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Selective Downregulation of the MDR1 Gene Product in Caco-2 Cells by Stable Transfection To Prove Its Relevance in Secretory Drug Transport

Constanze Hilgendorf,[†,‡,§] Hilde Spahn-Langguth,[†] Magdalena Rhedin,[§] Carl-Gunnar Regårdh,[‡] Björn Löwenadler,[§] and Peter Langguth*[,‡,||]

School of Pharmacy, Martin Luther University, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle/Saale, Germany, Department DMPK&BAC, AstraZeneca R&D, S-43183 Mölndal, Germany, Department of Molecular Sciences, AstraZeneca R&D, S-43183 Mölndal, Germany, and School of Pharmacy, Johannes Gutenberg University, Staudingerweg 5, D-55099 Mainz, Germany

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Abstract: Considerable interest is focused on overcoming multidrug resistance (MDR) in cancer chemotherapy. The in vitro experiments to characterize P-glycoprotein's (P-gp) function and to decrease its effects have led to a variety of strategies such as addition of competitors or supplementation of the medium with oligonucleotides complementary to the 5′-end of the MDR1-mRNA. For the Caco-2 cell line, an in vitro model for absorption screening, expressing multiple transporters including P-gp, which pumps substances back into the apical solution, P-gp activity might mask other relevant transport proteins' activity.The objective of the present study was to construct a Caco-2 subline with reduced P-gp expression level. Caco-2 cells were transfected by electroporation with two different mammalian expression vectors, and the obtained subclones were investigated at RNA (Northern blotting, RT-PCR), protein (FACS analysis), and functional (transport studies) levels for reduction in P-gp expression. Northern blotting showed that the levels of transcription of the inserted gene were different among the several clones, but those results did not completely correlate with the FACS analysis for P-gp expression. The clones with the strongest reduction in P-gp expression detected by the FACS analysis also showed the lowest secretory fluxes of the P-gp substrate talinolol in transport studies. Repetition of FACS analysis after 7 and 24 months on 20 to 30 passage older subclones still showed reduction in P-gp expression and indicated that they are stably transfected. The new cell lines constructed in the present study provide the possibility to perform in vitro absorption studies in a cell system composed of differentiated enterocytes growing as a monolayer like the normal Caco-2 cell line but with a lower down to almost lacking expression of P-gp.

Keywords: P-glycoprotein; P-gp-antisense DNA; MDR1-transfection; talinolol; Caco-2 cells; drug absorption; drug transport

Introduction

P-glycoprotein (P-gp) and its relevance in multidrug resistance in chemotherapy is a widely discussed issue,[1,2] and significant effort is being made worldwide to overcome this problem in cancer treatment. P-gp is an ATP-driven pump located at the apical membrane of epithelial cells of various organs (e.g., kidney, pancreas, testis, blood−brain barrier, and intestine) as well as in tumor cells. It supplies the cell with a potent mechanism to clear various xenobiotics

* Author to whom correspondence should be addressed: BioPharm & Pharm. Tech., Johannes Gutenberg University, Staudingerweg 5, D-55099 Mainz, Germany. Tel: +49-6131-392 5746. Fax: +49-6131-392 5021. E-mail: langguth@mail.uni-mainz.de.

[†] Martin Luther University.

[‡] Department DMPK&BAC, AstraZeneca R&D.

[§] Department of Molecular Sciences, AstraZeneca R&D.

[||] Johannes Gutenberg University.

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from cell lumen, resulting in decreased intracellular drug concentrations. P-gp accepts a wide variety of compounds with different chemical structures as substrates. This nonspecificity in substrate choice has been identified to be one of the causes of multidrug resistance (MDR) in cancer chemotherapy. Most therapeutic approaches to overcome MDR are mainly aimed at coadministration of drugs that bind to P-gp and block its excretory function. This is known for verapamil or cyclosporine, which themselves are pharmacologically active in concentrations necessary for P-gp inhibition.[3−5] New P-gp inhibitors have been tested in vitro[6−9] by several groups, and this research will be continued to find more specific inhibitors. Other approaches tested application of antisense oligonucleotides[10−13] and ribozymes[14] to reduce P-gp effects for an enhanced efficacy of cancer treatment.

Besides this frequent influence on cancer treatment, P-gp has turned out to influence drug oral absorption and pharmacokinetics[15−17] in the past few years due to its location in the apical membrane of intestinal epithelial cells. To monitor the influence of P-gp on the behavior of the compound of interest, the most common studies are intestinal perfusions and cell culture transport studies in the absence or presence of an inhibitor of the active secretion process.[18,20] As an in vitro model for the intestinal epithelium, Caco-2 cells are the most commonly used cell line as a high-throughput preclinical and in vitro screening method.[18,19] Caco-2 cells express a considerable amount of P-glycoprotein under standard culture conditions,[20−22] which makes them a widely used tool for investigations dealing with this transport system. However, with respect to quantitative measures, the contribution of one secretory process to the overall membrane permeability is hard to estimate with the help of chemical transport inhibitors and may cause problems in estimation of relative importance of different absorption and secretion pathways in humans from in vitro studies.

Studies on the therapeutical use of antisense oligonucleotide conjugates in the treatment of MDR had yielded a downregulation of the MDR1 mRNA expression to 10% of its control values.[23]

Therefore, in this study a transgenic Caco-2 cell line with all of the advantages of the parent cell line, such as growth as a polarized monolayer and expression of diverse trans-

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porters resembling the small intestinal phenotype, but with a reduced expression of P-glycoprotein has been created. This should help interpret drug permeability data where P-gp is involved among other active transport systems.

Caco-2 cells were transfected by electroporation with P-gp-antisense DNA, and clones were checked for their P-gp expression levels by RT-PCR or Northern blotting and flow cytometry. Transfected clones expressing antisense RNA were investigated for their suitability for transport experiments.

