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A New Intestinal Cell Culture Model To Discriminate the Relative Contribution of P-gp and BCRP on Transport of Substrates Such as Imatinib

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Abstract: P-glycoprotein (P-gp/MDR1/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) play an important role in transport of a wide variety of endogenous compounds, drugs and toxins. Transport of some drugs, for example the tyrosine kinase inhibitor imatinib, is influenced by both P-gp and BCRP. Establishing an intestinal Caco-2 cell culture model with specific knock-downs of P-gp and BCRP and double knock-down of both proteins, we aimed to elucidate the impact of each transporter on transport of imatinib. Stable single and double knock-downs of P-gp and BCRP were obtained by RNA interference (RNAi). Transporter expression was measured on RNA and protein level using real-time RT-PCR and Western blot, respectively. Functional activity was quantified by transport of specific substrates across Caco-2 cells. MDR1 and BCRP mRNA expression was reduced to 75% and 90% compared to wild-type control in single MDR1- and BCRP-knock-down clones, respectively. In double knock-down clones, MDR1 expression decreased to 95% and BCRP expression to 80%. Functional activity of P-gp and BCRP was diminished as transport of the P-gp-specific substrate ^{3}H-digoxin and the BCRP-specific substrate ^{14}C-PhIP was augmented in the opposite direction, when the respective transporter was knocked down. Similar effects were observed by chemical inhibition of the respective transporter. Bidirectional transport studies with ^{14}C-imatinib revealed an abrogation of asymmetric transport when P-gp was knocked down, either in single or double knock-down clones compared to wild-type cells. This was not observed in single BCRP-knock-down clones. In conclusion, this newly established cell system with single and concomitant knock-down of P-gp and BCRP can be used to quantify the specific partial impact of the transporters on transport of substrates that are transported by both proteins. For imatinib transport, the contribution of P-gp seems to be more important compared to BCRP in this Caco-2 cell system.

Keywords: P-glycoprotein; MDR1; ABCB1; BCRP; ABCG2; silencing; knock-down; Caco-2; intestine; transport; imatinib

Introduction

Gastrointestinal drug absorption is of major importance in drug development, since oral drug formulations are widely used. Drug solubility and permeability are physicochemical properties that influence drug absorption. Lipinski et al. proposed four important properties determining drug solubility and passive intestinal permeability:[1] octanol:water partition coefficient (log *P*), numbers of H-bond donors and acceptors and molecular weight. In recent years, intestinal metabolism and active efflux transport have been recognized in addition to the above factors as major determinants of

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drug bioavailability.[2] Active efflux into the intestinal lumen and/or metabolism in the intestinal cells can drastically lower the bioavailability of orally administered drugs. Active efflux is mainly mediated by proteins of the ATP-binding-cassette-transporters superfamily. Among these transporters, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) play an important role. Their expression is high at several excretory sites, predominantly in epithelial and endothelial cells. They are expressed at the apical brush-border membrane of the intestine and form a barrier for various drugs and toxins.[3−5]

Expression of P-gp and BCRP along the intestinal tract was described by several authors.[6−12] In human intestine, P-gp shows increasing expression along the small intestine with highest expression in the ileum and smaller expression in the colon.[6,8] In contrast to P-gp, BCRP expression is less varying. We found that BCRP mRNA expression is continuously decreasing from duodenum to ileum and colon,[7] while others stated a small increase from duodenum to ileum followed by a decrease in expression in the colon.[6] A full scan of the rat intestinal tract revealed increasing P-gp and Bcrp protein expression from proximal to distal small intestine with a decrease of BCRP expression in the colon.[11]

Both transporters have broad and partly overlapping substrate specificity. Simultaneous administration of drugs that are substrates of P-gp and BCRP could therefore lead to drug−drug interactions at the level of efflux-transport.

Imatinib has recently been described as a substrate of both human P-gp and BCRP.[13,14] It is an antiproliferative drug and is primarily used in Philadelphia chromosome positive chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) or gastrointestinal stromal (GITS) tumors. Imatinib is a tyrosine kinase inhibitor with activity against ABL as well as the BCR-ABL fusion protein, and the receptor tyrosine kinases DDR c-KIT and PDGFR.[15−18] Imatinib is orally taken, and its bioavailability is about 98%.[19] However, varying plasma area under the curve (AUC) has been described, which was attributed to metabolism and transport processes.[20,21]

The selective impact of P-gp and BCRP on imatinib transport in an intestinal cell culture model is not yet

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published. A suitable *in vitro* model for intestinal transport processes is Caco-2 cells.[22] The lack of defined high specific chemical inhibitors for P-gp and BCRP makes it difficult to detect the quantitative contribution of each transporter on transport of substrates that are transported by both transporters. The new technology RNA interference (RNAi) or silencing is a sequence specific knock-down of genes, which is based on the target specific recognition of the mRNA (mRNA) and its following cleavage. A selective and stable knock-down of P-gp and BCRP in Caco-2 cells would represent a good model for elucidating intestinal transport processes involving P-gp and BCRP.

Therefore, in this work, we aimed to establish an intestinal cell system with a stable and selective knock-down of the ABC transporters P-glycoprotein (P-gp, MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2), as well as of both transporters simultaneously. Using this system, the contribution of P-gp and BCRP to transport of substrates of both transporters such as imatinib can be elucidated. We aimed to investigate how imatinib is transported in the situation, where one or both of these transporters are inhibited and to quantify the specific impact of each transporter on drug transport.

Materials and Methods

Materials. ^{14}C-Imatinib mesylate (52.74 mCi/mmol) was a kind gift obtained from Dr. Hilmar Schiller and Dr. Albrecht Glaenzel (Novartis Pharma AG, Basel, Switzerland). ^{14}C-PhIP (10 mCi/mmol) was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). ^{14}C-Sucrose (588 mCi/mmol) and ^{3}H-digoxin (9 Ci/mmol) were from Perkin-Elmer (Schwerzenbach, Switzerland). Lucifer Yellow (LY) (Sigma-Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) (Invitrogen, Basel, Switzerland). Prazosin and verapamil (Sigma-Aldrich) were dissolved in DMSO and water, respectively.

