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RESEARCH PAPER

# Genomic Knockout of Endogenous Canine P-Glycoprotein in Wild-Type, Human P-Glycoprotein and Human BCRP Transfected MDCKII Cell Lines by Zinc Finger Nucleases

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## ABSTRACT

***Purpose*** To investigate whether it is possible to specifically suppress the expression and function of endogenous canine P-glycoprotein (cPgp) in Madin-Darby canine kidney type II cells (MDCKII) transfected with hPGP and breast cancer resistance protein (hBCRP) by zinc finger nuclease (ZFN) producing sequence specific DNA double strand breaks.

***Methods*** Wild-type, hPGP-transfected, and hBCRP-transfected MDCKII cells were transfected with ZFN targeting for cPgp. Net efflux ratios (NER) of Pgp and Bcrp substrates were determined by dividing efflux ratios (basal-to-apical / apical-to-basal) in over-expressing cell monolayers by those in wild-type ones.

***Results*** From ZFN-transfected cells, cell populations (ko-cells) showing knockout of cPgp were selected based on genotyping by PCR. qRT-PCR analysis showed the significant knock-downs of cPgp and interestingly also cMrp2 expressions. Specific knock-downs of protein expression for cPgp were shown by western blotting and quantitative targeted absolute proteomics. Endogenous canine Bcrp proteins were not detected. For PGP-transfected cells, NERs of 5 Pgp substrates in ko-cells were significantly greater than those in parental cells not transfected with ZFN. Similar result was obtained for BCRP-transfected cells with a dual Pgp and Bcrp substrate.

***Conclusion*** Specific efflux mediated by hPGP or hBCRP can be determined with MDCKII cells where cPgp has been knocked out by ZFN.

**KEY WORDS** ABC–transporter · breast cancer resistance protein · MDCKII · P-glycoprotein · zinc finger nucleases

## ABBREVIATIONS

| | |
|---|---|
| A-B | Apical-to-basal |
| ABC–transporters | ATP-binding cassette transporters |
| B-A | Basal-to-apical |
| cBcrp | Canine breast cancer resistance protein |
| cMrp | Canine multidrug resistance-associated protein |
| cPgp | Canine p-glycoprotein |
| ER | Efflux ratio |
| hBCRP | Human breast cancer resistance protein |
| hPGP | Human p-glycoprotein |
| ko-cell | cPgp knockout cell |
| LC-MS/MS | Liquid chromatography-tandem mass spectrometry |
| LQ | The limit of quantification |
| MDCKII | Madin-Darby canine kidney type II cell line |
| NER | Net efflux ratio |
| QTAP | Quantitative targeted absolute proteomics |
| SRM | Selected reaction monitoring |
| ULQ | Under the limit of quantification |
| ZFN | Zinc finger nuclease |
| ZFP | Zinc finger protein |

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## INTRODUCTION

Drug discovery and development programs in pharmaceutical industry normally include studies on interactions of drug candidates with ATP-binding cassette (ABC) transport proteins. For that purpose, transporter over-expressing cell lines are frequently used, such as the Madin Darby canine kidney cell line type II (MDCKII), which has been widely accepted for *in vitro* transport studies (1–3). MDCKII cells transfected with the human *MDR1* (ABCB1) gene encoding for P-glycoprotein (hPGP) (MDCKII-MDR1) and the human *BCRP* (ABCG2) gene (MDCKII-BCRP) are used as an established model in drug candidate selection for identification of hPGP or hBCRP substrates (4–7). The easy handling and their high proliferation rate explains the prevalent usage of these cell lines (1). However, a disadvantage is the expression of endogenous canine transport proteins (8), which might lead to interferences with the transfected transporter of interest due to efflux by the endogenous transporters (9).

Therefore, we recently developed subclones of hPGP over-expressing cells exhibiting reduced expression levels of canine Pgp (cPgp) and showing more reliable results with respect to hPGP transport (9). However, the improved cell line still showed interferences from the endogenous cPgp. Additionally, it was not possible to establish MDCKII cell lines transfected with other human transport proteins, like hBCRP, with a reduced endogenous cPgp activity.

Zinc finger nucleases (ZFN) can be used to suppress the expression of endogenous cPgp through a genomic knockout of the specific gene. ZFNs are a multifaceted tool for modifying genes by connecting the cutting domain of the restriction enzyme *FokI* with a sequence specific zinc finger protein (ZFP) (10,11). The ZFP belongs to protein motives, which are able to specifically bind to a DNA sequence. Among these motives are also the leucin-zipper and the helix-turn-helix motive. The cys2-his2 zinc finger motive contains of 20–30 amino acids with 2 cysteines and 2 histidines arranged around a zinc molecule. The number of zinc finger motives is variable (12). Examples vary from the precise modification of specific gene sequences to selective knockout of transcribed genes to integration of genes at specific sites of the DNA (13–17).

Cutting the DNA by ZFNs requires the dimerization of the restriction enzymes *FokI*, which is linked to the zinc fingers. As a monomer, they are inactive (18–20). The simultaneous binding of both ZFN pairs at their binding site guarantee a very high specificity. Genome editing with site-specific zinc-finger is becoming a new standard for targeted genome modification in cells and animals (21,22).

The purpose of this study is to investigate whether it is possible to specifically suppress the expression and efflux activity of cPgp making it negligible compared to those of the transfected hPGP and hBCRP in the MDCKII transfected cells. Therefore, it is important to show that cPgp was specifically knocked down and to determine how much lower the expression and activity of cPgp are compared to the hPGP- and hBCRP-transfected cells after the knock down by the ZFN technique. In case of the MDCKII-BCRP cells, specific knock down of cPgp can be evaluated by transport experiments (e.g. bi-directional transport assay) using P-gp specific substrates which are not transported by hBCRP. However, in case of the MDCKII-MDR1 cells, knockout of cPgp cannot be evaluated in transport experiments and western blot analysis because so far no substrates have been identified which are specifically transported by cPgp but not by hPGP.

Further, the antibody for Pgp cannot distinguish cPgp and hPGP proteins. Although cPgp and hPGP expression can be distinguished by PCR technique, the mRNA expression level does not always reflect protein expression levels and the activities. By contrast, protein expression levels of hPGP in the hPGP expressing cell lines correlate well with the efflux activities (23). Kamiie *et al.* have recently established the LC-MS/MS-based absolute quantification method to selectively determine the absolute protein expression levels of the target transporter proteins by using specific peptides for the target transporters (24). This technique, called "quantitative targeted absolute proteomics (QTAP)", enables us to distinctively quantify protein expressions of cPgp and hPGP, and to estimate the quantitative difference in the efflux activities from the measured protein expression levels in the hPGP transfected MDCKII cell lines.

