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Research paper

Effect of cell differentiation and passage number on the expression of efflux proteins in wild type and vinblastine-induced Caco-2 cell lines

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Abstract

The mRNA level expression of MDR1, MRP1-6, BCRP and CYP3A4 was determined by quantitative PCR in wild type (Caco-2WT) and vinblastine-treated (Caco-2VBL) Caco-2 cells at different passage levels (32–53). Differentiation increased the mRNA levels of MDR1, BCRP and all the MRPs except MRP4. Corresponding mRNA levels were observed in Caco-2WT and Caco-2VBL, except that the expression of MRD1 was higher in Caco-2VBL than in Caco-2WT cells. CYP3A4 was barely detected in either cell line.

MDR1 functionality was studied using rhodamine123 and verapamil as a substrate–inhibitor pair. Corresponding to the observed differences in mRNA levels, MDR1 activity was higher in the Caco-2VBL cells. In Caco-2WT, MDR1 functionality was elevated at low passage numbers (32–35) compared to higher ones (49–53). Verapamil inhibited MDR1 efflux except at higher passage Caco-2WT cells, where no MDR1 activity could be observed.

The results support the use of Caco-2VBL cells in MDR1 screening. The functional expression is higher than in Caco-2WT and remains consistent across the studied passages without major differences in mRNA levels of other efflux proteins. As both the passage number and the level of cell differentiation affect the expression profile of efflux proteins, short-term cell growth protocols should be evaluated accordingly.

Keywords: Caco-2 cell line; Efflux protein; Expression; Functionality; MDR1; Cell differentiation; Passage number

1. Introduction

The epithelium of the intestinal mucosa is the first significant physical and biochemical barrier to limit drug absorption from the gastrointestinal tract. This barrier has an important role as a defense mechanism against potentially harmful substances, but it also limits the bioavailability of perorally administered drugs. In addition to passive permeation properties, active uptake and efflux systems as well as intestinal metabolic enzymes have been recognized to have a significant effect on the extent of absorption [1].

The multidrug resistance protein MDR1 (P-glycoprotein) is probably the most studied efflux transporter and, although initially discovered in tumor tissues, is also present in normal human intestinal epithelium, blood–brain barrier and hepatic canalicular membranes [2]. Its physiological function in the small intestine is to protect tissues from toxins or xenobiotics by limiting their absorption. MDR1 has also been observed to limit the oral bioavailability of several drugs by the same mechanism [3]. Inhibition and induction of MDR1 are potential mechanisms for drug interactions. The presence of MDR1 at

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important barrier epithelia that control drug absorption, distribution and excretion makes its substrates likely candidates for drug–drug interactions. There is also considerable interindividual variation in the expression of MDR1 as well as the other efflux proteins and metabolic enzymes and, thus, in their role in limiting oral absorption. This is due to polymorphism and/or variable exposure to inductive or inhibitory food components and medicines.

The clinical importance of the above-described defense mechanisms has led to the increased interest to develop *in vitro* methods that can be used during drug discovery and development in order to recognize e.g. MDR1 substrates. The Caco-2 is a widely used cell line derived from human colorectal carcinoma [4]. Permeability across fully differentiated Caco-2 monolayers is considered to model intestinal absorption, since the cells represent many of the characteristics and functions (i.e. transporters and metabolic enzymes) similar to the epithelium of the small intestine [5]. Caco-2 monolayers are used to identify drugs with potential absorption problems and to select clinical drug candidates based on absorption characteristics [6].

Expression of MDR1 has been demonstrated in Caco-2 cells, but several factors have been shown to influence its expression levels and/or functionality [7]. These factors include the age in culture and level of differentiation, passage number, and exposure to modulators, such as some MDR1 substrates [8,9]. Typically, the expression may be quite high in the lower passages and then decline at higher passage numbers [8]. Induction of functional MDR1 expression in Caco-2 cells has been successful by adding e.g. vinblastine or vincristine to the culturing medium [10,11]. Vinblastine-induced Caco-2 cell line has even been proposed as a platform for screening MDR1 substrates [12]. However, the expression of several other efflux proteins (MRP1-6, BCRP) has been observed in Caco-2 cell lines, but the results tend to vary between different laboratories and cell batches and even cultivation lots originating from the same batch [13–15]. This creates a need for careful characterization of the Caco-2 cells in use in terms of efflux protein expression and function in order to develop a reliable *in vitro* model to study efflux substrates.