Cell Culture. The Caco-2 cell line was purchased from ATCC (Manassas, VA). Caco-2 cells were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, 50 μg/mL gentamycin (Invitrogen AG, Basel, Switzerland). Caco-2 clones additionally were treated with Geneticin (Invitrogen) 1 mg/mL as selection antibiotic. Cells were maintained in a humidified 37 °C incubator with 5% carbon dioxide in air atmosphere.

Reverse Transcription, Real-Time PCR. The procedure for measuring mRNA expression in Caco-2 cells was described before.[23] Briefly, cells were cultured as described above, total RNA was extracted and 0.5 μg was reverse transcribed. Real-time PCR analysis (TaqMan) was carried out on a 7900HT sequence detection system (Applied Biosystems) using qPCR Mastermix Plus from Eurogentec (Seraing, Belgium). Sequences of primers and probes are listed in Table 1. Expression of the respective gene was normalized to the expression of GAPDH. For absolute quantification external standard curves were used. Standards were gene-specific cDNA fragments that cover the TaqMan primer/probe area, and they were generated by PCR. Sequences of the corresponding primers are shown in Table 2.

Table 1. Primers and Probes for TaqMan Analysis

gene	probe
MDR1	5′-AAGCTGTCAAGGAAGCCAATGCCTATGACTT-3′
BCRP	5′-CCATTGCATCTTGGCTGTCATGGCTT-3′
GAPDH	5′-CGCCTGGTCACCAGGGCTGC-3′

gene	forward primer (FW) and reverse primer (REV)
MDR1	FW: 5′-CTGTATTGTTTGCCACCACGA-3′
	REV: 5′-AGGGTGTCAAATTTATGAGGCAGT-3′
BCRP	FW: 5′-CAGGTCTGTTGGTCAATCTCACA-3′
	REV: 5′-TCCATATCGTGGAATGCTGAAG-3′
GAPDH	FW: 5′-GGTGAAGGTCGGAGTCAACG-3′
	REV: 5′-ACCATGTAGTTGAGGTCAATGAAGG-3′

Western Blot. Protein extraction and Western blot were performed as described before.[23] 50 μg of total protein extract was separated on a 6.5% acrylamide gel and transferred to a nitrocellulose membrane. Antibody staining for P-gp and BCRP was performed with C219 antibody (Alexis Corporation, Lausen, Switzerland) and BXP-21 antibody (Alexis), respectively, in a dilution of 1:100 for both. Beta-actin mouse monoclonal antibody (abcam, Cambridge, U.K.) served as loading control and was used in a dilution of 1:1000.

Transport Assay. Caco-2 cells were seeded on type I collagen 5 μg/cm^2 (Becton Dickinson, Basel, Switzerland) precoated Transwell filters (polycarbonate 12 well, pore size 0.4 μm or polyester [clear], 12 well, pore size 0.4 μm) (Corning, Baar, Switzerland) in a density of 660 000 cells/cm^2. In one subset of experiments always the same filters were used. Cells were cultured for 3 days in DMEM high glucose (see Cell Culture). After 2 or 3 days medium was changed. Cells were incubated with Intestinal Epithelial Differentiation Medium (BD) and 0.1% MITO+ Serum Extender (BD). On the fourth or fifth day, medium was exchanged again, and on the fifth or sixth day, the transport

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Table 2. Primers for cDNA Standards

gene	forward primer	reverse primer
MDR1	5′-ACAGTCCAGCTGATGCAGAGG-3′	5′-CTTATCCAGAGCCACCTGAAC-3′
BCRP	5′-TTTCAGCCGTGGAACTCTTT-3′	5′-TGAGTCCTGGGCAGAAGTTT-3′
GAPDH	5′-ACATCGCTCAGAACACCTATGG-3′	5′-GCATGGACTGTGGTCATGAGTC-3′

assay was performed. This cell culturing procedure is based on the experiences of Yamashita et al.[24]

Before starting the assay, TEER (transepithelial electrical resistance) values were measured using the Millipore Millicell ERS (Volketswil, Switzerland) and the assay was started when the TEER values were at least 600 Ω cm^2. Next, both sides of the Transwell were washed 3 times with prewarmed HBSS (Gibco) supplemented with 1 mM pyruvate and 10 mM HEPES adjusted to a pH of 7.4 (HBSS-P). A preincubation with or without the respective inhibitor dissolved in HBSS-P on the apical side (0.5 mL) and HBSS-P on the basolateral side (1.5 mL) was performed for 15 min. For apical-to-basolateral (AB) transport, at time $t = 0$ substrates and tightness marker with or without inhibitor were given to the apical donor chamber and on the basolateral acceptor compartment HBSS-P was added. Transport was performed at 37 °C and 120 rpm over 2 h. Samples were taken after 10, 20, 40, 60, 90, and 120 min out of the basolateral acceptor compartment. Volume was replaced with HBSS-P. For basolateral-to-apical (BA) transport, the basolateral side acted as donor chamber and the apical side was the acceptor chamber. Thus, substrate and tightness marker were added to the basolateral side and samples were taken out of the apical side. However, if an inhibitor was used, it was added to the apical acceptor compartment. Samples were taken at indicated time points out of the acceptor compartment (apical), and volume was replaced.

After 2 h, transport buffer was removed and the Transwell was placed on ice. Both sides of the Transwell were washed 3 times with ice-cold HBSS-P. Transwell filters were cut out to detect the remaining substance in the cells. Insta Gel plus scintillation liquid was added to the radioactive samples, and analysis was performed on a scintillation counter (Packard TriCarb2000, Canberra Packard S.A.). To confirm tightness of the monolayer ^{14}C-sucrose (0.6 μCi/mL) (Perkin-Elmer, Schwerzenbach, Switzerland) or Lucifer Yellow (20 μM) (Sigma Aldrich, Buchs, Switzerland) was used. LY was measured at 428 nm excitation and 536 nm emission on a fluorescent reader (HTS 7000 Plus Bioassay Reader, Perkin-Elmer Ltd., Buckinghamshire, U.K.).