Materials and Methods

Materials. Cell culture media were purchased from Life Technologies, Paisley, U.K. Other cell culture materials were obtained through KeboLab, Göteborg, Sweden, and transwell cell culture dishes were supplied by Corning Costar. Molecular biology single-use plastic ware was purchased from Sarstedt, Nürnbrecht, Germany. Hybond-N membranes and ^{32}P-labeled CTP were purchased from Amersham, Buckinghamshire, U.K. All other chemicals were purchased from Sigma, Malmö, Sweden, or BDH, Poole, U.K.

Enzymes were obtained from Boehringer Mannheim, Mannheim, Germany, or Biolabs, Beverly, MA. The mammalian expression vectors used were from Promega, Madison, WI.

^{3}H-Talinolol was from J. Kix (Volxheim, Germany; www.isotopes.de).

Cell Line and Culturing Conditions. Caco-2 cells were obtained from the ATCC, Rockville, MD, and routinely grown in 75 cm^2 flasks in Dulbecco's modified Eagle medium containing 16.5% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. They were routinely subcultured once a week with trypsin−EDTA (0.25%, 0.3 mM) and seeded at a density of 4×10^5 cells per flask. In the experiments cells with passage numbers from 75 to 90 were used. For transport studies cells were seeded at a density of 3×10^5 cells per 24 mm polycarbonate culture insert (0.4 μm pore size) or at 1×10^5 cells per 12 mm filter. The medium was changed every other day.

Subclones obtained by transfection were cultivated in the same manner. Comparative studies on cell growth (number of cells per well under standard conditions) and TEER development ($\Omega \times$ cm^2) were performed during a period of 14 days. Confluence was reached to an extent of 80−90% after approximately 5 days in culture.

Plasmid Construction and Transfection. The human MDR1-cDNA[24] was obtained from the ATCC, Rockville, MD, ligated into two different mammalian expression vectors pMAM.neo and pEUK-C1, and transformed into *Escherichia coli*, according to standard procedures.[25] Briefly, the MDR1-cDNA was *Xba*I cut and ligated into the *Xba*I cloning site of the pEUK-C1 expression vector or the *Nhe*I-cut pMAM.neo expression system. The new plasmids were amplified in *E. coli*, and sense (MDR) and antisense (RDM) oriented plasmids were identified with restriction analysis and verified by sequencing on a 373 DNA sequencer (Applied Biosystems, Perkin-Elmer, Foster City, CA). The pEUK-C1-RDM plasmid contained an insert of cDNA identical to the positions 4487−327 of the consensus sequence of MDR1 ligated into the *Xba*I cloning site.

Table 1. Caco-2 Cell Lines Obtained by Transfection with Plasmids from pMAM.neo and pEUK-C1

plasmid	cloned cell lines	no. of colonies
pEUK-C1-RDM (cotransfected with pMAM.neo as G418-resistance donator)	Caco-2-R*n*	35
pMAMneo-RDM	Caco-2-RM*n*	18
pMAMneo-MDR (control)	Caco-2-S*n*	18

Prior to transfection into Caco-2 cells pMAMneo-RDM was linearized by a *Mlu*I cut. As the second vector system based on the pEUK-C1 vector does not contain any antibiotic resistance genes,[26] cotransfection with the pMAM.neo vector was performed. The plasmids were linearized prior to transfection with the restriction enzymes *Pvu*I (pEUK-C1-RDM) and *Nde*I (pMAM.neo), respectively.

Caco-2 cells were grown to 80% confluence, harvested by regular trypsinization, and transfected by electroporation with a GenePulser apparatus (BioRad, Hercules, CA) with a 250 V pulse at 960 μF. From the transfectants several clones were established as G418-resistant cell lines. As there was no experience in electroporation of Caco-2 cells, different voltages were used to compare mortality and efficiency (Table 2). Mortality increased in a linear manner with increasing voltage from 230 to 270 V, but higher voltage resulted in relatively more positive (Geneticin resistant) clones. For this reason for the following transfections a voltage of 250 V was used.

Cells were seeded in 100 mm dishes, and after 24 h G418 (Geneticin, Gibco-BRL, Täby, Sweden) was added as selective agent at a concentration of 950 μg/mL, which was determined to be the minimal lethal concentration for untransformed cells (data not shown). The medium was changed every other day and supplemented with fresh G418.

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Table 2. Transfection Efficiency (as Number of Resistant Clones from 1 × 10^6 Cells 14 Days after Transfection) and Mortality (%) as a Function of the Voltage Applied during Transfection by Electroporation in Caco-2 Cells

voltage at 960 μF	transfection efficiency: no. of clones	mortality [%]
230	22	60
250	31	68
270	86	95

Single colonies were picked from the dishes after 2 to 3 weeks and transferred to 24 well plates. Clones derived from pEUK-C1-RDM transfection were termed Caco-2-R*n* (*n* = number of clone), and cells derived from pMAMneo-RDM gave the Caco-2-RM*n* cell lines (Table 1). As control experiment Caco-2 cells were also transfected with a plasmid containing the normal MDR1 gene. Sublines which developed promisingly were grown to higher cell numbers and examined for P-glycoprotein expression on mRNA, protein, and functional levels.