In this study, we examined the effects of cell age (passage number) and level of differentiation on the mRNA expression levels of efflux proteins MDR1, MRP1-6 and BCRP in Caco-2 wild type (WT) and vinblastine-treated (VBL) cell models. As considerable overlap has been observed for substrates and inhibitors of efflux proteins, variability in the expression levels of efflux proteins would affect the suitability of the cell culture for efflux activity studies of any individual efflux protein. Of similar interest was the selectivity of the induction of MDR1 with vinblastine as the co-regulation of efflux proteins and metabolizing enzymes has been suggested [16–18]. Therefore, the mRNA levels of CYP3A4 were also studied. In addition, transport experiments with rhodamine123 across differentiated Caco-2 monolayers were performed to relate mRNA results with information on the functionality of the MDR1 in Caco-2WT and Caco-2VBL cells within the studied passage range.

2. Materials and methods

2.1. Reagents and materials

Cell culturing reagents were purchased from Euroclone (Pero, Italy) except for fetal bovine serum and HBSS/PBS 10× concentrates from Gibco Invitrogen Corporation (Carlsbad, CA, USA) and Hepes from Sigma (St. Louis, MO, USA). All the plasticware were obtained from Corning B.V. Life Sciences (Schiphol-Rijk, Netherlands). Rhodamine123 and vinblastine were from Fluka (Buchs, Switzerland) and verapamil from MP Biomedicals (Aurora, OH, USA). Materials and reagents for qRT-PCR were purchased from Applied Biosystems (Foster City, CA, USA), except for probes and primers for MDR1, MRP2, BCRP, CYP3A4 and β-actin, which were kindly provided by the University of Kuopio [19]. Reagents for qRT-PCR sample preparation were from Sigma, Ambion (Austin, TX, USA), Molecular Probes (Eugene, OR, USA), MBI Fermentas (Vilnius, Lithuania), Pharmacia Biotech (Amersham Biosciences AB, Uppsala, Sweden) and Promega (Madison, WI, USA). All the other chemicals were of analytical grade, in qRT-PCR also RNAse free.

2.2. Cell cultures

Caco-2WT (wild type) cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained at +37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity, in a medium consisting of DMEM (Dulbecco's modified Eagle's medium, high glucose 4.5 g/l), 10% HIFBS (heat-inactivated fetal bovine serum, inactivation at +56 °C for 30 min), 1% NEAA (non-essential amino acids), 1% L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The medium was changed three times a week during cell growth and differentiation.

The cells were grown in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. For transport experiments, the cells were seeded onto polycarbonate filter membranes (pore size 0.4 μm, filter area 1.1 cm^2) in 12-well Transwell® insert plates at 6.8×10^4 cells/cm^2 and grown for 21–28 days before the experiments [5].

Caco-2VBL (vinblastine-treated) cells were handled according to similar procedures as Caco-2WT cells, except that 10 nM vinblastine was added to the culturing medium. Since the initiation of vinblastine treatment substantially disturbed cell growth and attachment, Caco-2VBL cells were used for experiments only after their behaviour in culture had normalized, which took at least four to five passaging cycles (recuperation was determined by visual examination during the growth period).

The cells were studied at two levels of differentiation: undifferentiated (cultured to near confluency in flasks)

and fully differentiated (cultured on polycarbonate filter membranes for at least 21 days). The studied Caco-2WT cells were within the passage range P32...P53. For Caco-2VBL cells, the passage range was P33/9...P49/20, expressed as overall passage number of the respective Caco-2WT/passage under VBL treatment.