The apparent permeability coefficient (P_{app}) was calculated for tightness markers in order to determine the tightness of the monolayers.[25]

Design of shRNA. ABCB1 (MDR1) (Acc. No. NM_000927) and ABCG2 (BCRP) (Acc. No. NM_004827) small hairpin RNA (shRNA) sequences were designed using the small interfering RNA (siRNA) design algorithm of Whitehead Institute for Biomedical Research (http://jura.wi.mit.edu/bioc/siRNAext/home.php). The proposed sequences were aligned using the NCBI Blast tool. The iRNAi program (http://mekentosj.com/irnai/) was used to obtain shRNA sequences which fit into the pSUPER vector between the restriction sites *Bgl*II and *Hin*dIII.[26] As negative control, a nontargeting but functional sequence was taken. This sequence was described earlier by Taniguchi et al.[27] Sequences for the shRNA are listed in Table 3.

Plasmid Preparation. pSUPER-vector containing the neomycin resistance gene was digested with the restriction enzymes *Bgl*II and *Hin*dIII (Fermentas Inc., Ontario, Canada). The previously described designed shRNA sequences were synthesized by Invitrogen (Invitrogen AG, Basel, Switzerland). The forward and reverse strands were annealed and then ligated into the pSUPER-vector (OligoEngine, Seattle, WA) at the *Bgl*II and *Hin*dIII sites with T4 DNA ligase (Fermentas).

These obtained plasmids were subsequently transformed into Sure2 Supercompetent cells (Stratagene, La Jolla, CA) following the manufacturer's protocol, and sequences were analyzed by Microsynth (Microsynth AG, Balgach, Switzerland) using T3 seq primer. Plasmid isolation was performed with the Nucleobond AX500 Kit (Macherey-Nagel, Oensingen, Switzerland) according to the manufacturer's protocol.

Transfection and Generation of Stable Caco-2-Silencing Clones. Caco-2 cells were transfected using Lipofectamine 2000 (Invitrogen). One day before transfection, Caco-2 cells were seeded on 6-well plates in DMEM (see Cell Culture). Transfection was started when cells reached a confluence of ~60%. Transfection was performed following the manufacturer's protocol using 4 μg of plasmid DNA and 10 μL of Lipofectamine 2000. In the case of simultaneous silencing of MDR1 and BCRP, equal amounts of both plasmids, siMDR1 and siBCRP, were mixed and transfected into the cells. After 72 h, cells were diluted 1:40 into 10 mm Petri dishes and the selection antibiotic Geneticin (Invitrogen) was added in a concentration of 1.5 mg/mL. Every 3−4 days DMEM with Geneticin was replaced. After 2 weeks clones

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Table 3. Sequences for shRNA

	siMDR1
target sequence	5′-TTGGAGGATTATGAAGCTAAATT-3′
sequence for pSUPER	FW: 5′-GATCCCC **GGAGGATTATGAAGCTAAA** TTCAAGAGA **TTTAGCTTCATAATCCTCC** TTTTTGGAAA-3 REV: 5′-AGCTTTTCCAAAAA **GGAGGATTATGAAGCTAAA** TCTCTTGAA **TTTAGCTTCATAATCCTCC** GGG-3
	siBCRP
target sequence	5′-GTGGAGGCAAATCTTCGTTATTA-3′
sequence for pSUPER	FW: 5′-GATCCCC **GGAGGCAAATCTTCGTTAT** TTCAAGAGA **ATAACGAAGATTTGCCTCC** TTTTTGGAAA-3′ REV: 5′-AGCTTTTCCAAAAA **GGAGGCAAATCTTCGTTAT** TCTCTTGAA **ATAACGAAGATTTGCCTCC** GGG-3′
	si-Scrambled
target sequence	5′-GCTATCGCTACGTGTAAGT-3′
reference	Taniguchi, H. *Development.* **2006**, *133*, 1923−31, Supporting Information
sequence for pSUPER	FW: 5′-GATCCCC **GCTATCGCTACGTGTAAGT** TTCAAGAGA **ACTTACACGTAGCGATAGC** TTTTTGGAAA-3′ RV: 5′-AGCTTTTCCAAAAA **GCTATCGCTACGTGTAAGT** TCTCTTGAA **ACTTACACGTAGCGATAGC** GGG-3′

were picked using Scienceware Cloning discs (Sigma-Aldrich) and further cultured with a Geneticin concentration of 1 mg/mL.

Each clone was passaged over 6 passages, and quantitative mRNA expression analysis of the gene of interest was performed. The clone with the lowest expression level of the gene of interest was chosen for further experiments. Evaluation of the silencing clones was performed on the mRNA level using real time RT-PCR (Taqman), on the protein level by Western blot analysis and on the activity level using different functional assays such as uptake, efflux and transport assay. During cell culture, the stable knock-down of MDR1 and BCRP was tested regularly during different passage numbers on mRNA expression level without loss of their specific properties. Experiments were carried out at passage 57−79.

Statistics. Groups were compared to control group by analysis of variance (ANOVA). If this analysis revealed significant differences and more than one treatment group was included in the analysis, pairwise comparisons of treatment groups with the control group were performed subsequently using Dunnett's two-sided multicomparison test. All tests were performed using the SPSS for Windows software (version 15.0). The level of significance was $P < 0.05$.

Results

Evaluation of the Caco-2 Knock-Down Model. *Knock-Down of MDR1 and BCRP.* In the single siMDR1-transfection silencing of MDR1 mRNA expression resulted in a reduction of 75% compared to wild-type cells (Figure 1A). In order to measure unspecific off-target effects, BCRP mRNA expression was additionally measured in the siMDR1 knock-down clone. BCRP expression resulted in a slightly reduced mRNA expression compared to wild-type cells (Figure 1B).

With the single BCRP transfection, siBCRP, BCRP silencing effects on mRNA expression were achieved, leading to a reduction of 90% compared to wild-type (Figure 1B). To quantify possible off-target effects of the siBCRP knock-down clone, we measured MDR1 mRNA expression, in addition. The siBCRP knock-down had no significant effect on MDR1 mRNA expression compared to wild-type cells (Figure 1A).