Thus, we aimed to clarify whether the endogenous cPgp in the MDCKII transfectants can be knocked out by the ZFN technique, and to establish useful over-expressing systems *in vitro* for the functional analysis of the hPGP and hBCRP. We transfected the ZFN to the wild-type, hPGP-transfected, and hBCRP-transfected MDCKII cell lines, and then selected cell populations showing a knockout of cPgp by PCR. In these populations, we evaluated the knockdown of functional cPgp not only by western blotting and bi-directional transport assay but also by the QTAP technique. Consequently, specific and sufficient knock down of cPgp protein against the transfected human transporters was demonstrated and confirmed in the measurements of drug efflux activities of the transfected hPGP and hBCRP in bi-directional transport assays. These data suggest that new MDCKII cell lines were established for the functional evaluation for hPGP and hBCRP with significant reduction of cPgp activity.

## MATERIALS AND METHODS

### Cell Lines and Cell Culture

Wild type MDCKII cells (MDCKII), MDCKII cells transfected with human P-Glycoprotein (hPGP, MDR1, ABCB1) (MDCKII-MDR1) and MDCKII cells transfected

with human breast cancer resistance protein (hBCRP, ABCG2) (MDCKII-BCRP) were provided by Prof. Piet Borst (the Netherlands Cancer Institute, Amsterdam, Netherlands). The cells were cultured in DMEM containing 3.7 g/l $NaHCO_3$, 4.5 g/l D-Glucose and L-glutamine. The medium was supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/ml streptomycin as final working concentration. The medium and the supplements were obtained from Biochrom AG (Berlin, Germany). The cells were grown under standard conditions (37°C, a humidified air-5% $CO_2$ atmosphere) and were used for >50 passages.

In our previous publication (9) we described a cell line called MDR1_high, which was generated by preparative FACS sorting to get a MDCK-MDR1 cell line expressing higher levels of hPGP. This cell line was the starting point for the MDR1_ZFN cell line.

### ZFN Induced Knockout of cPgp

The sequence specific ZFN were manufactured and purchased from Sigma-Aldrich®. As transfection reagent GenJet™ *In Vitro* DNA Transfection Reagent for MDCKII Cells (Ver. II) from SignaGen® Laboratories was used according to the manufacturers protocol. For transfection 2 µl plasmid DNA and 8 µl GenJet™ reagent were diluted each into 100 µl serum-free DMEM with High Glucose. Afterwards the diluted GenJet™ reagent was added to the diluted plasmid DNA mix. This reaction mix incubated for 15 min at room temperature to allow GenJet™-DNA transfection complexes to form. $1.2 \times 10^6$ cells were immediately resuspended in the 200 µl transfection complex and incubated at 37°C for 20 min. At the end of incubation, 2.0 ml of pre-warmed fresh complete cell growth medium were added to the cells and they were plated onto one well of a 6-well plate.

### Preparative FACS Sorting

Culture medium was removed from MDCKII cells and cells were washed twice with phosphate-buffered saline (PBS). Cells were incubated with 1 µM Calcein-AM for 30 min in serum-free medium. Then, they were trypsinized and counted; 1, 000,000 cells were taken and centrifuged for 10 min at 100 g. The pellet was resuspended in 1 mL PBS with 2% FBS and again centrifuged. This step was repeated 3 times. Preparative sorting was done with a FACSAriaTM flow cytometer (Becton Dickinson, Heidelberg, Germany). Single cells were sorted for low activity of Pgp in the wild type MDCKII cells and in the MDCKII-BCRP cells using Calcein-AM as a fluorescent marker to increase the probability of finding the ko-cell lines. For the MDCKII-MDR1 cells this pre-selection could not be used, because Calcein-AM is not able to discriminate between human and endogenous canine Pgp.

### PCR

For RNA isolation RNeasy® Mini Kit and QIAshredder™ columns (Qiagen, Hilden, Germany) were used according to the manufacturers protocol. The PCR was performed with a total reaction volume of 25 µl containing 2.5 µl 10× Taq-buffer, 0.5 µl dNTPs, 2 µl Primer forward (GCCATCAGCC CTGTTCTTG), 2 µl Primer reverse (ACAGACCATGCC ATCTGTGG), 0.5 µl $MgCl_2$, 1 µl DNA, 0.25 µl Taq-polymerase, 16.25 µl water. The program was set with an initial denaturation at 94°C for 3 min. followed by 35 cycles with 30s at 94°C, 45 s at 52°C and 1 min at 72°C. At the end final elongation took place at 72°C for 3 min. Afterwards completed PCR product was loaded on a 1% agarose gel containing ethidiumbromide with 1 µl loading dye and run for 30 min. at 100 V. This PCR method was used for genotyping the sorted cell lines.

### Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from the cells using RNeasy® Mini Kit and QIAshredder™ columns (Qiagen, Hilden, Germany) according to the manufacturers protocol. RNA concentrations and purity were measured spectrophotometrically at 260 and 280 nm using a NanoDrop 2000 photometer (PeqLab, Erlangen, Germany). For qRT-PCR RNA was reversed transcribed using iscript™ Reverse Transcription for qRT-PCR (Bio-Rad Laboratories GmbH, Munich, Germany) according to the instructions of the manufacturer. QuantiFast® SYBR® Green PCR Kit (Qiagen, Hilden, Germany) was used for qRT-PCR reaction. According to the protocol the following cycling conditions were used: 95°C for 5 min; 95°C 10s, 60°C 30s (for 40 cycles). After every run a melting curve analysis was performed to verify the specificity of the PCR products. The qRT-PCR analysis was performed on a LightCycler carrousel based system using LightCycler 3 software (Roche Applied Science, Mannheim, Germany). To quantify qRT-PCR gene results, the standard curve method according to manufacturer specifications of the software was utilized. Results were normalized to the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh). Additionally, a standard with defined dilutions of cDNA amplified with Gapdh were measured on every run to quantify the amount of the gene of interest for a better normalization. Primer for analysis were used as previously described (9,25).

### Western Blot Analysis

Membrane proteins were isolated from cultured cells using the ProteoExtract® Native Membrane Protein Extraction Kit (Calbiochem® Merck, Dramstadt, Germany). Membrane proteins were separated on a NuPage 4–12% Bis-Tris Gel

(Novex Life Technologies GmbH, Darmstadt, Germany) and transferred to a PVDF membrane (Millipore Merck-Millipore, Darmstadt, Germany). The blotting took place at 300 mA for 90 min. Primary antibodies for Pgp (C129, dilution 1:250) and $Na^+$-$K^+$-ATPase (M7-PB-E9, 1:500) were used in combination with anti-mouse IgG-HRP (dilution 1:10,000) (Enzo, Lörrach, Germany). Bands were detected on a BIO-RAD Universal Hood II (Bio-Rad Laboratories GmbH, Munich, Germany) using Western Lightning Plus-ECL (PerkinElmer®, MA, USA). Band intensities were measured utilizing Quantity One 1-D Analysis software (Bio-Rad Laboratories GmbH, Munich, Germany).