Northern Blotting and RT-PCR. Total RNA was isolated from Caco-2 cells and the transfectants by the guanidinium-thiocyanate method (Trisolv, Biotecx Laboratories Inc., Houston, TX). Each RNA sample was analyzed by Northern blotting in duplicate. Briefly, 10 μg of each RNA was separated on two 6% formaldehyde−1% agarose gels and blotted onto Hybond-N transfer membranes by vacuum blotting at 4 bar under 10× SSC buffer (VacuBlot, BioRad). On one membrane the introduced antisense RNA was detected with a ^{32}P-labeled riboprobe, and on the other membrane it was probed for the expression of the sense MDR1-mRNA. Probe synthesis was carried out with linearized pGEM3Zf(−)Xba-MDR1 vector as a template (cut with *Dra*I for the sense probe and with *Msc*I for the antisense probe) using the Promega Riboprobe SP6/T7 Kit (Promega, Madison, WI). Hybridization was for 24 h at 65 °C in 50% formamide, 10% 0.5 M PIPES (pH 6.5), 25% 20× SSC, 2% 0.5 M EDTA (pH 8), 5% 50× Denhardt's, and 0.1 mg/mL salmon sperm DNA. Washing was performed in two steps of 5 and 50 min with 0.2× SSC and 0.1% SDS at 65 °C. The filter was exposed to a Kodak-XAR film with an intensifying screen at −80 °C.

RNA from transfectants bearing the pEUK-C1-derived DNA was analyzed by RT-polymerase chain reaction (RT-PCR). This vector system contains a 935 bp intron downstream of the SV40 late promoter, allowing discrimination between the transcribed mRNA and the cloned plasmid. RT-PCR was carried out using specific primers (forward primer 5′-CGCCAGGCCTCCGTTAAGGT-3′ and reverse primer 5′-TTTAACATTTCCTCAGTCAAGT-3′) synthesized by SGS (Köping, Sweden). As controls primers for the ubiquitous gene of the human glycerylaldehyde 3-phosphate dehydrogenase (GAPDH) were used for verification of the RT process. The transfection vector in the presence of genomic background was always used for verification of the PCR process. The reaction was performed in 50 μL of 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). Forty cycles were run with the following cycle conditions: 60 s at 96 °C, 60 s at 50 °C, and 90 s at 72 °C. The respective products were 1275 (from the vector including the intron sequence), 342 (from anti-MDR1-RNA), and 280 bp (GAPDH) DNA fragments.

Flow Cytometry. Caco-2 cells and Caco-2 transfectants were grown for 5 days to 80−90% confluency and trypsinized carefully. Labeling was performed according to the protocol of Anderle et al.,[27] yet with slight modifications. Cells were washed once with PBS and then resuspended in PBS containing 5 mM D-glucose and 1% (m/v) bovine serum albumin (PBS-BSA-G) to 2 × 10^6 cells in 100 μL. They were incubated with 5 μL of MRK16 anti-human P-glycoprotein monoclonal antibody (Kamiya Biomedical Company) for 30 min on ice. As controls, cells (a) without any antibody labeling, (b) only stained with the secondary antibody, and (c) incubated with 2.5 μL of IgG2α isotype control were prepared. After washing with PBS-BSA-G, cells were resuspended in 100 μL of PBS-BSA-G, mixed with 2 μL of goat anti-mouse Ig-FITC, and incubated on ice for another 30 min. After subsequent washing, labeled cells were resuspended in 500 μL of PBS-BSA-G. Flow cytometry was run on 10000 cells using a FACSCalibur (Becton-Dickinson, Glostrup, Denmark).

Talinolol Transport Characteristics and Influence of Verapamil on Its Effective Permeabilities in Different Anti-MDR1-Transfected Subclones. In all transport experiments the hydrophilic β-blocking agent talinolol was used as a model compound, since it had previously been shown to be a P-gp substrate with an efflux ratio in Caco-2 cell monolayers significantly higher than unity.[15] All cell monolayers were used after 14−18 days in culture. During this time, development of the monolayer was monitored by TEER (transepithelial electrical resistance) measurements and visual control under a light microscope (observation of cells seeded parallel on a regular 6-well plate). Hanks' balanced salt solution (HBSS, obtained from Gibco LifeTechnologies) supplemented with 10 mM MES adjusted to pH 6.5 was used throughout as transport medium. Each experiment was performed in triplicate. Talinolol donor concentrations were 500 μM racemic compound. Bidirectional transport studies were performed for selected subclones (R11, R19, R34, RM12, RM13, RM15, parental Caco-2 as control). Transport inhibition experiments, employing 500 μM verapamil as secretion inhibitor, were performed for R11, R19, R34, and RM12.

Sampling and Drug Assays. Samples were taken from the respective donor compartment at the beginning and the end of the experiment. Samples of 600 μL from the acceptor

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compartments were taken at time points of 0, 15, 30, 45, and 60 min, and the volume was replaced by fresh HBSS. The integrity of the monolayer was checked at the beginning and the end of the experiment by measurement of the TEER and ^{14}C-mannitol flux. Mannitol flux was determined by liquid scintillation counting (WinSpectral 1410, Wallac, Turku, Finland). Validation parameters included a relative standard deviation of less than 3%.

Talinolol concentrations were determined by HPLC analysis following enantiomeric separation performed on a Chiraspher NT, 250 × 4 mm i.d. (Merck, D-Darmstadt). The mobile phase was a mixture of ethanol/triethylamine (1000: 0.5, v:v) at a flow rate of 0.45 mL/min. Sample preparation was modified from Wetterich.[28] The HPLC system was composed of a Jasco PU-980 intelligent pump (Jasco, D-Gross-Zimmern), a Jasco 851-AS intelligent autosampler, and a Jasco UV-975 UV/vis detector. UV absorbance was monitored at 245 nm. Data acquisition and integration was carried out using Borwin software (JMBS Developments, Le Fontanil, France).

Validation yielded precision and accuracy below 8% over the calibration range and below 15% at the limit of quantification (2.5 ng/mL).[29] Intraday coefficients of variation were always below 9%.