2.3. qRT-PCR

Total RNA was extracted from differentiated ($6 \times 1.1\ cm^2$ polycarbonate filters) and undifferentiated (75-cm^2 culture flasks) Caco-2WT and Caco-2VBL cells using TRI-Reagent® (Sigma) protocol. The samples were DNAse treated with Ambion's DNA-Free kit and total RNA concentrations were determined with RiboGreen RNA® Quantitation Reagent and Kit (Molecular Probes). Total RNA (2 μg/sample) was reverse-transcribed to cDNA with 30 U M-MuLV reverse transcriptase (Fermentas) in a reaction mixture containing 4 μl of 5× First-Strand buffer (Fermentas), 1 mM dNTPs (Fermentas), 3 μg $pd(N)_6$ primers (Pharmacia Biotech) and 20 U RNAse inhibitor (Fermentas) in a total volume of 20 μl. The cDNA was diluted to a concentration of 8 ng/μl for qRT-PCR.

The mRNA expression levels of MDR1, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, BCRP and CYP3A4 were determined with AbiPrism7500 system (Applied Biosystems) and normalized to β-actin using the comparative Ct (threshold cycle) method. FAM-labeled Assay on Demand TaqMan® Gene Expression Assay reaction mixes were used for MRP1 and MRP3-6. The primers and probes for MDR1, MRP2, BCRP, CYP3A4 and β-actin were kindly provided by the University of Kuopio [19]. The samples were analyzed in triplicate using TaqMan Universal PCR Master Mix and 40 μg cDNA in each 15 μl reaction mix. Negative controls for PCR mix, cDNA synthesis of each sample and cDNA synthesis reaction (without RNA) were included in all the qRT-PCR runs. The reaction profile consisted of 10 min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C, except for MDR1 and MRP2, for which an extra annealing step of 15 s at 55 °C was added to the cycle. Analysis of qRT-PCR data was performed using Qgene software (BioTechniques).

2.4. Transport experiments

Transport of a fluorescent MDR1 substrate, rhodamine123 (Rho123), was studied under sink conditions both in apical to basolateral (A → B) and basolateral to apical (B → A) directions. Before the experiments, the cell monolayers were washed twice with preheated HBSS–Hepes (10 mM Hepes, pH 7.4) and then equilibrated for 30 min at 37 °C. The integrity of the cell monolayers was verified by measuring transepithelial electrical resistance (TEER) across each monolayer before the experiment using Millipore Millicell®-ERS device (Bedford, MA, USA).

In the experiments, Rho123 in HBSS–HEPES was placed in apical (50 μM) or basolateral (5 μM) chamber. The initial concentrations were verified by immediately taking a sample from the donor compartment, so that 500 μl (A) or 1.5 ml (B) of Rho123 solution was present at the beginning of the experiment. Samples (400 μl apical, 1.5 ml basolateral) were taken from the receiver compartment at 15-min (A → B) or 5-min (B → A) intervals and replaced immediately with buffer. Sampling intervals and initial concentrations were optimised for each direction to ensure sample concentrations above the limit of quantification while maintaining sink conditions (no significant change in the amount of Rho123 in the donor compartment during the study). Preliminary experiments were performed in either direction at 5, 20 and 50 μM initial concentrations to ensure that the use of different apical and basolateral concentrations would not affect the observed efflux.

In the inhibition experiments, the donor solutions contained 200 μM verapamil in addition to Rho123 (50 μM A → B and 5 μM B → A). These experiments were conducted according to similar protocols as the other transport experiments.

Fluorescence of the samples was analyzed in 96-well plates using Wallac Victor® 1420 multilabel counter (Turku, Finland) at 485 nm excitation and 535 nm emission wavelengths. The analytical method was calibrated for each analysis over the concentration range 0.001–2 μM ($R^2 > 0.99$). Apparent permeability coefficients (P_{app}, cm/s) were calculated based on Eq. (1):

$$P_{app} = \frac{(dQ/dt)}{(A * C_0 * 60)} \quad (1)$$

where dQ/dt, cumulative transport rate (nmol/min); A, surface area of the cell monolayer (1.1 cm^2); and C_0, initial concentration in donor compartment (nmol/ml).

3. Results

3.1. Expression of the efflux proteins in Caco-2WT and Caco-2VBL cells

The expression of MRP1 was at about similar level in all the samples (Table 1). The expression levels of MDR1, MRP2, MRP3 and MRP6 were higher in differentiated than undifferentiated cells (Fig. 1). Interestingly, the mRNA level expression of MRP4 was lower in differentiated than undifferentiated cells. Differentiation did not affect the expression of MRP5, but it was somewhat higher in older passages. The expression of BCRP was somewhat lower in the undifferentiated cells. In contrast to differentiation, no obvious trends were observed between passage number and most of the studied efflux proteins (Table 1).