## Bi-Directional Transport Studies

Cells were seeded onto 96-well Transwell® plates (polycarbonate membrane with a pore size of 0.4 μm; Millipore Merck Millipore, MA, USA) at a density of 300,000 cells/$cm^2$ and the assay was performed on day 5. At the beginning of each experiment the culture medium was removed and the cells were washed with Hank's Balanced Salt Solution adjusted to a pH of 7.4 (HBSS; Gibco Life Technologies GmbH, Darmstadt, Germany). Depending on desired transport direction, substrate solution was added to the apical compartment (A to B direction) or to the basolateral compartment (B to A direction), respectively. As substances quinidine, vinblastine (Sigma Aldrich, Taufkirchen, Germany), sumatriptan, nelfinavir, paclitaxel, saquinavir and topotecan (AbbVie Deutschland GmbH & Co. KG, Ludwigshafen, Germany) were used with a concentration of 1 μM each. Samples were taken at 0 and 60 min with a volume of 50 μl of each compartment. For analytics an Acquity UPLC system (Waters GmbH, Eschborn, Germany) and an API5000 mass spectrometer (AB Sciex, MA, USA) were used (see below). Transport study data were analyzed as follows:

$$P_{app}(nm/s) = (\Delta Q/\Delta t)*(1/(A \times C_0)) \quad (1)$$

where $P_{app}$ is the apparent permeability, $\Delta Q$ is the amount of drug solute transported (μmole), $\Delta t$ represents the incubation time (sec.), A is the monolayer surface area ($cm^2$), $C_0$ is the mean of starting-end drug concentration in the donor well (μM).

$$\text{Efflux ratio(ER)} = P_{app,\ B-A}/P_{app,\ A-B} \quad (2)$$

$$\text{Net efflux ratio(NER)} = ER_{MDCKII-MDR1}/ER_{MDCKII},$$
$$\text{or } ER_{MDCKII-BCRP}/ER_{MDCKII},\ \text{or} ER_{MDR1_Z\,FN}/ER_{MDCKII_Z\,FN}, \quad (3)$$
$$\text{or } ER_{BCRP_Z\,FN}/ER_{MDCKII_Z\,FN}$$

Net efflux ratios were used to show the difference between the parental and the ko cell lines, because NER take respect to efflux of the endogenous transport protein. Here, the efflux of the transfectants is normalized to the efflux of the respective wild type cells.

## Quantification of Substrate Concentrations by LC-MS/MS

The generic analytical method is based on a protein precipitation to separate the analytes from matrix. An aliquot of sample (25 μL) was pipetted in a 384 well polypropylene plate and the proteins were precipitated by addition of 100 μL acetonitrile/H2O containing the internal standard carbutamide. The plate was centrifuged at 2000 rpm for 10 min (4°C). Samples were injected (6.0 μL) on a Waters BEH C18 50×2.1 mm, 1.7 μm column with an acetonitrile 0.1% formic acid/0.1% formic acid gradient at a flow rate of 0.70 mL/min. Run time was 1 min. The column temperature was held at 50°C.

Mass spectrometric detection was performed on a AB SCIEX Triple Quad™ 5000 with a turbo-ion spray interface by multiple reaction monitoring (SRM/MRM) in positive mode. All samples were run and analyzed using DiscoveryQuantTM . Evaluation was based on peak area.

## Quantitative Targeted Absolute Proteomics (QTAP)

Absolute protein expression levels of target molecules were determined by a multiplexed SRM/MRM analysis in the LC-MS/MS using the peptide probes to quantify the target molecules as described previously with minor modifications (24,26). In the present study, two and three peptide probes were used for Pgp and Bcrp, respectively, to distinguish the canine proteins from the other animal ones, uncover the existence of canine Pgp or canine Bcrp, and estimate their protein expression levels (supplemental table 1). For Pgp, one (Pgp peptide-1) is a peptide probe with the shared amino acid sequence among at least canine, human and monkey Pgp proteins, and another (Pgp peptide-2) is that among at least human and monkey Pgp except for canine Pgp. For Bcrp, two probes (Bcrp peptide-1, and -2) are those among at least canine, human and monkey Bcrp proteins, and another (Bcrp peptide-3) is that among at least human and monkey Bcrp except for canine Bcrp. All peptides were designed on the basis of in silico peptide selection criteria (24,27), and synthesized by Thermoelectron Corporation (Sedanstrabe, Germany) with over 95% peptide purity.

Fifty μg protein of the membrane fractions isolated from the cultured cells was dissolved in denaturing buffer containing 7 M guanidium hydrochloride, 500 mM Tris–HCl (pH 8.5) and 10 mM EDTA. The denatured proteins were reduced with dithiothreitol at room temperature for 60 min,

and then S-carbamoylmethylated with iodoacetamide at room temperature for 60 min in dark. The alkylated proteins were precipitated with a mixture of methanol, chloroform, and water. The precipitates were suspended in the buffer containing 6 M urea and 100 mM Tris–HCl (pH 8.5), diluted five-fold with 100 mM Tris–HCl (pH 8.5) to reach 1.2 M urea, and then sonicated in the ice-cold water bath of Branson 2510 sonicator to completely dissolve the protein precipitates. ProteaseMax surfactant (Promega, Madison, WI, USA) and lysyl endopeptidase (Wako Pure Chemical Industries, Japan) were added to the samples at 0.05% and an enzyme/substrate ratio of 1:100 as the final concentrations, respectively, and then incubated at 30°C for 3 h, followed by the digestion with TPCK-treated trypsin (Promega) at an enzyme/substrate ratio of 1:100 at 37°C for 16 h. The tryptic digests were mixed with stable-isotope labelled peptides (internal standard) so that an injection amount per LC-MS/MS measurement was 500 fmol, acidified with formic acid, and centrifuged at 15, 000 rpm for 5 min at 4°C.