In order to cross-validate the results for unlabeled talinolol, a set of a → b and b → a transport studies through Caco-2 cell monolayers were performed as described above at initial total concentrations of 100, 500, and 1000 μM talinolol, yet containing 5% *rac*-^{3}H-talinolol (specific activity: 27 Ci/mmol) at the respective donor side. LSC data did not deviate more than 7.4% from HPLC data, indicating the reliability of the enantiospecific method and the possibility to use labeled compound for faster sample analyses when total talinolol levels as opposed to drug enantiomer levels are of interest. For both methods, accuracy and precision were acceptable and in the same range as described in previous studies.

Permeability Calculations. Transport Parameters. The effective permeability coefficients were calculated from concentration−time profiles of the respective data according to the following equation:

$$P_{\text{eff}} = \frac{\frac{dC}{dt}V}{AC_0} \qquad [\text{cm/s}]$$

where dC/dt is the flux across the monolayer (mM/s), V is the volume of the acceptor chamber (cm^3), A is the surface area of the monolayer (cm^2), and C_0 is the initial concentration (mM) in the donor compartment. The flux across the monolayer is calculated from the slope of the amount transported versus time.

Efflux ratios were calculated as $(P_{\text{eff,ba}})/(P_{\text{eff,ab}})$. The efflux ratio represents a measure of the extent of directed transport (efflux ratio = 1 means no difference between a → b and b → a transport; efflux ratios higher than 1 point to higher b → a than a → b transport rates = net efflux).

The net secretory flux was calculated as difference between b → a and a → b transport.

Results

Anti-MDR1-Transfection, Cell Growth, and Antisense mRNA Detection. The expression vector pMAM.neo contains a G418 resistance gene, which helps to select mammalian cells after transfection with the gene construct in the presence of G418 (Geneticin) in the growth medium. For selection of the Caco-2 clones (Table 1), which have successfully taken up the new plasmid, the macrolide-type antibiotic G418 was used at a concentration of 950 μg/mL medium.

Clones were monitored in terms of Caco-2 like growth in a monolayer, displaying polygonal shape and forming the typical domes of epithelial cell monolayers, i.e., blisterlike structures where some adjacent cells of the monolayer form water-filled cysts, which can be observed for original Caco-2 cells. Epithelial cultures resembling clones were investigated for their expression of the antisense RNA with a reduction of P-glycoprotein expression. Transcription of the antisense gene was investigated by means of Northern blotting and RT-PCR. The expression of sense MDR1 mRNA in the transfectants was also investigated by Northern blot. The extent was unchanged in the untransfected Caco-2 cells whereas a higher extent was seen in cells transfected with a vector containing the sense MDR1 gene (Figure 1). This gives evidence that the expression vectors work satisfactorily in Caco-2 cells. In antisense transfectants the autoradiography band of the antisense mRNA is expected in the same region on the filter membrane. The rate of expression of this transcript was not constant among the cell lines transfected with a given plasmid (Figures 1 and 2). Because all lanes were loaded with 10 μg total RNA, after the blot the relative amount of mRNA was estimated. It was found that the amount of antisense mRNA varied among the positive clones, ranking with a descending order from clone RM6, RM3, R11, RM12, RM15, R34.

RT-PCR on RNA from pEUK-C1-transfection vector derived cells in most cases did not give a link to positive clones, though the control reactions both worked satisfactorily (Figure 3). Only clones R11 and R34 were clearly verified as positively bearing the antisense DNA and transcribing it into mRNA (lanes 2 and 5, Figure 3B). This experiment was performed on mRNA of early passages (passages 4 and 5) and 7 months as well as 24 months later on late passages (Caco-2-R11 passage 27, Caco-2-R34 passage 38), in order to investigate stability of expression.

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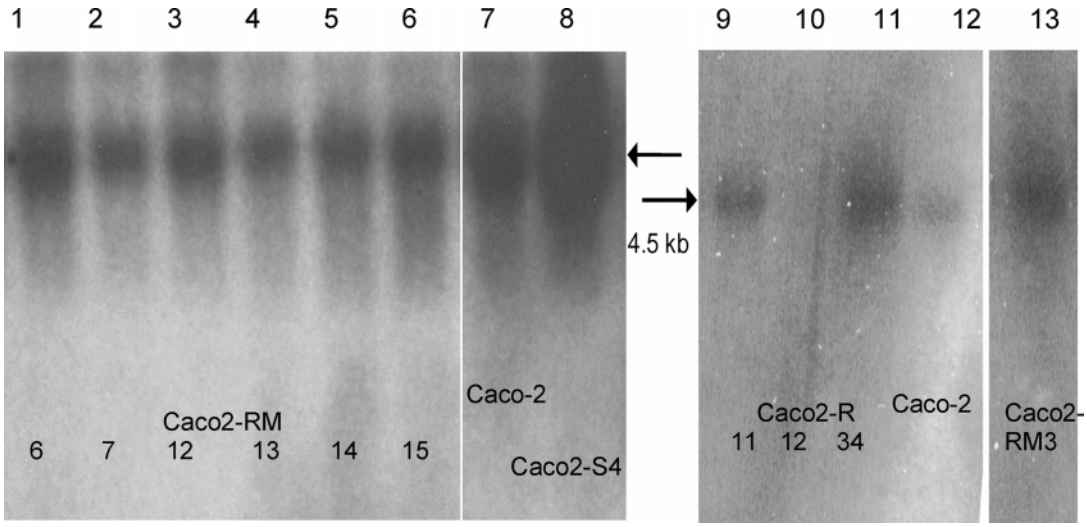

Figure 1. Northern blots of total RNA isolated from transfectants (lanes 1−6, 9−11, and 13), untransfected Caco-2 cells (lanes 7 and 12), and cells transfected with a vector containing the sense MDR1 gene (lane 8). In all lanes the approximately 4.5 kb mRNA coding for the MDR1 gene product P-glycoprotein was found to the same extent in antisense transfectants and parent Caco-2 cells and to a higher extent in sense-transfected cells (S).