In the co-silencing clone (*cosil*), where both siMDR1 and siBCRP were transfected at the same time, silencing of MDR1 and BCRP mRNA expression resulted in a reduction of 95% and 80% compared to wild-type, respectively (Figures 1A and 1B).

In order to measure unwished side effects of transfection, a negative control, *si-scrambled,* with a nontargeting sequence was generated and mRNA expression of both genes of interest, MDR1 and BCRP, was measured. The negative control exhibited no significant effects on MDR1 and BCRP mRNA expression compared to wild-type (Figures 1A and 1B).

Western blot analysis was performed to confirm the knock-down of the respective transporters on protein level. As expected, in the MDR1-silenced clones (siMDR1 and cosil), no P-gp protein expression was detectable, while in the wild-type Caco-2 cells, in the negative control (si-scrambled) and in the BCRP-silenced clone (siBCRP) a clear MDR1 protein expression at 170 kDa was observed (Figure 2A).

It is known from the literature[28] that BCRP forms homodimers to be functionally active. Typically, BCRP protein expression is localized at 72 kDa, but since our

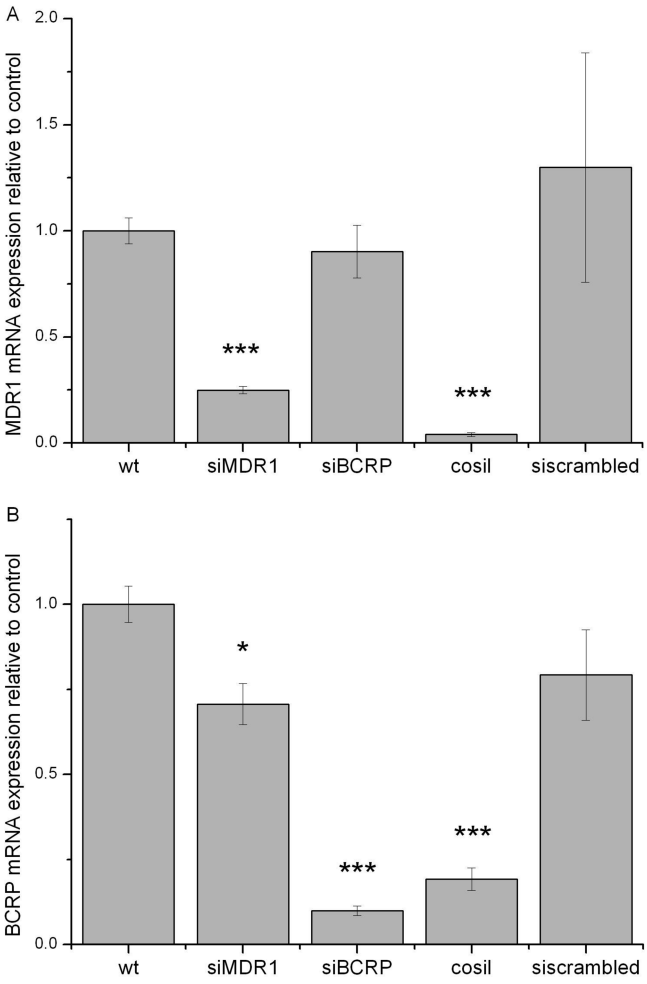

Figure 1. mRNA expression of MDR1 (A) and BCRP (B) in stable silenced clones. mRNA expression was determined by quantitative real-time PCR, and results are normalized to wild-type (wt) cells and expressed as mean ± SEM ($n = 3-5$). *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ vs wild-type cells. Statistics in (A) were performed after exclusion of si-scrambled.

samples were not heated with reducing agents, dimers of BCRP could be detected at a molecular weight of ~150 kDa. In Figure 2B, protein expression of BCRP monomers and dimers is clearly detectable. At 150 kDa, BCRP dimers are only detected in wild-type cells, in the negative control (si-scrambled) and in the MDR1-silencing clone. In BCRP-silenced and co-silenced clones no BCRP protein dimers, which represent the functional active form, are present. Nevertheless, BCRP in its monomeric form could be seen in all clones at more or less the same intensity (Figure 2B).

Functional Activity of MDR1 and BCRP. Functional activity of MDR1 and BCRP was assessed by transport experiments using specific substrates for the respective transporter. Transport was performed in the direction apical to basolateral with or without an inhibitor for the respective protein. Since MDR1 and BCRP are working as efflux transporters on the apical side, silencing or inhibition of the transporters should result in an increase in the apparent permeability (P_{app}). This was clearly shown in the experiments shown in Table 4. The MDR1 specific substrate ^{3}H-digoxin showed significant higher transport rates from apical to basolateral when MDR1 was inhibited by verapamil. In

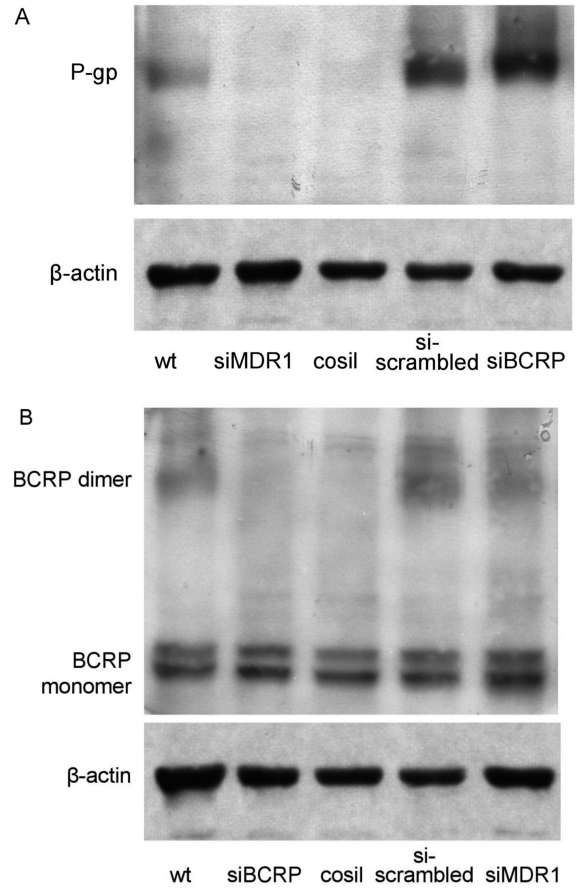

Figure 2. Protein expression of MDR1 (A) and BCRP (B). Beta-actin served as loading control. Protein was extracted 14 days after seeding, and analysis was performed using Western blot technique.