As expected, the mRNA expression level of MDR1 was constantly higher in the vinblastine-induced Caco-2 cell line (Caco-2VBL) than in Caco-2WT cell samples (Fig. 2). On all occasions, the levels were lower in the

Table 1
mRNA expression levels of MDR1, MRP1-6, BCRP and CYP3A4 vs. β-actin in differentiated and undifferentiated Caco-2WT and Caco-2VBL samples over the passage range typically used for transport experiments

Cell line	P	Diff	MDR1	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	BCRP	CYP3A4
Caco-2WT	32	D	6.3	17.0	56.2	75.8	66.4	3.0	50.1	74.7	0.02
Caco-2WT	33	D	3.8	7.4	166.6	46.4	20.3	7.3	40.4	45.0	0.18
Caco-2WT	43	D	8.3	10.0	100.4	64.0	37.2	4.3	36.3	53.1	0.41
Caco-2WT	32	U	0.7	4.0	17.4	0.7	25.8	4.3	6.9	4.9	0.0004
Caco-2WT	33	U	5.8	7.9	30.8	2.5	117.4	2.6	6.9	13.1	0.02
Caco-2WT	44	U	1.9	7.2	24.8	2.9	142.7	4.2	15.7	20.3	0.01
Caco-2VBL	42/13	D	21.2	8.9	181.3	63.9	21.6	3.6	33.2	36.2	0.17
Caco-2VBL	48/19	D	12.9	10.8	94.0	72.9	43.7	4.1	31.3	15.1	0.33
Caco-2VBL	35/11	U	10.9	6.8	30.9	2.7	162.8	2.5	10.1	17.0	0.03
Caco-2VBL	43/14	U	11.4	4.9	33.0	4.4	62.7	3.3	4.9	6.2	0.01
Caco-2VBL	49/20	U	9.7	12.6	16.2	9.2	181.8	7.5	19.6	13.9	0.04

Levels expressed as average ($n = 3$) normalized with β-actin $\times 10^3$.
P, passage number (for VBL-treated cells: overall passage of the respective Caco-2WT/passage under VBL treatment); D, fully differentiated cells, grown on inserts for 21–28 days; U, undifferentiated cells, grown to near confluency (7 days) in a culturing flask.

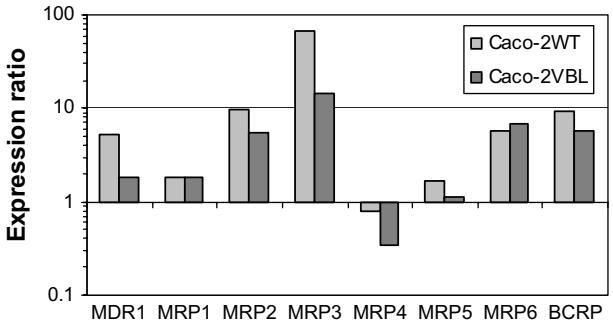

Fig. 1. The expression ratios of different efflux proteins in differentiated vs. undifferentiated Caco-2WT and Caco-2VBL cells. 1 = the same mRNA expression level in differentiated and undifferentiated cells.

undifferentiated than the respective differentiated sample. There was considerable variation between the different Caco-2WT cultivation lots, whereas the expression in Caco-2VBL cells remained more consistent. Vinblastine did not appear to affect the expression levels of any of the other studied efflux proteins or CYP3A4, except for a slight decrease in BCRP in differentiated Caco-2VBL cells compared to Caco-2WT. The mRNA expression of CYP3A4 was very low compared to the efflux proteins.