The supernatants were injected onto a HPLC system (Agilent1100 system; Agilent Technologies, Santa Clara, CA) connected to an ESI–triple quadrupole mass spectrometer (API5000; AB SCIEX, Framingham, MA) operated in positive ionization mode. A Waters XBridge BEH130 C18 column (1.0 mm ID × 100 mm, 3.5 µm particles; Waters, Milford, MA) was used. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Linear gradients were applied to separate and elute the peptides at a flow rate of 50 µl/min as follows; 99%A:1%B for 5 min after injection, then a linear gradient to 40%A:60%B at 65 min, switch to 0%A:100%B at 66 until 68 min, then a linear gradient to 99%A:1%B at 70 and continue until 130 min. Total run time was 130 min.

Each target (standard) peptide was monitored with four SRM transitions (Q1/Q3-1, Q1/Q3-2, Q1/Q3-3, Q1/Q3-4), and the corresponding internal standard peptide was monitored with the four SRM transitions corresponding to those of the target peptide (supplemental table 1). The ion counts of chromatograms were determined by using the data acquisition procedures in Analyst software version 1.5 (AB SCIEX). Selecting the target peaks and quantification of target peptides were performed as described previously (24,27). Signal peaks with a peak area count of over 5000 detected at the same retention time as an internal standard peptide were defined as positive. When positive peaks were observed in three or four SRM transitions, the target molecules were considered to exist in samples, and then the absolute protein expression levels were determined as the average of three or four quantitative values. If positive peaks were detected in two SRM transitions or less, the amounts of peptides in the samples were considered to be under the limit of quantification (ULQ), and then the values of the limit of quantification (LQ) were determined as described by Uchida *et al.* (28).

In the present study, the brain microvessels isolated from the frozen cerebral cortex of adult rhesus monkey were analyzed as quality control samples to check that the quantitative values of Pgp or Bcrp proteins determined from the different peptides (e.g. two peptides for Pgp) were comparable. Briefly, the monkey brain microvessels express only monkey proteins such as monkey Pgp and Bcrp, and all the peptide probes for Pgp (two) and Bcrp (three) enable us to quantify the monkey Pgp and Bcrp expression, respectively. Therefore, the determined expression levels of Pgp or Bcrp proteins in the microvessels should be comparable among different peptides. The quantitative analysis for the monkey brain microvessel sample was conducted according to the same experimental procedure as the MDCKII samples.

### Statistics

Statistical data are expressed as mean ± SD. Statistical analysis was performed as two tailed, nonparametric Mann–Whitney t-test.

## RESULTS

### Preselection of Cell-Lines

ZFNs cause a sequence specific double strand break. Due to DNA repair mechanisms, 1–20% of the cells showed miss-match repairs resulting in non-functioning cPgp. Since only a small percentage of the whole cell population was affected, single cells were sorted in order to identify the desired cPgp ko-cells. For selection, the parental wild type MDCKII cells (MDCKII) and cells transfected with hBCRP were pre-sorted by FACS based on a Calcein assay. Since both cell lines express only endogenous cPgp it could be assumed that ko-cells exhibit a very high fluorescence signal corresponding to a lack of export pump activity. Therefore, a narrow range of highest fluorescence intensity was defined in the FACS experiments in order to sort and identify the ko-cells. The MDCKII-MDR1 cell line could not be pre-selected, because the fluorescent marker was not able to discriminate between human and endogenous canine Pgp.

### Identification of ko-Cells by Genotyping

Selected cells were cultured and then the genetic knockout verified in the following manner. Primer pairs were generated, which flank the cutting site of the ZFNs. Cell populations with

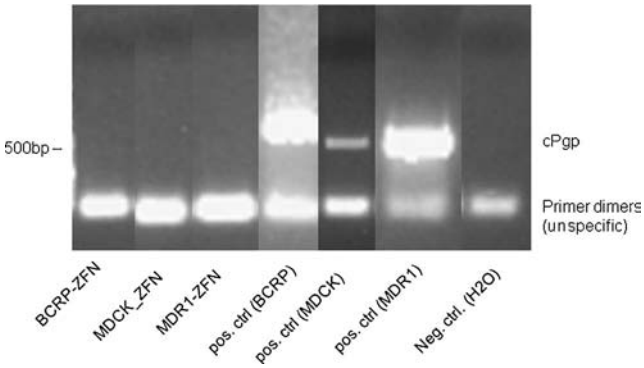


**Fig. 1** Identification of ko-cells by PCR. Genotyping by PCR was performed to identify ko-cell lines. The absence of a PCR product indicates the genomic knockout of cPgp. The upper band shows a PCR product of 500 bp. The lower bands represent unspecific primer dimers.

knockout were identified by the lack of a PCR product after separation on an agarose gel. Primers for genotyping were designed to flank the cutting site of the ZFN. Figure 1 shows the result of the genotyping for the ZFN-transfected cells as well as the parental populations.

The parental cell lines showed a signal for cPgp at 500 bp, whereas the ko-cell populations showed no signal. They were named MDR1_ZFN, MDCKII_ZFN and BCRP_ZFN.

## mRNA Expression Analysis of ABC Transporters by qRT-PCR

The mRNA pattern of the created ko-cell lines was determined and compared with that of the parental cell lines (Fig. 2).

All ko-cell lines showed a significant reduction of mRNA expression of cPgp. Interestingly, ZFN-treated cells showed also a decrease of Mrp2 mRNA expression, which was particularly high in the MDCKII_ZFN population and the MDR1_ZFN cells, were the expression decreased by 37-fold and by 13-fold, respectively. Thus the possibility cannot completely be neglected that a non-specific depletion of cMrp2 gene occurs by the ZFN for cPgp.

Some expression of cPgp was still observed in all ZFN cell populations. To verify this finding, Western Blot and QTAP analysis were performed to investigate if the functional protein might be expressed.

## Protein Expression Analysis of Pgp by Western Blotting

Compared to the parental MDCKII and hBCRP-transfected cells, the MDCKII_ZFN and the BCRP_ZFN cells showed a significant reduction but not a complete suppression of cPgp protein expression, respectively (Fig. 3). Both the parental hPGP-transfected and