Table 4. Apparent Permeability (P_{app}) of Apical-to-Basolateral Transport of ^{3}H-Digoxin and ^{14}C-PhIP across Caco-2 Monolayers[a]

cells	P_{app} (cm/s) ($\times 10^{-6}$)	
	^{3}H-digoxin	^{14}C-PhIP
wt	2.06 ± 0.09	8.72 ± 0.66
wt + inhibitor	4.11 ± 0.04***	17.01 ± 0.27**
siMDR1	4.14 ± 0.16***	
siBCRP		18.54 ± 0.96**
cosil	4.84 ± 0.14***	20.02 ± 2.95**
si-scrambled	1.74 ± 0.05	10.56 ± 0.34

[a] Apical-to-basolateral transport of ^{3}H-digoxin (66.6 nM, 0.6 μCi/mL) and ^{14}C-PhIP (4 μM, 0.04 μCi/ml) across Caco-2 monolayers over 2 h. Verapamil 100 μM and prazosin 50 μM were used as inhibitors of P-gp and BCRP, respectively. Data represent means of $n = 3$ ± SEM. **$p < 0.01$, ***$p < 0.001$ vs wild-type cells.

(28) Kage, K.; Tsukahara, S.; Sugiyama, T.; Asada, S.; Ishikawa, E.; Tsuruo, T.; Sugimoto, Y. Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization. *Int. J. Cancer* **2002**, *97*, 626–630.

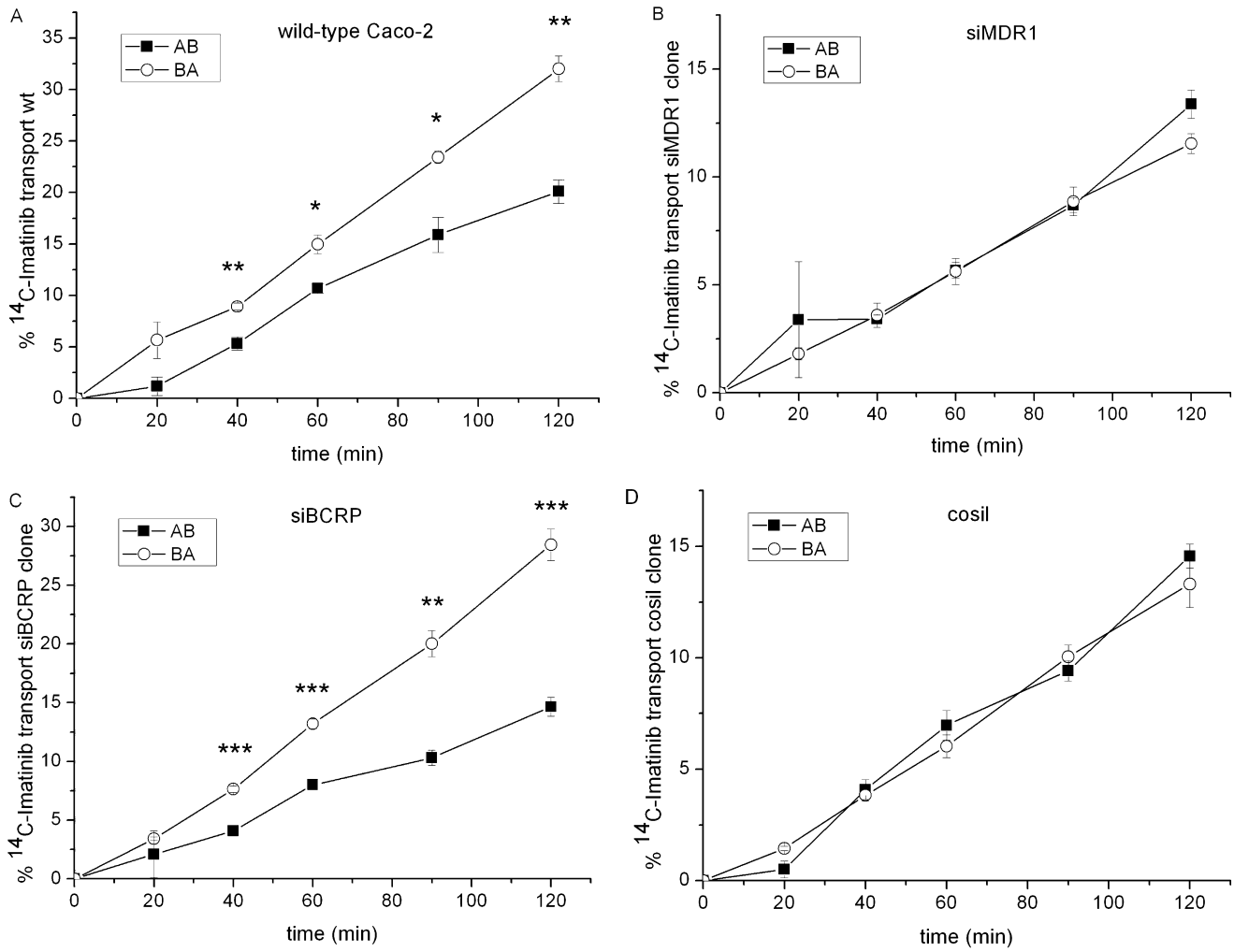

Figure 3. Bidirectional transport of ^{14}C-imatinib (11.4 μM, 0.6 μCi/mL) in Caco-2 wild-type cells (A) and silencing clones (siMDR1 (B), siBCRP (C) and cosil (D)). Percentage of transport apical to basolateral (AB) and basolateral to apical (BA) is plotted against time. Data represent mean ± SEM ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the case of MDR1-silencing, the transport rate from the apical to the basolateral compartment is similarly increased as in the chemically inhibited wild-type cells as shown in the clones siMDR1 and cosil. ^{14}C-Sucrose was used as tightness marker for monolayer integrity, and all clones had a comparable tightness. After 2 h, only 2.5−3.25% sucrose was transported from the apical to the basolateral side. The apparent permeability coefficient (P_{app}) for sucrose was determined and was around $1.7-2.1 \times 10^{-6}$ cm/s (data not shown).