3.2. Functionality of MDR1: effect of passage number on the transport of rhodamine123

In Caco-2WT cells, rhodamine123 (substrate for MDR1 and BCRP) transport was observed to be higher in basolateral to apical ($B \rightarrow A$) than in apical to basolateral ($A \rightarrow B$) direction (permeability ratios ranging between 2 and 5 at passages <40), indicating some P-glycoprotein efflux activity (Fig. 3). The difference between $B \rightarrow A$ and $A \rightarrow B$ transport was greater in Caco-2VBL cells

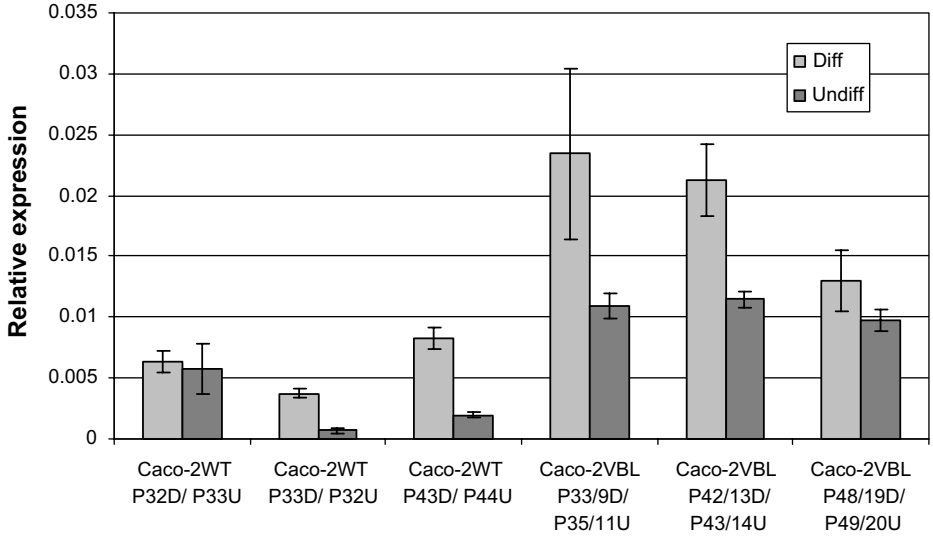

Fig. 2. mRNA expression level of MDR1 vs. β-actin in differentiated (D, Diff) and undifferentiated (U, Undiff) Caco-2WT and Caco-2VBL samples ($n = 3$) over the passage range typically used for transport experiments in our laboratory.

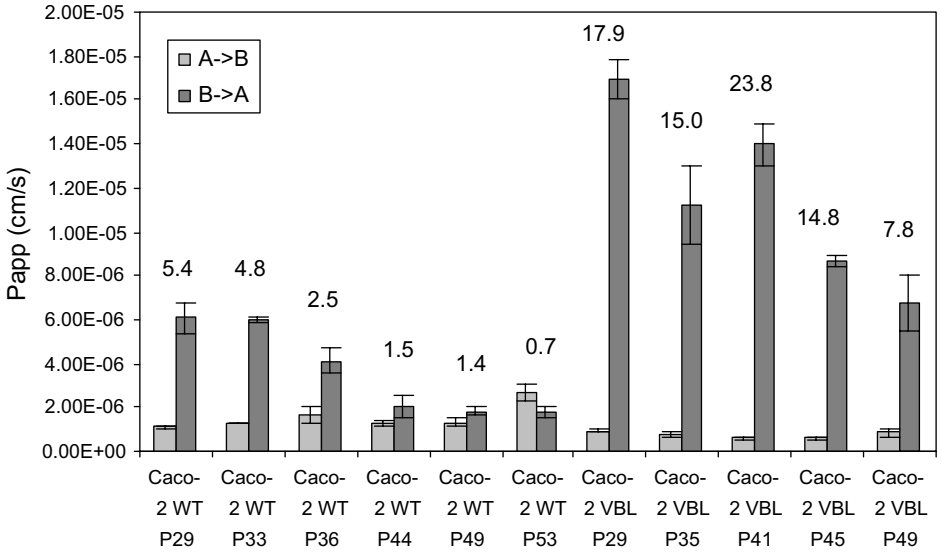

Fig. 3. The effect of passage number on the transport of rhodamine123 ($P_{app} \pm SD$, $n = 3$) across differentiated monolayers of wild type (WT) and vinblastine-induced (VBL) Caco-2 cells. Permeability ratios (B → A vs. A → B) for each pair of experiments are presented above the respective bars.

(permeability ratios of 8–24). These findings are consistent with the results of mRNA expression levels.

