are expressed as mean ± SEM which are relative to the control group (no inhibitor), n=3 for duodenum, jejunum and colon, n=6 for ileum (data for ileum include the data for CP 100356 and quinidine obtained in the experiments where the six inhibitors were tested). NI, no inhibition.

**3.4.2. Inhibitory potencies of various P-gp inhibitors**

The inhibitory potency of six P-gp inhibitors on R123 accumulation in ileum was compared using various concentrations of the inhibitors. As shown in **Fig. 6**, all six inhibitors enhanced R123 accumulation in the PCIS concentration-dependently, indicating their inhibition of P-gp.

An approximately 2.5 to 3.0-fold increase of R123 accumulation at maximum inhibition was observed. The inhibition response vs. concentration curves were used to calculate the
corresponding IC\textsubscript{50} values. The results clearly indicate that the potency of P-gp inhibition, estimated by the IC\textsubscript{50} value, varied greatly. PSC833 and CP100356, two potent P-gp inhibitors, showed strong inhibition with IC\textsubscript{50} values at 0.60 µM and 0.66 µM, respectively. The other four classical P-gp inhibitors showed a lower potency of P-gp inhibition: quinidine (23.57 µM), cyclosporine A (2.34 µM), verapamil (5.21 µM), and ketoconazole (8.22 µM) (Fig. 6 and Table 1). The IC\textsubscript{50} values of these inhibitors found for rat PCIS were generally higher than those reported before for rat P-gp expressed in the LLC-PK1 cell line [27]. However, the rank order was the same. In contrast, the rank order of inhibition efficacy was different for rat and human P-gp, with respect to the rank order of ketoconazole and verapamil, indicating species differences in P-gp substrate specificity [25].

![Fig. 5](image)

**Fig. 5** The influence of P-gp inhibition by quinidine and CP100356 on R123 accumulation in PCIS of the different intestinal regions. The results were expressed as mean ± SEM. N = 3 for duodenum, jejunum and colon, n = 6 for ileum (data for ileum include the data for CP 100356 and quinidine obtained in the experiments where the six inhibitors were tested). Two-way ANOVA with Bonferroni post-hoc test was used to compare multiple groups with two factors, i.e. region and inhibitor treatment. In the control group, R123 accumulation was found significantly different between ileum and colon, however, with P-gp inhibition, no regional difference in R123 accumulation was found. Besides, there was a significant increase of R123 accumulation after P-gp inhibition in ileum slices. ns = not significant; ** significant with \(p<0.01\), *** significant with \(p<0.001\)

4. Discussion

Intestinal transporters are major determinants of the absorption, and thus of the efficacy and safety profile of drugs and in addition to that, determine the local concentration in the intestinal epithelium. There is no doubt that they need to be taken into account in drug development, as reviewed by the international transporter
consortium [33]. In this review Caco-2 or P-gp-overexpressing polarized epithelial cell lines were recommended as methods to screen for drug candidates that are substrates and/or inhibitors of transporters, especially P-gp. However, it is generally known that cell lines do not express the transporters at physiological levels nor can represent the different regions of the intestine [34, 35]. The data of the present study show that the PCIS model can be a better and more physiologically relevant alternative for these cell lines.

Fig. 6 Effect of 6 different P-gp inhibitors on R123 accumulation in PCIS. The results are expressed as mean ± SEM, which was relative to the control group (no inhibitor), n=3 for each inhibitor.

In the past decade we and others have developed PCIS as ex vivo model to study drug metabolism and toxicity [15-20]. Because PCIS are prepared from fresh tissue, the expression of transporters and metabolic enzymes is at physiological levels. Furthermore it is possible to make >100 slices from each region of the intestine within one experiment, thus, the number of animals needed is largely reduced, which is in good accordance with the 3Rs (Reduction, Replacement and Refinement). In previous studies we have shown that PCIS remain viable and retain their metabolic activity for at least 8 hours of culturing, which is substantially longer than other intact tissue preparations [15-17]. However the only study on transport up to now was the study of Possidente et al. showing the applicability of PCIS for transport studies, but without
information on gradients along the length of the intestine and on the cellular localization of the accumulated substrates.

Table 1 Comparison of IC$_{50}$ values of the tested P-gp inhibitors and their rank order among different models and species

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<tr>
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Ref.1: [27]; Ref.2: [25]; Ref.3: [36]; Ref.4: [28]; Ref.5: [26]

In the present study, we used R123 to study P-gp function along the length of the intestine and to investigate inhibitory potency of drugs and to identify the cells involved in the transport of the P-gp substrate. The accumulation of R123 in PCIS was linear until 120 min, and concentration dependent (Fig. 3 a&b). Furthermore, inhibition of P-gp resulted in a further increase of the intracellular concentration. This increase was mainly due to increase in R123 content of the enterocytes as shown by fluorescence microscopy (Fig. 2). Interestingly, it appeared that the estimated average concentration of R123 in the tissue was 6-8 times higher than the medium concentration after incubation for 2 h. This might be explained by the high affinity of R123 to mitochondria and/or the formation of R123 micelles [37]. By this intracellular compartmentalization of R123 the cytoplasmic concentration apparently remains low, ensuring a driving force for passive diffusion into the cell even when the efflux of R123 is inhibited.