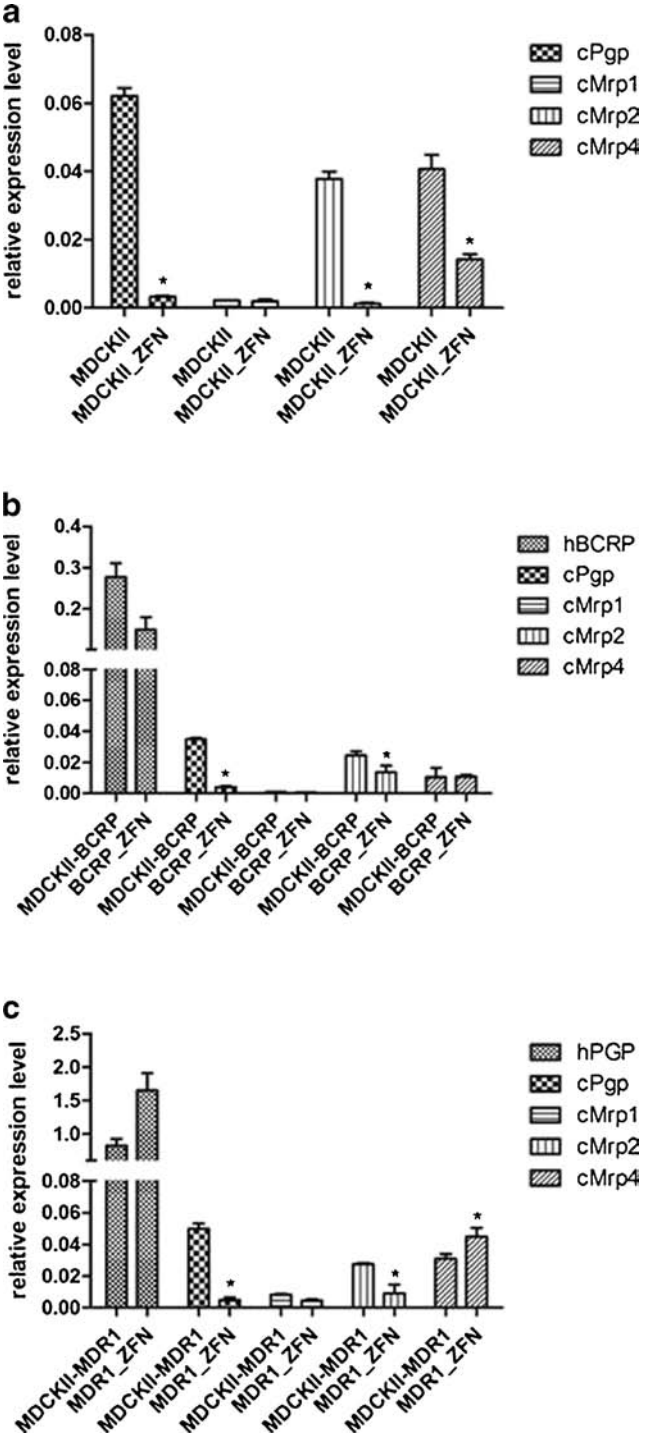


**Fig. 2** mRNA expression levels of transport proteins in parent cells and ZFN-treated cells. Relative mRNA expression of different export pumps in ko-cells and their parental cell populations. Expression was normalized with respect to the house keeping gene GAPDH. (**a**) MDCKII_ZFN cells and MDCKII cells, (**b**) hBCRP_ZFN cells and hBCRP cells (**c**) MDR1_ZFN cells and MDR1 cells. All cells were at passage $P = 15$ ($n = 3$, mean values ± SD; $p$ * < 0,05).

MDR1_ZFN cells showed the high expression levels of Pgp proteins.

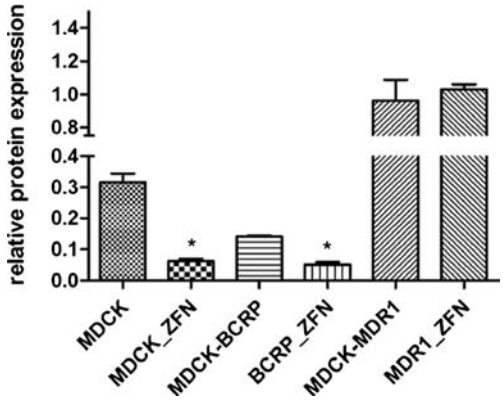


**Fig. 3** Western Blot analysis for protein expression of Pgp in ko-cells and parental cells. Band intensity in ko-cell lines compared to parental cell lines was measured for Pgp. (normalized to the house keeping protein $Na^+/K^+$-ATPase). All cells were at passage $P = 15$ ($n = 3$, mean values ± SD; $p$ *< 0,05).

## Animal Species-Specific and Quantitative Protein Expression Analysis of Pgp and Bcrp by QTAP Technique

Since the antibody of Pgp cannot discriminate cPgp from human hPGP, it was questionable whether the cPgp protein expression was knocked out in the MDR1_ZFN cells in the Western Blot analysis. The Western Blot analysis also lacks a quantitative performance, thereby being unclear whether the cPgp protein expressions were sufficiently knocked out in the three ZFN-transfected cell lines to be able to measure the specific efflux activities of hPGP and hBCRP without interference by cPgp. Furthermore, it is also important to clarify the protein expression levels of cBcrp in the individual MDCKII cells. Therefore, we conducted a species-specific and quantitative protein expression analysis of Pgp and Bcrp by means of a QTAP technique recently established by Prof. Tetsuya Terasaki (24,28,29) (Table I).

First, we evaluated the quality of the peptides by using the monkey brain microvessel samples to clarify the question whether the two peptides of Pgp or three peptides of Bcrp selected from different regions of these proteins can give comparable quantitative values. As shown in Table I, no significant difference was observed for the expression levels of monkey Pgp determined by 2 Pgp peptides in the microvessels. The 3 peptides of Bcrp also did not show any significant difference in the expression levels of monkey Bcrp. These data suggest that there was no problem in the quality of peptides such as incompleteness of trypsin digestion efficiency and inaccuracy of quantification , although we cannot strictly exclude the possibility that the stability and digestion efficiency might be different among the different species or between cell lines and brain vessels.

Pgp peptide-1 detected the endogenous expression of cPgp protein in the parental MDCKII and MDCKII-BCRP cells, but did not detect it in the MDCKII_ZFN and BCRP_ZFN cells (Table I). Based on the limit of quantification, the expression levels of cPgp in the MDCKII_ZFN and BCRP_ZFN cells were at least 48-fold and 29-fold smaller than those in the parental MDCKII and hBCRP-transfected cells, respectively. In the parental hPGP-transfected cells, the expression level of hPGP determined by the peptide-2 was significantly smaller than that of Pgp determined by the peptide-1, suggesting that the cPgp protein was expressed in the parental hPGP-transfected cells although the expression level was smaller than that of human hPGP protein. However, in the MDR1_ZFN cells, no significant difference was observed in the expression levels determined by the peptide-1 and peptide-2, and the expression levels were rather greater than those in the parental hPGP-transfected cells. Therefore, these data demonstrated that the cPgp protein expression was specifically knocked down in the MDR1_ZFN cells.