In Caco-2WT cells the functionality of MDR1 (P-glycoprotein) appeared to be strongly dependent on the passage number, although differences in the mRNA expression levels were less evident. The efflux activity was higher in wild type cells with low passage numbers compared to the later passages, as evidenced by higher permeability ratios at lower passage numbers (P29 > P36 > P44 > P49 > P53) (Fig. 3). This supports our previous findings, where loss of MDR1 activity was observed at high passage number Caco-2WT cells of the same origin [20]. In Caco-2VBL cells the effect of passage number was less evident and remained also at high passages above the activities observed even in lower passage WT cells, indicating a more consistent functionality of P-glycoprotein across all the passages.

Co-administration of 200 μM verapamil, inhibitor of MDR1, MRP1 and MRP3 but not MRP2 or BCRP [21–23], in the donor chamber in rhodamine123 transport experiments reduced the B → A transport of rhodamine123 in Caco-2VBL cells, indicating extensive inhibition of P-glycoprotein-mediated efflux (Fig. 4). In P53 Caco-2WT cells, the presence of verapamil had no significant effect on rhodamine123 transport, demonstrating the absence of functional P-gp in wild type cells at later passages.

4. Discussion

During the selection of clinical drug candidates, Caco-2 cell lines are often used as platforms for screening for potential interactions related to efflux mechanisms such as MDR1 (P-glycoprotein). However, considerable overlap

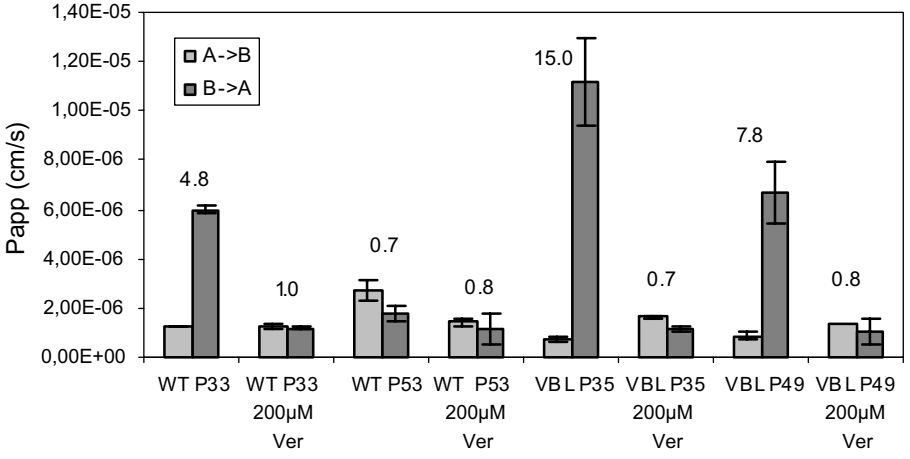

Fig. 4. The effect of presence of 200 μM verapamil on the transport of rhodamine123 ($P_{app} \pm SD$, $n = 3$) across wild type (WT) and vinblastine-induced (VBL) Caco-2 cell monolayers. Permeability ratios (B → A vs. A → B) for each pair of experiments are presented above the respective bars.

has been reported in the specificity of substrates and inhibitors of different efflux proteins and metabolizing enzymes. Therefore, an essential part of designing study protocols is to elucidate the effect of the growth protocol on the transporters involved in the system.

The present work showed that cell differentiation had a distinct effect on the mRNA level expression of the studied efflux proteins, especially in the wild type cells. In particular, many of the efflux proteins for which Caco-2 cells could provide a screening system appear to have up to 10-fold higher mRNA expression levels in the fully differentiated cells compared to the undifferentiated cells.

Overall, the results indicate that the expression of efflux proteins is higher in the differentiated cells (except for MRP4), which may have implications for higher throughput methods. Transport studies are usually conducted with fully differentiated cells grown on inserts for at least 21 days, while most of the current screening methods (e.g. Calcein AM test for MDR1) are based on shorter growth protocols, where the cells are grown for no more than 7–8 days [11,24–27]. The expression profile of different efflux mechanisms may then resemble that of undifferentiated rather than differentiated cells. This may in turn affect the sensitivity and specificity of efflux screening methods. Cell samples for transporter expression studies are also often collected from plastic flasks instead of permeable supports, which ensure enterocyte-like polarization of the cell monolayer by allowing uptake and secretion of molecules on both surfaces.