^{14}C-PhIP, which is according to the literature[29,30] a specific substrate of BCRP, was used in the transport studies for BCRP. When BCRP was inhibited with prazosin in wild-type cells, an increase in transport from the apical to the basolateral compartment was observed compared to not-inhibited wild-type Caco-2 cells. A comparable increase in transport was also seen in the silenced clones siBCRP and cosil. The increased transport rate is shown as an increase in P_{app} in Table 4. In these transport experiments, Lucifer Yellow (LY) was used as tightness marker. The amount of LY transport after 2 h was between 2 − 4.5%. Calculations of P_{app} were about $1.3-2.9 \times 10^{-6}$ cm/s and confirmed that transport studies were performed across cells forming a tight monolayer (data not shown).

The negative control (si-scrambled) was also used in these transport experiments and showed similar transport of ^{3}H-digoxin and ^{14}C-PhIP transport compared to Caco-2 wild-type cells.

Transport of Imatinib. Imatinib was described to be a substrate of both intestinal efflux transporters P-gp and BCRP. Using ^{14}C-imatinib we investigated in a Caco-2 cell culture model with specific and double knock-down of P-gp

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(30) Pavek, P.; Merino, G.; Wagenaar, E.; Bolscher, E.; Novotna, M.; Jonker, J. W.; Schinkel, A. H. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 144–152.

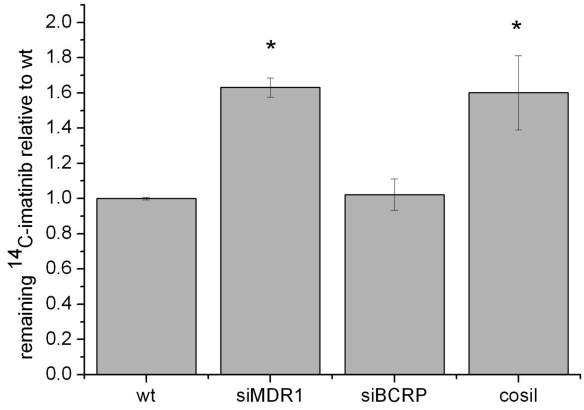

Figure 4. Remaining ^{14}C-imatinib (11.4 μM, 0.6 μCi/mL) in wild-type Caco-2 and silencing clones after 2 h transport. Data represent means ± SEM ($n = 3$). *$p <$ 0.05.

and BCRP, whether these two transporters can compensate for imatinib transport if one of both is down-regulated.

Transport assays were performed to study bidirectional transport of ^{14}C-imatinib through a monolayer. LY served as tightness marker, and calculations of $P_{app,A-B}$ values were about 2.1×10^{-6} to 1.3×10^{-5} cm/s and $P_{app,B-A}$ 1.1×10^{-6} to 8.7×10^{-6} cm/s. Transport in both directions from apical to basolateral (AB) and from basolateral to apical (BA) was measured. Active efflux on the apical membrane is given when the transport rate form basolateral to apical is higher than the transport rate from apical to basolateral. In Figure 3, we can see that Caco-2 wild-type cells exhibit an asymmetry in transport. More ^{14}C-imatinib is transported to the apical compartment, which represents active efflux on the apical side. This asymmetry is eliminated in P-gp- and co-silenced clones (siMDR1 and cosil). In BCRP-silenced clone (siBCRP) the asymmetry of transport is still present, indicating that the efflux activity at the apical side still exists. These results indicate that P-gp exhibits a higher affinity to imatinib transport and can compensate for BCRP-silencing. However in the P-gp-silenced clone, as well as in the co-silenced clone, where both transporters are knocked down, no active efflux of imatinib is detectable, indicating that BCRP cannot fully compensate P-gp mediated efflux.

Measurement of remaining ^{14}C-imatinib in wild-type, MDR1-, BCRP- and co-silencing clones revealed a comparable result (Figure 4). Higher intracellular levels of ^{14}C-imatinib are indicative of reduced efflux function. In clones where P-gp activity was abolished by knock-down, significantly more imatinib accumulates in the cells. In the clone where only BCRP is silenced, the intracellular accumulation is not significantly increased. These results indicate also that P-gp can compensate the loss of BCRP with respect to imatinib transport, but not vice versa.

In a control experiment, we tested ^{14}C-PhIP-transport, a substrate of BCRP but not of P-gp, across wild-type Caco-2 cells and Caco-2 wild-type cells inhibited with imatinib and in BCRP-silenced clone (Figure 5). P_{app} values of the tightness marker LY was calculated and were about $P_{app,A-B}$ $1.0 \times 10{-}6$ to $1.9 \times 10{-}6$ cm/s and $P_{app,B-A}$ 6.5×10^{-7} to 3.7×10^{-6} cm/s. Transport of PhIP across Caco-2 wild-type cells shows a wide asymmetry, while, in cells that are