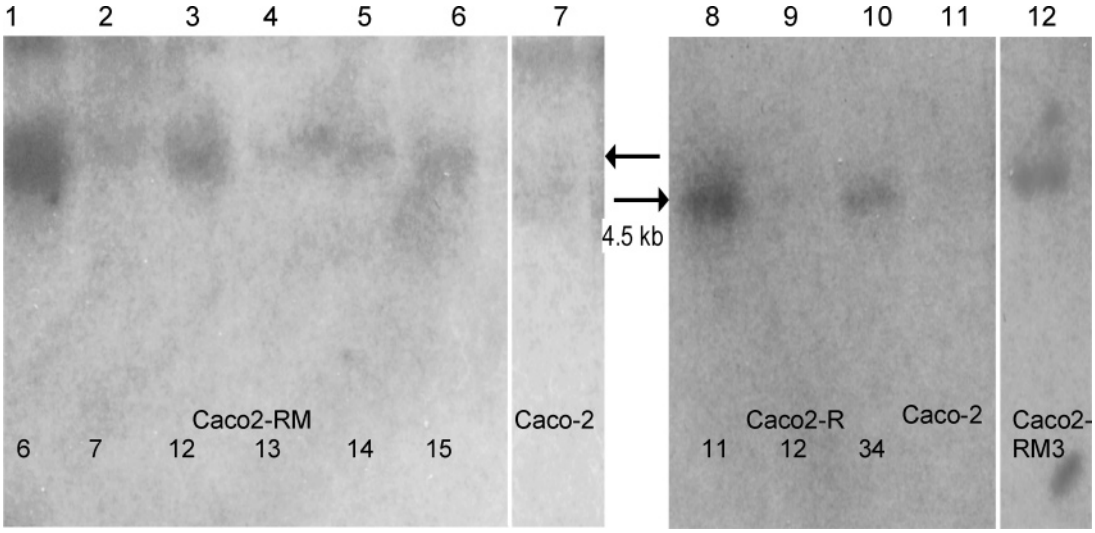

Figure 2. In Northern blots of total RNA isolated from transfectants (lanes 1−6, 8−10, and 12) the autoradiography band of the antisense mRNA showed in the same region on the filter membrane as the MDR1-RNA (arrows). The rate of expression of this transcript decreases from clone RM6, RM12, R11, RM3, RM15, to R34. Nontransfected Caco-2 cells (lanes 7 and 11) do not give any autoradiographic signal, as they do not carry the antisense MDR1 transcript.

FACS Analysis. FACS analysis on the clones showing a high level of antisense MDR1 mRNA expression in the Northern blot gave the result that the amount of mRNA detected in the blot did not correlate with the reduction in P-gp expression at the protein level (Figures 4 and 5). Clone Caco-2-RM6, with the strongest band in the Northern blot, did not show any difference from nontransfected Caco-2 cells in the FACS analysis (figure not shown) whereas Caco-2-R34 cells had a significantly reduced P-gp expression though their antisense MDR1 mRNA expression did not seem to be very high. Repetition of FACS analysis after 7 months on later passages (passage 27, passage 36, as well as passage 44) was performed to compare the results with the early passages and to determine long-term downregulation in protein expression levels. After this period of time there is still a significant decrease in P-gp expression levels, so clones Caco-2-R11 and Caco-2-R34 are to be considered as stably transfected.

Characterization of Cell Growth and TEER. TEER development during a 2-weeks' period and growth characteristics are summarized in Table 3. The results indicate higher TEERs for higher passages, but an overall comparability of TEER development for parental and transfected cells. Also the comparison of growth characteristics yielded comparable cell number ranges.

Transport and Transport Inhibition in Monolayers. Transport characteristics of the clones investigated so far show that antisense transfection results in reduced secretory

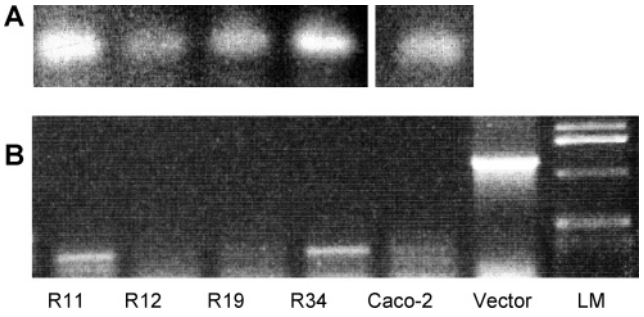

Figure 3. RT-PCR reactions on mRNA from Caco-2 cells and subclones from the Caco2-R series, generated by transfection with antisense gene cloned into the pEUK-c1 vector. (A) Positive control shows the amplified product of PCR with primers for the ubiquitary GAPDH-gene. (B) Specific bands (arrow) of RT-PCR on the antisense P-gp mRNA were found in clones R11 and R34. The clones R12 and R19 and parental Caco-2 cells did not show this amplification product. The positive control PCR on the pEUK-c1-RDM vector gives a product 965 bases longer, as the vector contains an intron. (LM = ladder marker, representing different molecular lengths).

flux of P-gp substrates compared to parent Caco-2 cells (for examples, see Table 4). P_{eff} values for both transport directions are summarized in Table 4. For the investigated subclones R11, R19, and R34, yet not for RM12, RM13, and RM15, apparent permeability of a → b transport was higher than that observed with control Caco-2 cells, while the TEER was in a comparable range. For b → a transport P_{eff} values were smaller for all subclones except for RM12. Addition of verapamil always reduced b → a and enhanced (except for R19) a → b fluxes. There appeared to be a tendency for measured TEER values to be smaller when verapamil was added as transport inhibitor.