Bcrp peptide-1 and peptide-2 did not detect the endogenous expression of cBcrp protein in the parental MDCKII, MDCKII-MDR1, MDCKII_ZFN, and MDR1_ZFN cells (Table I). Both in the parental MDCKII-BCRP and hBCRP_ZFN cells, the expression levels of hBCRP determined by the peptide-3 were not significantly different from those of Bcrp determined by the peptide-1 and peptide-2. These data suggest that the protein expression level of cBcrp was negligible small compared to that of the human hBCRP. In the BCRP_ZFN cells, the protein expression level of human hBCRP was 5.91 fmol/μg protein, and at least 281-fold greater than that of the endogenous cPgp on the basis of the limit of quantification for cPgp (0.021 fmol/μg protein).

## Bidirectional Transport Experiments

Several efflux transporter substances were used to confirm the suppression of cPgp expression and thereby the improvement of the determination of efflux transport, specific to human hPGP and hBCRP (Fig. 4). A Net Efflux Ratio (NER) of two indicates the threshold to identify a substance as a substrate of the transfected human transporter (hPGP, hBCRP) (30). The two hPGP substrates quinidine and vinblastine showed a strong improvement of the NER using the MDR1_ZFN and MDCKII_ZFN cell lines. For quinidine, the NER was about five-fold higher and for vinblastine it was even 30-fold higher compared to the parental cell lines. Vinblastine actually exhibited a NER below two for the parental cell lines which would indicate that vinblastine is not a substrate for hPGP using these cell lines. A similar improvement in NER was also achieved for nelfinavir showing a 10-fold higher NER for the ko-cell lines. For the substance sumatriptan an opposite effect occurred, demonstrating a NER around 2 for the parental cell lines and a lower NER for the ko-cell lines. Paclitaxel and sequinavir displayed similar effects compared to vinblastine with a NER below 2 for the parental cell lines and an increased NER for the ZFN-cell lines. For all these substances,

**Table I** Absolute Protein Expression Levels of cPgp, cBcrp, Transfected hPGP and hBCRP in the Membrane Fractions of Parental-Cell and ZFN-Transfected ko-Cell Lines.

| Molecular names (Specificity of peptides) | Absolute protein expression level (fmol/$\mu$g protein) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Parental-cell lines | | | cPgp ko-cell lines | | | Rhesus monkey brain microvessels |
| | MDCKII | PGP | BCRP | MDCKII-ZFN | PGP-ZFN | BCRP-ZFN | |
| Pgp | | | | | | | |
| Peptide-1 (human, canine, monkey Pgp) | 0.909 ± 0.053 | 4.62 ± 0.27 | 0.615 ± 0.099 | U.L.Q. (<0.019) | 6.70 ± 0.21 | U.L.Q. (<0.021) | 3.66 ± 0.28 |
| Peptide-2 (human, monkey Pgp) | U.L.Q. (<0.040) | 3.71 ± 0.10* | U.L.Q. (<0.044) | U.L.Q. (<0.038) | 6.05 ± 0.16 | U.L.Q. (<0.039) | 3.04 ± 0.21 |
| Bcrp | | | | | | | |
| Peptide-1 (human, canine, monkey Bcrp) | U.L.Q. (<0.254) | U.L.Q. (<0.267) | 9.52 ± 0.16 | U.L.Q. (<0.251) | U.L.Q. (<0.255) | 5.26 ± 0.13 | 11.2 ± 0.36 |
| Peptide-2 (human, canine, monkey Bcrp) | U.L.Q. (<0.210) | U.L.Q. (<0.208) | 7.36 ± 0.27 | U.L.Q. (<0.192) | U.L.Q. (<0.201) | 4.49 ± 0.30 | 8.75 ± 0.23 |
| Peptide-3 (human, monkey Bcrp) | U.L.Q. (<1.02) | U.L.Q. (<1.09) | 10.2 ± 0.41 | U.L.Q. (<1.01) | U.L.Q. (<1.02) | 5.91 ± 0.25 | 9.75 ± 0.53 |

The membrane fractions (50 $\mu$g protein each) of the cell lines were digested with lysyl endopeptidase and trypsin. The protein expression levels were measured by subjecting the digests (33.3 $\mu$g protein) to LC-MS/MS with 500 fmol of stable-isotope labeled peptide mixture. As a quality control sample, the monkey brain microvessels were isolated from the frozen cerebral cortex of adult rhesus monkey by means of nylon mesh method as described previously (52). Briefly, after homogenizing the cerebral cortex, the brain microvessels in the homogenate were precipitated with the buffer containing 17.5% dextran, and then applied to the 45 $\mu$m nylon mesh. The brain microvessels retained on the mesh were collected, and the quantitative analysis was conducted with the whole tissue lysates according to the same experimental procedure as the MDCKII samples. Peptide-1 of Pgp is a common peptide able to quantify human, canine and monkey Pgp proteins. Peptide-2 of Pgp is a common peptide able to quantify only human and monkey Pgp proteins except for canine Pgp protein. Peptide-1 and -2 of Bcrp are common peptides able to quantify human, canine and monkey Bcrp proteins. Peptide-3 of Bcrp is a common peptide able to quantify only human and monkey Bcrp proteins except for canine Bcrp protein. The data represent mean ± S.E.M. ($n = 3$). * $p < 0.01$, significantly smaller than the value obtained by Pgp peptide-1 (student's t-test). U.L.Q., under the limit of quantification. The limits of quantification (fmol/$\mu$g protein) are in parentheses

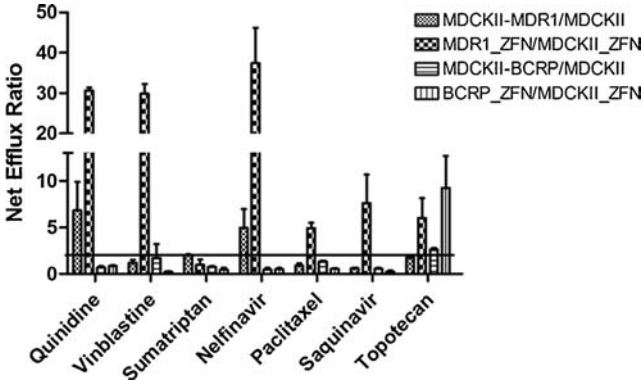


**Fig. 4** Bidirectional transport analysis of various substances in parental and ko-cell lines. Net Efflux Ratios (NER) are demonstrated for the different substances in parental cell lines (MDR1/MDCKII and hBCRP/MDCKII) and in ko-cell lines (MDR1_ZFN/MDCKII_ZFN and hBCRP_ZFN/MDCKII_ZFN). Data are taken from Table II, and NER was calculated according to the Eq. 3 described in the Materials and Methods section. The line passing through the NER of 2 indicates the threshold to identify a substance as a substrate of the transfected human transporter. ($n = 3$, mean values ± SD).

both hBCRP-cell lines, parental and BCRP_ZFN, showed NERs below two except for vinblastine, showing a NER around 2 using the parental hBCRP cell population. Topotecan demonstrated an increased NER compared to their parental cell populations for both ko-cell lines, MDR1_ZFN and BCRP_ZFN.