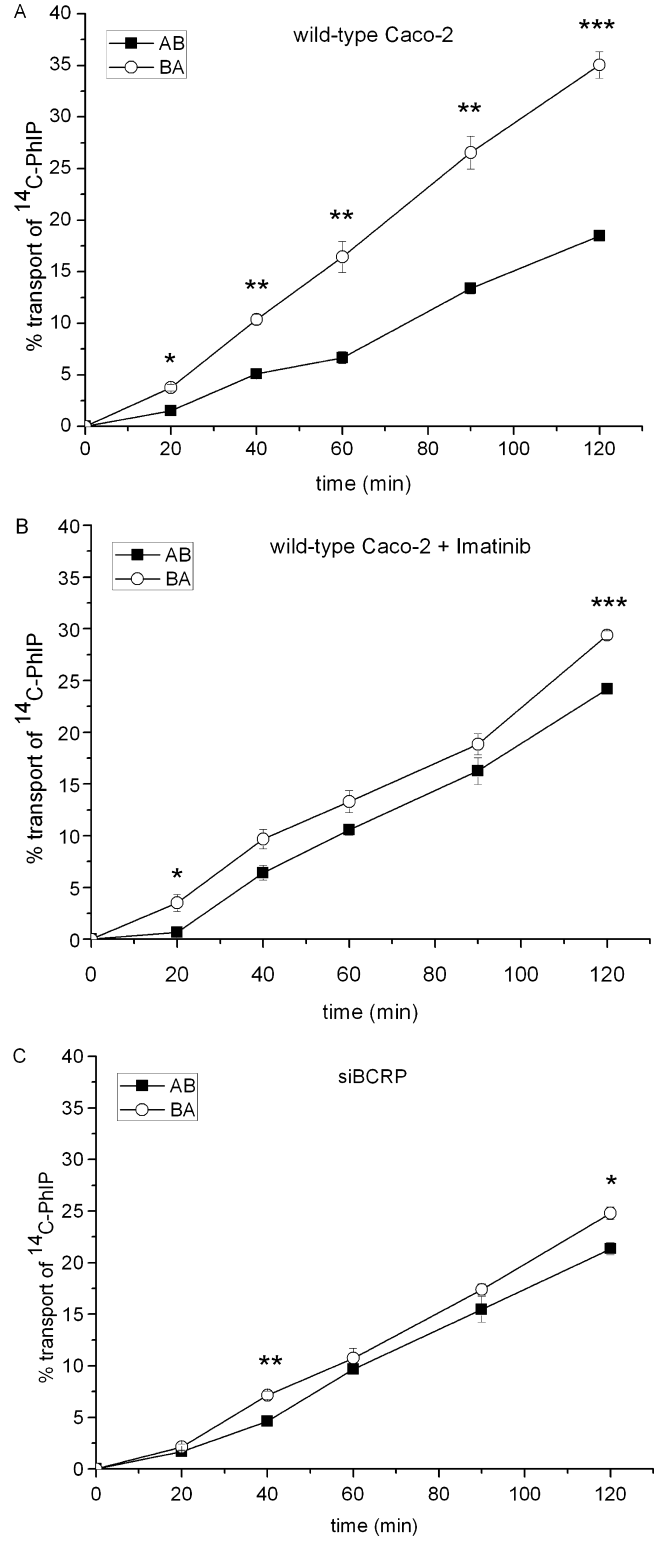

Figure 5. Bidirectional transport of ^{14}C-PhIP (4 μM, 0.04 μCi/mL) across Caco-2 wild-type cells without inhibitor (A), with imatinib (100 μM) as inhibitor (B) and in BCRP-silencing clone (siBCRP) (C). Percentage of transport apical to basolateral (AB) and basolateral to apical (BA) is plotted against time. Data represent mean ± SEM ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

inhibited with imatinib, this asymmetry decreases, indicating specific inhibition of BCRP efflux activity. In the BCRP-silenced clone the asymmetry in PhIP transport is also eliminated, indicating loss of BCRP-mediated efflux transport.

Discussion

In this study we successfully established a model of single and concomitant knock-down of P-gp and BCRP in the intestinal cell culture model Caco-2 cells. Using this model, we evaluated transport of imatinib when one or both transporters are silenced.

Silencing of MDR1 in Caco-2 cells was already earlier published by Celius et al.[31] and Watanabe et al.[32] They achieved a down-regulation of MDR1 mRNA expression of 96 and 90%, respectively. We approached these results, since our knock-down of MDR1 mRNA was between 75 and 95%. On protein level, no MDR1 could be detected by Western blot analysis. Also, the functional activity assays confirmed the knock-down of MDR1.

Stable knock-down of BCRP in Caco-2 cells has until now not been reported. Stable BCRP silencing was earlier performed in BeWo cells by Evseenko et al.;[33] a reduction of BCRP mRNA expression of about 65% was reported. In our model, we achieved 80−90% reduction of BCRP mRNA expression, which even exceeded this result. In Western blot analysis, dimers of BCRP were detected at ∼150 kDa and monomers at 72 kDa, which was described previously for Caco-2 cells.[34] Our protein data show a reduction of BCRP dimer expression in the silencing clones compared to controls, but the monomer at 72 kDa varied ambiguously. However, further analysis of BCRP activity clearly demonstrated a reduction of BCRP function in our clones. A slight decrease in BCRP mRNA expression was also measured in siMDR1 and si-scrambled clones, but on the protein level BCRP dimer expression still was clearly detectable.

Knock-down of both MDR1 and BCRP together in a single clone is described for the first time. Since both transporters have overlapping substrate specificity, a possible compensation by one transporter must be taken into account while the other is inhibited. Relevant interaction of drug absorption by MDR1 and BCRP in the intestine can be predicted, while working in Caco-2 cells, which is a common model for intestinal drug absorption.[35] The impact of MDR1 and BCRP on the transport of any substrate can be determined in this model with single and double knock-down of P-gp and BCRP, especially for drugs that are transported by both proteins, such as imatinib.

Imatinib is an orally administered anticancer drug, which can evoke therapy resistance after long-term treatment. Although resistance is frequently due to the emergence of clones expressing mutant, imatinib-insensitive BCR-ABL,[36] an additional cause of therapy resistance might be the overexpression of active efflux-transporters. The role of ABC-transporters in therapy resistance is widely accepted. Several studies showed that imatinib is a substrate of P-gp.[13,37,38] Studies in Caco-2 cells showed that imatinib induced P-gp and BCRP expression, which led to reduced intracellular drug accumulation.[39] We could confirm that P-gp plays a certain role in transport of imatinib in the intestinal cell culture model Caco-2. When P-gp is silenced, only around half of the amount of imatinib is transported to the apical compartment (Figure 3) and almost twice as much remains in the cells compared to wild-type Caco-2 cells (Figure 4).