Discussion

P-glycoprotein's presence in Caco-2 cells has been reported by numerous groups,[3,16,20,26] and there is evidence that its transport function influences permeation studies for several kinds of compounds, e.g., calcium antagonists such as verapamil, cytostatic drugs (vinblastine), or β-blocking agents (celiprolol, talinolol). Extensive studies with Caco-2 cells revealed, as well, that P-glycoprotein expression levels were influenced by culturing conditions and media composition.[27] P-gp levels varied with time in culture, in a way that cells with higher passage numbers had higher P-gp levels. In contrast, subculturing at late confluency resulted in decreased P-gp. Addition of P-gp substrates, e.g., vinblastine, to the culture medium led to an increase in transporter numbers on the cell surface.[27,30] Consistency in culturing and experimental conditions seems to be crucial for reproducibility in Caco-2 permeation experiments.

Table 3. Overview on TEER Development and the Growth Characteristics of Anti-MDR1-Transfected as Well as Parent Caco-2 Cells

A. TEER Development

days in culture	TEER [Ω cm²]	
control Caco-2	p75	
4	555 ± 98	
10	974 ± 83	
12	1068 ± 50	
15	1127 ± 58	
18	1194 ± 61	
26	1167 ± 57	
Caco-2-R11	p15	p28
4	495 ± 102	795 ± 102
10	824 ± 66	642 ± 57
12	802 ± 72	1066 ± 50
15	788 ± 89	996 ± 65
18	669 ± 48	898 ± 71
26	597 ± 97	795 ± 102
Caco-2-R34	p16	p44
4	807 ± 93	1557 ± 163
10	1188 ± 105	1489 ± 85
12	1099 ± 87	1572 ± 79
15	1205 ± 93	1634 ± 68
18	1100 ± 136	1535 ± 227
26	1073 ± 151	1301 ± 144

B. Growth Characteristics

days in culture	cells per well (×106)
Caco-2	
4	0.26 ± 102
10	1.45 ± 66
12	2.20 ± 72
14	3.99 ± 89
Caco-2-R11	
4	0.31 ± 102
10	2.43 ± 66
12	4.18 ± 72
14	4.85 ± 89
Caco-2-R34	
4	0.28 ± 102
10	2.84 ± 66
12	3.17 ± 72
14	3.97 ± 89

The results of the present studies show clearly that the antisense approach to decrease P-glycoprotein levels is a possibility to modify Caco-2 cells in their expression pattern. A stably transfected Caco-2 cell line with lower P-glycoprotein expression can give more insight into contribution of P-gp in intestinal absorption processes, and permeabilities obtained from those cells can help unraveling other transporters accepting the same substrates. Especially basic compounds which appear to have a low permeability in regular permeation studies often are suspected to be substrates for P-gp. A transgenic Caco-2 cell line with low P-gp expression level may help to complement the results obtained

(30) Döppenschmitt, S.; Spahn-Langguth, H.; Regardh, C. G.; Langguth, P. Radioligand binding assay employing P-gp overexpressing cells: Testing drug affinities to the secretory intestinal multidrug transporter. *Pharm. Res.* **1998**, *16*, 1067−1072.