## DISCUSSION AND CONCLUSION

One important compound characteristic in drug development is a potential interaction of compounds with ABC-transport proteins, e.g. Pgp. Cell culture models are commonly used to study such interactions. However, results are often inconsistent due to substrate overlap between transfected and overexpressed human export protein and endogenous export proteins. In case of transfected MDCKII cells, hPGP is expressed in parallel to canine P-glycoprotein.

So far, no differences of substrates have been described for hPGP and cPgp. However, this is an important safety issue, since novel compounds tested for P-glycoprotein interactions may interact with both proteins in a different way. One possibility to prevent interactions with endogenous cPgp is the suppression of its expression with zinc finger nucleases. In contrast to a transient post transcriptional transporter knockdown with siRNA, ZFN offer the tremendous advantage of inducing a permanent gene deletion (10,11,31). Here, ZFNs were specifically designed for the sequence of the cPgp gene with strict avoidance of an overlap with the sequence of hPGP.

After successful identification of the ko-cell lines by PCR genotyping, the expression profile was studied by qRT-PCR and Western Blot analysis. All cell lines (MDR1_ZFN, MDCKII_ZFN and BCRP_ZFN) showed a significantly decreased expression of cPgp, although deletion was not absolutely complete. Quantitative analysis of the Pgp and Bcrp protein expressions of the various cell lines with LC-MS/MS by the QTAP technique demonstrated that the cPgp protein was specifically knocked down, the protein expression levels of cPgp left in the ZFN cell variants were sufficiently small compared to those of the transfected hPGP and hBCRP, and the expression levels of endogenous cBcrp were also negligible (Table I). In general, there is a possibility that quantitative values determined by using different peptide probes are not precisely identical due to the limitation for the accuracy of quantification. The difference between P-gp peptide-1 and -2 was 0.62 fmol/μg protein (3.66 – 3.04) in the monkey brain microvessels only expressing monkey P-gp. The difference in the MDR1_ZFN was 0.65 fmol/μg protein (6.70 – 6.05) and consistent to that in the monkey brain microvessels. This implies that the slight difference in the MDR1_ZFN could be caused by the limitation for the accuracy of quantification and the expression of cPgp protein was sufficiently knocked down. This was also suggested by the statistical analysis.

Several reasons may explain the residual expression of cPgp detected by qRT-PCR and Western Blots. Different transcription variants of the m*dr1*-genes are known within the animal family (canis lupus familiaris), from which the cell culture was orginally generated (www.ensembl.org). From these transcripts several translated proteins may result, which can be detected in Western Blots with a Pgp-specific antibody because of the presence of the same epitopes. As variants of the *mdr1*-gene, these transcripts have a very high sequence homology compared to the complete Pgp-mRNA, making it possible to detect them by qRT-PCR with the used primers. It may also be possible that single-nucleotide polymorphisms (SNP) occur. These SNPs may lead to an exchange of amino acids or a stop codon in the translated protein, which then loses its functionality. For the human MDR1-gene, several SNPs have been identified (32,33).

Another possibility is the presence of several copies of the *mdr1*-gene in the cell line (34–36). By treatment with ZFN, only one copy may be deleted and a residual activity may result in protein translation. In order to eliminate this residual activity, the cells need to be treated again with ZFNs. Genetic details of the MDCKII cell lines are not known in detail, but the original cell line was established in 1958. It was modified several times and several variants have been established (37). Our own work showed a relatively high heterogeneity, and, therefore, it is likely that over time, multiple allelity and gene copy variants of Pgp have evolved.

Even more important is the functionality of the new developed cell lines. To ascertain the improvement of the ko-cell lines bidirectional transport assays were conducted and net

efflux ratios (NER) calculated. A threshold of two was defined to identify substances as a substrate of the specific transfected human transporter using this transport assay.

Quinidine and vinblastine are well-known substrates of Pgp (38–41). The ko-cell lines MDR1_ZFN and MDCKII_ZFN demonstrated a strong improvement for both reference substrates. Especially vinblastine showed a very encouraging result. For vinblastine using the parental cell lines a NER below two was calculated and therefore showed a false negative result. In contrast, the NER for the ZFN-cell lines was about 30-fold higher and reliably identified vinblastine as a substrate for hPGP.

A similar result was obtained with paclitaxel and saquinavir. Both substances were previously described as substrates of Pgp (42–44). The parental cell lines showed a NER below two and the ZFN-cell lines clearly identified paclitaxel and saquinavir as substrates of hPGP. Sumatriptan was excluded as a substrate of Pgp with the ko-cell lines whilst the parental cell lines were not able to clearly exclude this compound. Referring to previous studies sumatriptan is not a substrate of Pgp (45). Nelfinavir demonstrated a similar result compared to quinidine with a strong increase of the NER using ko-cell lines and, according to the literature, nelfinavir is a substrate of Pgp (46). For all these substances, the NER for the parental hBCRP and the BCRP_ZFN cell line were quite similar and indicating none as a substrate of the hBCRP. Topotecan showed an increase in both ko-cell lines, MDR1_ZFN and BCRP_ZFN, identifying topotecan clearly as a substrate of PGP and hBCRP. The parental cell lines were not able to clearly identify this substance as a substrate. Topotecan is a well-known substrate of hBCRP, but is also a substrate of hPGP (43,47,48). Topotecan exemplifies the influence of the endogenous cPgp on the efflux transport results of human transport proteins. Due to its wide substrate spectrum Pgp often overlaps in recognizing substances with other transporters like Bcrp or Mrp2 (49–51). Atenolol as a paracellular marker showed the high integrity of all cell lines, so that changes of NER were not due to disruptions of the cellular monolayer (Table II). The $P_{app}$-values provided in Table II underline, that the increase of NERs occur because of less Pgp activity in the ko-cell lines. It has to be noted thought, that residual endogenous efflux was still observed for vinblastine and paclitaxel in the MDCKII_ZFN. More studies would be needed to clarify the nature of the efflux pumps and characterize in particular which efflux proteins might possibly interfere with these substances and accompanying with that the expression of the efflux protein in both cell lines, parental and ZFN, accountable for the residual activity.