The passage number of Caco-2 cells has also been found to affect many of the features of the cell line, such as growth in culture, viability and efflux protein expression [8,28]. Therefore, a certain passage range (e.g. P30. . .P40) is normally used for the experiments conducted in any laboratory. Based on these results, the functionality of MDR1 is considerably lower in the Caco-2WT cells at passages 45+ than at around P30 in our laboratory. The mRNA expression of the efflux proteins varies and raises the need to thoroughly characterize the cell line and passage range in use in each laboratory.

A wide overlap is recognized in the specificity of substrates and inhibitors of e.g. MDR1, BCRP and the MRPs, as well as MDR1 and CYP3A4 [29]. For example, the apical MDR1, MRP2 and BCRP are known to act in cooperation, whereas the basolateral MRP3 may have the opposite effect [30,31]. Therefore, validation of study protocols for any efflux mechanism is typically conducted using a selection of compounds with different affinities to different efflux proteins. Another approach is to develop a system where the change in response is essentially due to the studied mechanism, e.g. use of cells or vesicles transfected with the desired efflux system or a cell line where the functionality of different efflux proteins has been well established.

Based on the mRNA expression level results, vinblastine treatment induces the higher expression of MDR1, while no obvious inductive effects on other apical or basolateral efflux proteins were observed. The activity of MDR1 is higher in Caco-2VBL than Caco-2WT cells and, due to the relatively consistent functionality across numerous passages, Caco-2VBL cells can be used over a prolonged passage span as compared to the wild type cells. In addition, based on the qRT-PCR results, vinblastine treatment does not induce any other apical or basolateral efflux mechanisms at mRNA level. Since the expression of CYP3A4 is insignificant in Caco-2 cells, its interplay with MDR1 will not confuse the studies, either. The presented results support the Caco-2VBL cell line as a very promising candidate for screening of potential MDR1 substrates, especially when higher throughput is desired.

The differences observed in the rhodamine123 efflux results and the effect of verapamil inhibition in Caco-2VBL and Caco-2WT cells should explicitly represent MDR1 activity, since verapamil does not inhibit MRP2 or BCRP, the other apical efflux proteins assumed to be active in the Caco-2 cells. What is more, the expression of neither MRP2 nor BCRP at mRNA level was higher in the Caco-2VBL than in the Caco-2WT cells. Therefore, the observed increase in apical efflux is due to the increase in MDR1.

Of the presently studied efflux proteins, the relationship between mRNA and protein levels and functionality has been studied in the case of MDR1 [32]. For MDR1, mRNA expression was found to correlate with protein abundance and efflux activity. On the other hand, MRP1 has been found in Caco-2 cells at protein level, but has been localized in cytosolic compartments instead of the basolateral membrane (as *in vivo*), suggesting that the mRNA expression may not be indicative of MRP1 activity [13,33]. However, these studies also showed localization of the MRP2-4 in Caco-2 monolayers reflective of the *in vivo* situation with a corresponding activity of at least MRP2 and MRP3. Since the mRNA expression levels of the other studied efflux proteins were not significantly affected by vinblastine treatment, it was assumed that their functionality would also be similar in Caco-WT and Caco-VBL cells.

Assuming that the mRNA levels of MRP2 reflect the presence of functional protein, the relative ease of culturing would make Caco-2WT the cell line of choice for the screening of e.g. MRP2 efflux substrates compared to Caco-2VBL. The expression levels of most efflux proteins are similar in Caco-2WT and Caco-2VBL, except for MDR1 and BCRP. The lower expression of BCRP in Caco-2VBL may increase the specificity of Caco-2VBL-based MDR1 screening methods, since the cooperation of these efflux mechanisms should be less significant. The qRT-PCR results also indicate a potential increase in MRP2 levels at higher passage numbers of Caco-2WT cells.

In conclusion, not only the choice of cell line, passage number and modulators but also the growth protocols and the level of differentiation of the cells at the time of study have a pronounced effect on the expression profile of efflux proteins and, thus, the specificity and sensitivity of screening methods. Therefore, the cell line used in efflux studies should always be thoroughly characterized at the relevant passage and level of differentiation.

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