The intestine is a heterogeneous organ in which differences in structure and function are prominent among its regions. Each of the intestinal efflux transporters has its unique expression profile along the intestine [5, 38]. However, it has not been studied up
to now to what extent these expression levels also represent functional differences. A discrepancy between protein expression and transport activity can be anticipated as it has been demonstrated that the transporters are not solely localized at the plasma membrane but also intracellularly, such as in intracellular membrane vesicles [39]. In the present study, we used slices from the different intestinal regions to study the regional differences in P-gp activity. It should be noted that in vivo there are also other physiological differences between the different intestinal regions that were not accounted for in the ex vivo studies. For example the pH in the rat intestinal lumen is different in the different regions, but was kept the same (pH 7.4) in the ex vivo incubations. Nevertheless, the increasing gradient of P-gp activity from jejunum to ileum and lower activity in colon was consistent with the reported P-gp expression profile. The highest inhibition effect was found in the ileum compared to the duodenum and jejunum (Fig. 4). Similarly, using the everted gut sac model, Yumoto et al. observed a higher transport rate by P-gp in the ileum than in duodenum and jejunum with a similar fold difference as found in the current study [40]. The inhibition effect was lowest in colon, which is consistent with the lower P-gp abundance in human colon quantified by mass spectrometry-based targeted proteomics [41]. The maximum inhibition effect of the P-gp inhibitors was approximately 2 to 2.5 fold in ileum and 1.5 fold in the other regions, which is similar to that in vivo [42, 43]. Fenner et al. summarized data from 123 clinical trials where P-gp inhibitors were co-administrated with digoxin, and found that the AUC and C_max increased not more than 2 fold [42]. In contrast, with cell lines a 6 - 10 fold decrease in efflux rate was commonly found [44]. This suggests that the slices ex vivo mimic the in vivo situation better than the cell lines.

The inhibition potency of P-gp inhibitors, usually estimated by the IC_{50} value, varies in different in vitro models, mainly because of the different intracellular concentrations of the inhibitors and the different P-gp substrates used as well as different calculation methods [45]. On the other hand, the rank order of inhibition ability is expected to be the same among models and different substrates [28, 46]. In the present study, the rank order of the IC_{50} values of the used inhibitors agreed very well with published data on the K_i of these compounds for rat P-gp. Compared with human cell data, we found a difference in the rank order of verapamil and quinidine, which is probably due to a species difference, which is in line with the data of Suzuyama et al. who found different relative IC_{50} values for different animal species using quinidine and verapamil as P-gp inhibitors [47]. Since the PCIS technique can be applied to human intestine as well [17, 29], it will be very interesting to study the
inhibitory potency of inhibitors in human PCIS prepared from human intestine. These experiments are currently in progress.

Nevertheless, the absolute IC\textsubscript{50} value is important for correct prediction of DDIs and according to the FDA, if the ratio of the systemic steady-state C\textsubscript{max}, [I] to its \textit{in vitro} inhibitory potency (K\textsubscript{i} or IC\textsubscript{50}), is \( \geq 0.1 \) a clinical trial should be performed to indicate the risk of a DDI for potential P-gp inhibitors. Since the absolute IC\textsubscript{50} value is guiding for these predictions, it is important that \textit{in vitro} systems do not only rank the potency of possible P-gp inhibitors correctly. If the \textit{in vitro} IC\textsubscript{50} values are lower than \textit{in vivo}, the predicted values of the ratios of [I]/IC\textsubscript{50} are too high. This might be one of the reasons why many false positive interactions were found [48].

The IC\textsubscript{50} values of P-gp inhibitors found in our study were substantially higher than found with cell lines and P-gp overexpressing cells (Table 1). The cause of the different IC\textsubscript{50} values could be a different concentration at the target (the transporter) in the different systems. Since target concentrations of substrate and inhibitors are influenced by metabolism and influx/efflux in the target cells it can be expected that IC\textsubscript{50} values are closer to \textit{in vivo} when predicted in a system where transporters and drug metabolizing enzymes are expressed at physiological levels and original tissue/organ structure is preserved, such as in PCIS. Therefore predictions for DDIs made with the PCIS are possibly more relevant for the \textit{in vivo} situation in the intestine than those obtained with cell lines.

In the FDA guidelines for DDI prediction, the influence of regional differences in the intestine and the overexpression of P-gp in Caco-2 or other P-gp-expressing polarized epithelial cell lines are ignored. However, the influence of inhibition on the absorption and as such on the AUC of drugs is largely determined by P-gp expression and regional differences are therefore relevant. As the majority of drugs is already largely absorbed in duodenum and jejunum where P-gp expression is low, systems that highly express P-gp, such as Caco-2 cells might overestimate the effect of P-gp inhibitors on AUC \textit{in vivo}. Furthermore, it should be noted that the local exposure to a P-gp substrate in the presence of an inhibitor is largely determined by the local P-gp expression itself. The use of PCIS of different intestinal regions can reflect these regional differences, and thus represent the physiology of the intestine to a larger extent.

In conclusion, the present study shows that rat PCIS are a reliable and efficient \textit{ex vivo} model to study the activity of intestinal transporters, and to assess the inhibitory potential of new drugs in each of the different regions of the intestine. In addition we confirmed for the first time that in the PCIS this inhibitory action is exerted in the enterocytes specifically. The IC\textsubscript{50} values of P-gp inhibitors measured \textit{ex vivo} in PCIS show the same rank order as those
measured in cell lines, but the absolute values are much higher and expected to be closer to the \textit{in vivo} values. Therefore, the PCIS model has the potential to make a better prediction of transporter-mediated DDIs in the intestine and the toxicity derived from this type of DDIs. Furthermore, its future application in human intestinal slices could overcome difficulties in extrapolation from experimental animals to humans.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

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