In many studies, interactions between endogenous transport proteins, especially Pgp, and the transfected human transport proteins in animal cell lines were reported (9,25). The results of the present study underline these findings with more sensitive and improved results in the cell lines carrying a

**Table II** Efflux Values.

| Cell type | $P_{app}$ (A-B) +/− SD | $P_{app}$ (B-A) +/− SD | ER | Substance |
|---|---|---|---|---|
| MDR1 | 0.8 +/_ 0.03 | 0.65 +/_ 0.07 | 0.82 | Atenolol |
| | 5 +/_ 0.26 | 78.5 +/_ 2.93 | 15.77 | Quinidine |
| | 0.6 +/_ 0.27 | 19.3 +/_ 0.18 | 32.19 | Paclitaxel |
| | 1.7 +/_ 0.49 | 39.7 +/_ 1.99 | 23.25 | Vinblastine |
| | 1.1 +/_ 0.05 | 13.3 +/_ 0.72 | 12.28 | Topotecan |
| | 1.3 +/_ 0.2 | 50.9 +/_ 9.98 | 39.15 | Nelfinavir |
| | 0.5 +/_ 0.04 | 1.9 +/_ 0.2 | 3.8 | Sumatriptan |
| | 0.6 +/_ 0.16 | 14.2 +/_ 2.26 | 23.67 | Saquinavir |
| MDR1_ZFN | 1 +/_ 0.18 | 0.95 +/_ 0.06 | 1 | Atenolol |
| | 4.5 +/_ 1.3 | 78.3 +/_ 3.54 | 17.32 | Quinidine |
| | 0.4 +/_ 0.07 | 37.2 +/_ 0.72 | 93 | Paclitaxel |
| | 0.6 +/_ 0.18 | 77.4 +/_ 7.42 | 126.43 | Vinblastine |
| | 1.8 +/_ 1.4 | 16.7 +/_ 3.84 | 9.24 | Topotecan |
| | 0.8 +/_ 0.27 | 43.6 +/_ 2.75 | 54.5 | Nelfinavir |
| | 1.6 +/_ 1.4 | 2.4 +/_ 0.54 | 1.5 | Sumatriptan |
| | 0.5 +/_ 0.14 | 40.2 +/_ 2.7 | 80.4 | Saquinavir |
| BCRP | 0.5 +/_ 0.04 | 0.52 +/_ 0.12 | 1.15 | Atenolol |
| | 19.5 +/_ 2.37 | 35.6 +/_ 0.94 | 1.82 | Quinidine |
| | 0.5 +/_ 0.19 | 23.1 +/_ 1.03 | 46.89 | Paclitaxel |
| | 1 +/_ 0.82 | 23.3 +/_ 3.73 | 22.19 | Vinblastine |
| | 0.6 +/_ 0.1 | 11.4 +/_ 0.62 | 18.08 | Topotecan |
| | 2.6 +/_ 1.31 | 7.7 +/_ 2.5 | 2.96 | Nelfinavir |
| | 0.5 +/_ 0.22 | 0.6 +/_ 0.04 | 1.2 | Sumatriptan |
| | 0.9 +/_ 0.18 | 22.7 +/_ 6.81 | 25.22 | Saquinavir |
| BCRP_ZFN | 0.6 +/_ 0.08 | 0.51 +/_ 0.04 | 0.79 | Atenolol |
| | 37.5 +/_ 0.07 | 24.7 +/_ 2.29 | 0.66 | Quinidine |
| | 1.3 +/_ 0.28 | 14.7 +/_ 0.63 | 10.9 | Paclitaxel |
| | 4.7 +/_ 1.87 | 4.4 +/_ 0.25 | 0.94 | Vinblastine |
| | 1.2 +/_ 0.02 | 12.9 +/_ 0.53 | 10.59 | Topotecan |
| | 3.9 +/_ 0.45 | 2.2 +/_ 0.08 | 0.56 | Nelfinavir |
| | 1.1 +/_ 0.53 | 0.7 +/_ 0.06 | 0.64 | Sumatriptan |
| | 3.7 +/_ 0.36 | 6.1 +/_ 0.46 | 1.65 | Saquinavir |
| MDCK | 0.5 +/_ 0.11 | 0.54 +/_ 0.05 | 0.98 | Atenolol |
| | 21.2 +/_ 4.08 | 45 +/_ 2.53 | 2.12 | Quinidine |
| | 0.9 +/_ 0.31 | 33.1 +/_ 1.55 | 35.02 | Paclitaxel |
| | 2.2 +/_ 0.9 | 37.1 +/_ 8.28 | 16.88 | Vinblastine |
| | 1.6 +/_ 0.06 | 10.9 +/_ 0.66 | 6.97 | Topotecan |
| | 1.9 +/_ 0.68 | 11.3 +/_ 1.9 | 5.95 | Nelfinavir |
| | 0.6 +/_ 0.06 | 1.0 +/_ 0.09 | 1.67 | Sumatriptan |
| | 0.8 +/_ 0.3 | 31.3 +/_ 4.5 | 39.13 | Saquinavir |
| MDCK_ZFN | 0.8 +/_ 0.03 | 0.69 +/_ 0.17 | 0.85 | Atenolol |
| | 29.8 +/_ 0.94 | 21.9 +/_ 5.03 | 0.73 | Quinidine |
| | 1.1 +/_ 0.09 | 23.9 +/_ 4.58 | 20.8 | Paclitaxel |
| | 3 +/_ 0.59 | 12.8 +/_ 1.01 | 4.28 | Vinblastine |
| | 2.6 +/_ 0.76 | 3 +/_ 0.18 | 1.16 | Topotecan |
| | 5.2 +/_ 1.7 | 4.8 +/_ 3.17 | 0.92 | Nelfinavir |
| | 1.2 +/_ 0.5 | 2.8 +/_ 10 | 2.33 | Sumatriptan |
| | 3.2 +/_ 0.07 | 16.5 +/_ 5.76 | 5.16 | Saquinavir |

$P_{app}$-vales and efflux ratios (ER) of different substances for each cell line, parental and knockout. ($n = 3$, mean values ± SD)

genomic knockout of the endogenous canine Pgp. Thus, this methodology offers an opportunity to eliminate the problems caused by endogenous transport proteins in interpreting the results of exogenous transporter interactions.

Overall, these results demonstrate a significant improvement for the study of human transporter interactions in transfected cell lines. The higher reliability and sensitivity in testing substance interactions with specific human transport proteins was very well established in this work. In contrast, the parental cell lines mighty falsely identify compounds as either efflux substrate or non-efflux substrates. This rate of false results could be reduced with the ZFN-cell lines and improve the assessment of human transporter interactions.

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