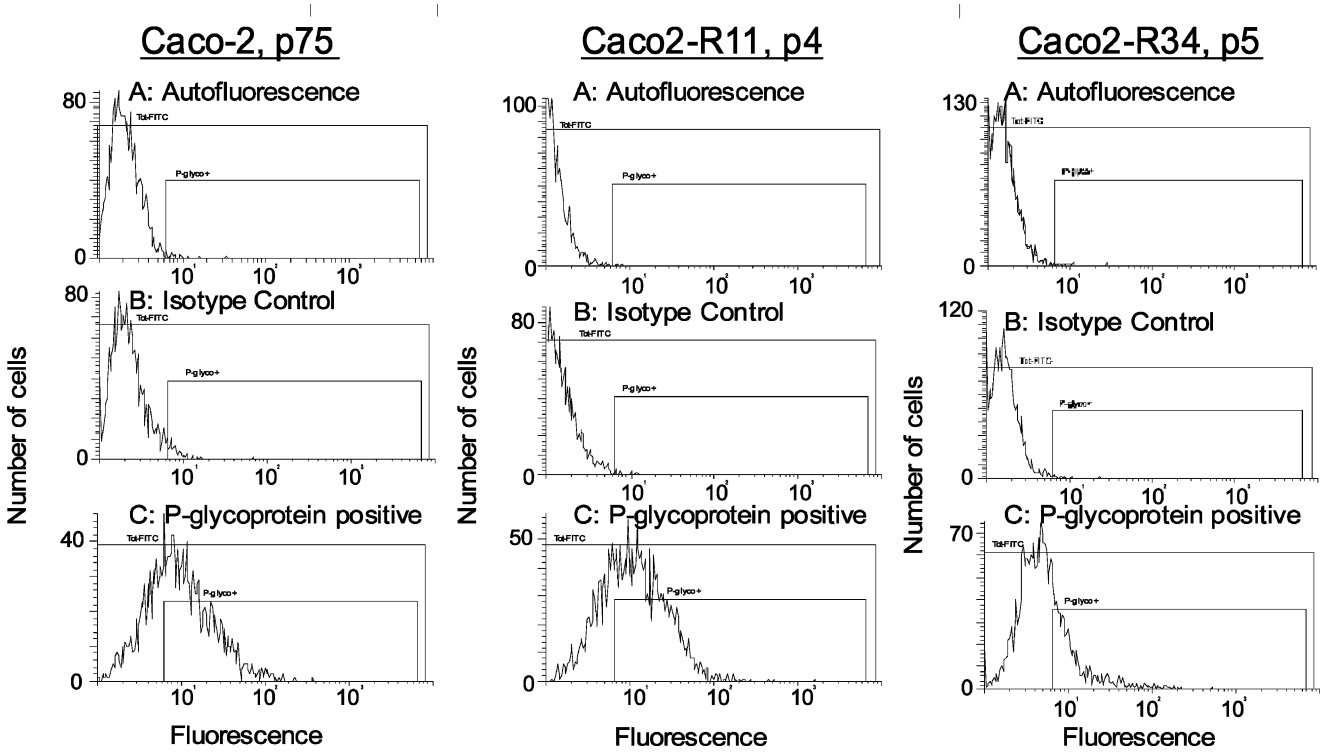

Figure 4. FACS analysis of Caco-2 cells, p75 (left), and the anti-MDR1-transfectants Caco-2-R11, p4 (middle), and Caco-2-R34, p5 (right), for the expression of P-glycoprotein. The cells were analyzed for their autofluorescence (A), incubated with an unspecific mouse-antibody and FITC-labeled anti-mouse-antibody (isotype control, B), and specifically labeled with the monoclonal antibody MRK16 and FITC-labeled anti-mouse-antibody (P-gp expression, C). Autofluorescence and isotype control analysis did not reveal significant numbers of cells that would be considered P-gp positive (1.2% and 5% of the cells respectively appear in the gate "P-glyco+"). Clone R11 shows only minor reduction in P-gp expression levels 74.9% of the cells detected as "P-glyco+"), compared to nontransfected cells (79.4%), while the P-gp protein expression on clone Caco-2-R34 was significantly reduced (41.8%).

in parent Caco-2 cells to get a quantitative estimate of the physiologically relevant contribution of P-gp to epithelial membrane permeability of the respective compound.

The observed variability in expression level of the antisense MDR1 RNA suggests that the expression of the gene might depend on the site of integration of the plasmid in the host genome. In a second step after transcription the MDR1-mRNA and the inserted complementary antisense construct had to pair in a way suppressing further protein synthesis. To achieve this, hybridization had to be stable and there had to be equal numbers of transcripts of the regular sense and the antisense gene. Translation from heterodimers from antisense and target mRNA is less efficient or abolished.[31] From studies with antisense oligodeoxynucleotides (ASO) it is known that the dimers formed from ASO and mRNA are degraded by ribonucleases.[31] Hence, we can assume that the reduction in P-gp levels in several of our clones is due to a reduced translation effectiveness of the MDR1-mRNA.

Other approaches to reduce P-gp effects in cell culture models have been undertaken with ASO treatment.[13] Some success in terms of decrease in P-gp expression levels was reported, but the stability of ASO within the cells was limited and, furthermore, the reduction in MDR1-mRNA translation is reversible.[13] FACS analysis of parent Caco-2 cells shows variation in P-gp expression among cells within the same population. The idea of selecting a subclone by limiting dilution has been tested by Woodcock and co-workers[32] for Caco-2 cells with increased taurocholic acid transport, but these authors reported that the subclone still had the potency to vary in expression of the transport protein.

Although creation and identification of Caco-2 clones with stably reduced P-gp expression levels through antisense transfection is time-consuming and requires specific equipment, the availability of a stably transformed cell line allows systematic investigations in a homogeneous cell system. The created clones can be used over at least 30 passages as a complementary in vitro model to the established Caco-2 cell line. The good comparability of the parental cells with the selected antisense MDR1 transfected clones is shown in

(31) Woolf, T. M.; Jennings, C. G. P.; Rebagliati, M.; Melton, D. The stability, toxicity and effectiveness of unmodified and phosphothioate antisense oligodeoxynucleotides in Xenopus oocytes and embryos. *Nucleic Acids Res.* **1990**, *18*, 1763−1769.

(32) Woodcock, S.; Williamson, I.; Hassan, I.; Mackay, M. Isolation and characterization of clones from the Caco-2 cell line displaying increased taurocholic acid transport. *J. Cell Sci*. **1991**, *98*, 323−332.