Interactions between imatinib and BCRP are intensively discussed in the literature. Several authors demonstrated that imatinib is a substrate of BCRP while others concluded that it is only an inhibitor of BCRP. Jordanides et al.[40] and Houghton et al.[41] both showed that imatinib is an inhibitor of BCRP function, but is not transported by BCRP. In contrast to these results several authors revealed that imatinib

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is not only an inhibitor, but also a substrate of BCRP.[14,42] Shukla et al. demonstrated that imatinib interacts at the transport-substrate binding sites of BCRP and P-gp.[43] Furthermore, Nakanishi et al. showed that imatinib-induced cytotoxicity in BCRP-transfected cell lines is lower than in nontransfected cell lines.[44]

Our results confirm the fact that imatinib is an inhibitor of BCRP function, since it inhibited the transport of the specific BCRP-substrate PhIP (Figure 5). Transport studies of ^{14}C-imatinib in our cell model show interesting findings. In Caco-2 cells, where only BCRP is silenced, there is no difference in ^{14}C-imatinib transport compared to wild-type cells. Remaining imatinib in cells after two hour transport shows a slight but not significant increase in BCRP-silenced clones. An explanation for this observation might be P-gp-function. In the BCRP-silencing clone P-gp still is expressed and possibly compensates the loss of BCRP-function. However, on the other hand, in P-gp-silencing clones, a compensation of BCRP-transport when P-gp activity is lacking was not observed. However, in the co-silenced clone, where both P-gp and BCRP are silenced, remaining ^{14}C-imatinib after two hour transport is higher compared to the single P-gp-silenced clone. This result indicates that P-gp and BCRP both transport imatinib but P-gp seems to contribute to a higher degree to this transport than BCRP.

These conclusions are in accordance with results of Bihorel et al.[45,46] In a mouse model, knockout of mdr1a/1b (−/−) increased the brain-to-blood ratio but knockout of bcrp1-(−/−) showed the same ratio compared to wild-type mice. However, inhibition of P-gp and BCRP in wild-type mice with elacridar increased the brain-to-blood ratio compared to single inhibition of P-gp with valspodar or zosuquidar.

Similar results were obtained by Zhou et al.[47] in a mouse model with single and concomitant knockout of mdr1a/1b-(−/−),(−/−) and bcrp(−/−). The imatinib brain concentration increased 3- to 4-fold in the mdr1a/1b(−/−),(−/−) mice, and no increase was observed in bcrp(−/−) mice, whereas 19- to 50-fold increased imatinib brain concentration was measured in the double knockout mice mdr1a/1b(−/−),(−/−)bcrp(−/−) compared to FVB mice. In addition, *in vitro* transport experiments confirmed the fact that imatinib is a common substrate of both P-gp and BCRP, with P-gp-mediated transport exhibiting a higher affinity to imatinib than BCRP.

Summarizing our findings with imatinib, we could see that single silencing of P-gp had more potent effects on bidirectional transport changes of imatinib than single silencing of BCRP. These findings are in accordance with the findings in the literature, which used several knockout mouse models.

Concerning the extent of the impact of P-gp and BCRP in the Caco-2 cell culture model, we observed an up to ∼2-fold increased uptake or transport of imatinib when transporter function is lacking. This is only a small effect in comparison to the described increased uptakes across the blood−brain barrier. Nevertheless, the clinical importance of intestinal transporter function in imatinib uptake has to be further investigated.

Regarding the *in vivo* situation, the different expression patterns of P-gp and BCRP along the intestinal tract have to be considered. We have shown that BCRP mRNA expression decreases from proximal to distal parts of the human intestinal tract and P-gp shows high alterations in mRNA expression with an increase from proximal to distal small intestine followed by a decrease in colonic expression.[8,7] Other groups stated that BCRP expression also increases in the small intestine from proximal to distal parts followed by a decrease in the colon.[6] In addition, a full scan of the rat intestinal tract revealed increasing P-gp protein expression from proximal to distal parts, while BCRP showed a curved pattern with highest expression at the end of the small intestine.[11] Therefore site specific differences in transporter expression could additionally influence drug absorption in the *in vivo* situation.

The absolute expression of P-gp and BCRP in the intestine compared to Caco-2 cells was investigated by several authors and revealed ambiguous results. Seithel et al.[9] concluded that, in human jejunum and colon, MDR1 is expressed at high levels, while BCRP is expressed in jejunum biopsies at intermediate levels and in colon at low levels. In addition, they investigated the expression in Caco-2 cells and classified MDR1 as intermediately expressed and BCRP as at low levels expressed. Englund et al.[6] stated that BCRP is the most prevalent ABC-transporter in all measured intestinal segments and is expressed at lower levels in Caco-2 cells

(42) Breedveld, P.; Pluim, D.; Cipriani, G.; Wielinga, P.; van Tellingen, O.; Schinkel, A. H.; Schellens, J. H. The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer Res.* **2005**, *65*, 2577–2582.

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compared to the intestine. Taipalensuu et al.[48] stated that transporter expression between Caco-2 cells and jejunum correlate well with the exception of BCRP showing lower expression levels in Caco-2 cells. We have shown in jejunal biopsies that MDR1 expression was slightly elevated in comparison to BCRP. In addition, we found comparable BCRP expression in human duodenum and Caco-2.[7] Therefore, we assume that the Caco-2 model is a suitable *in vitro* model to predict BCRP transporter expression also in humans.

Our model is the first *in vitro* model showing transport of imatinib with single and concomitant knock-down of human MDR1 and BCRP in an intestinal cell culture model. The findings confirm the relative impact of these two transporters, which was recently published at the blood−brain barrier in mice.

Furthermore, the presented new model, including selective P-gp respectively BCRP, as well as co-silenced Caco-2 cells, provides a valuable tool to determine the relative impact of each transporter on transport of a substrate, which is a shared substrate of both transporters in the intestine.

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