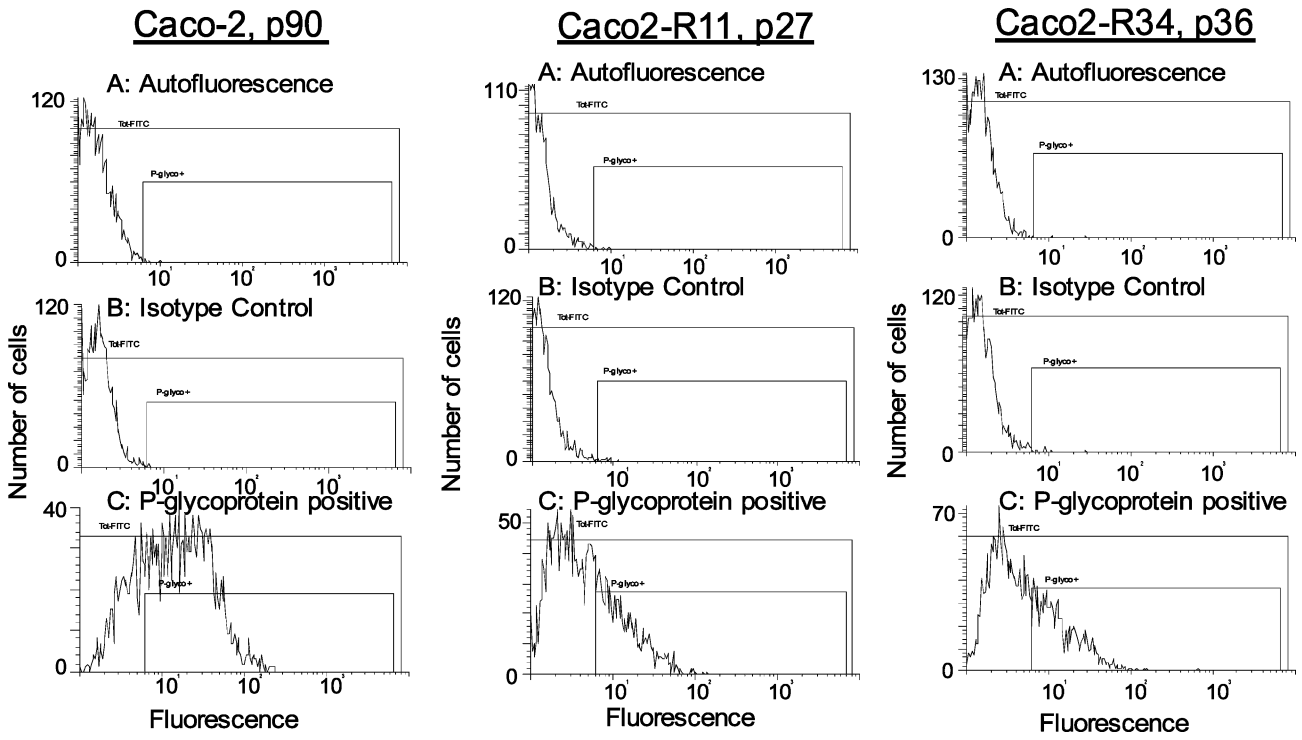

Figure 5. FACS analysis of Caco-2 cells, p90 (left), and the anti-MDR1-transfectants Caco-2-R11, p27 (middle), and Caco-2-R34, p36 (right), for the expression of P-glycoprotein. The cells were analyzed for their autofluorescence (A), incubated with an unspecific antibody and FITC-labeled (isotype control, B), and specifically labeled with the monoclonal antibody MRK16 and FITC-labeled anti-mouse-antibody (P-gp expression, C). Autofluorescence and isotype control analysis did not reveal significant numbers of cells that would be considered P-gp positive (1.7% and 2.2% of the cells respectively showing up in the gate "P-glyco+"). Both clones show a significant reduction in P-gp expression levels compared to untransfected cells after more than 20 passages post-transfection.

Table 4. Transport Characteristics (P_{eff}, 10^{-6} cm/s) for Talinolol in Different Clones vs Control in Apical-to-Basolateral (a → b) and Basolateral-to-Apical (b → a) Direction and Influence of Verapamil on P_{eff} for Selected Clones [P_{eff} in 10^{-6} cm/s]

(A) Transport Characteristics

selected clone	talinolol $P_{eff,ab}$ [10^{-6} cm/s]	S/R_{ab}[a]	talinolol $P_{eff,ba}$ [10^{-6} cm/s]	S/R_{ba}[a]	av TEER[b] [Ω cm^2]
Caco-2 control	0.19 ± 0.058	0.90	1.98 ± 0.085	1.02	1737 ± 412
anti-MDR1					
R11	0.24 ± 0.027	1.04	0.80 ± 0.027	1.00	750*** ± 244
R19	0.26 ± 0.021	1.00	0.67 ± 0.015	0.96	658*** ± 122
R34	0.42 ± 0.052	0.91	1.21 ± 0.132	0.86	562*** ± 142
RM12	0.16 ± 0.012	0.85	2.13 ± 0.373	0.99	1650[n.s.] ± 323
RM13	0.16 ± 0.003	0.99	1.22 ± 0.930	0.95	234*** ± 38
RM15	0.17 ± 0.003	1.00	0.53 ± 0.002	0.96	719*** ± 55
MDR1(S4)	0.021 ± 0.009	0.78	1.27 ± 0.067	0.98	1653[n.s.] ± 14

(B) Influence of Verapamil

	reduction/increase of talinolol P_{eff} or TEER by verapamil addition[c]		
selected clone	increase of a → b flux	red. of b → a flux	TEER red.
control	×2.75	/3.33	/1.19
R11	×2.30	/2.33	=1
R19	×1.62	/0.66 (×1.51)	/1.27
R34	×1.37	/1.25	/1.46
RM12	×4.83	/4.40	/1.35

[a] S/R gives the enantiomeric ratio of (S)- and (R)-talinolol. [b] n.s.: not significantly different from the control ($p > 0.05$; $n = 5-6$). ***: significant difference between the test and the control ($p < 0.05$; $n = 5-6$). [c] In relation to reference/control; control = 1 = 100%; $n = 5-6$.

similar growth characteristics as well as their capability to form tight epithelial layers shown by high TEER values.

Transport studies through Caco-2 cell monolayers obtained with the P-gp model substrate talinolol[33,34] clearly indicated—in addition to the cell characterization with direct quantification of P-gp expression—that the secretory part contributing to the effective permeabilities decreases together with the efflux ratio in antisense MDR1 transfected sublines. And, as expected, verapamil addition yields a smaller inhibitory effect on talinolol secretion in clones anti-MDR1 R11 and anti-MDR1 R34 than in parental Caco-2 cells, demonstrating that the residual secretion can be inhibited.

Overall, the present comparative concept including the selective inhibition of transporter expression on the gene level may be superior to the use of selective or semiselective inhibitors or to the sole use of cell lines with increased transporter expression following transfection (e.g., ref 35).

Abbreviations Used

MDR, multidrug resistance; P-gp, P-glycoprotein; cDNA, complementary DNA; bp, base pairs; PBS, phosphate-buffered saline pH 7.4; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; LSC, liquid scintillation counting; SSC, 0.15 M sodium chloride/0.05 M sodium citrate; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; HBSS, Hanks' balanced salt solution; MES, morpholinoethanesulfonic acid; TEER, transepithelial electrical resistance.

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