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Stable suppression of MDR1 gene expression and function by RNAi in Caco-2 cells

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Abstract

Vector-based RNAi was used to establish a stable Caco-2 cell line with a persistent knockdown of multidrug resistant gene 1 (MDR1) and P-glycoprotein (P-gp). Several positive clones were collected, many of which showed significantly reduced levels of MDR1 mRNA and P-gp compared to wt Caco-2 cells. Selected clones were sub-cultivated for six passages and real-time PCR showed that MDR1 expression remained significantly reduced (up to 96%) over this period of time. RNAi-MDR1 clones frozen long term also kept their low MDR1 expression levels when re-cultured. Permeability studies were performed across RNAi-MDR1 clone cell monolayers, and the efflux of cyclosporine A, digoxin, vinblastine, and vincristine showed 58%, 61%, 91%, and 78% decrease in active transport, respectively, compared to wt Caco-2 cells. This stably modified Caco-2 cell line provides a novel tool for studies on MDR1 and other ABC transporter protein gene cellular functions.

Keywords: Vector-based; RNAi; P-glycoprotein; MDR1; Stable cell line; Permeability; Active transport; ABC transporter proteins

P-glycoprotein (P-gp) (170 kDa), the product of the multidrug resistant gene 1 (MDR1), is a member of the ATP-binding cassette (ABC) transporter superfamily. P-gp (ABCB1) is mainly responsible for the phenomenon known as multidrug resistance in which cells become resistant to drugs due to cellular overproduction of P-gp followed by efflux of the drug [1]. The distribution of P-gp is tissue-specific and the anatomical localization indicates that P-gp may affect absorption, distribution, metabolism, and excretion of a variety of clinically important drugs and this can have dramatic consequences for the pharmacological behaviour of many drugs [2]. P-gp is present in the epithelial cells throughout the body and is primarily localized in the plasma membrane, although it has also been detected in the nuclear envelope and in the membranes of cytoplasmic organelles where it possibly regulates intracellular traffic of drugs [3,4]. P-gp is located in the apical domain of the enterocytes of the gastro-intestinal (GI) tract, thereby limiting the absorption of compounds from the GI tract. In the liver and kidney the expression of P-gp on the apical side of various cell membranes enhances the secretion of drugs into bile and urine [5]. P-gp is also an important component of the blood–brain barrier (BBB) where it is sited on the apical side of the endothelial cells facing the blood. The observed poor BBB permeability of some drugs is mainly due to the efflux function of P-gp [2]. Mice lacking the mdr1-type P-gp show drastic alteration in the pharmacological distribution of drugs into the brain [6].

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism where double-stranded small interfering RNA (siRNA) induces degradation of mRNA in a sequence-specific manner. Physiologically, RNAi is initiated by a dsRNA-specific RNase III enzyme known as Dicer that processes long dsRNA into siRNA. These siRNAs are components of a protein

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complex that recognizes and cleaves its target RNA [7]. One problem to achieve RNAi in mammals is that dsRNAs (longer that 30 bp) will activate an antiviral response, followed by a non-specific degradation of mRNA and a general shutdown of the host cell protein translation [8,9]. This problem is overcome by introducing siRNAs shorter than 30 bp (typically 19–23 nt) into mammalian cell cultures [10]. SiRNAs can either be synthetic, in vitro transcribed, or expressed from a plasmid as RNA hairpin loops (RNA with a self-complementary stem loop). Chemically synthesized siRNAs are widely used for silencing of genes in mammalian cell lines; however, recent reports have described DNA vector-based expression of hairpin RNAs as an alternative for delivery of siRNAs into mammalian cells [11]. Transfection of synthetic- or in vitro transcribed siRNAs causes only transient knockdown of target genes and is often limited to cell lines that are easily transfected. Stable suppression of gene expression by RNAi in mammalian cells overcomes these disadvantages and studies indicate that the vector-based knockdown is maintained over long periods and that the hairpin transcript is not toxic to the cells [12,13].

P-gp is the most studied drug efflux transporter protein and transports a variety of structurally and functionally different substrates including many pharmaceuticals [4]. Developing assays that can identify compounds that are P-pg modulators (substrates/inhibitors/inducers) will aid in the optimization and the selection of new drug candidates [14]. P-gp substrate specificity overlaps with those of other ABC-transporter proteins; however, less is known about the significance of the other efflux proteins in drug transport. Due to the extensive substrate overlap between P-gp and other ABC-transporters it is possible that these proteins may compensate for any loss of P-gp activity following P-gp inhibition or knockout. The use of RNAi to downregulate expression levels of MDR1 in Caco-2 cells may allow studies on the importance of other ABC transporter proteins following exposure to pharmaceuticals.

In two reports, siRNAs were used to transiently modulate MDR1 expression levels in human carcinoma cell lines. Inhibition of P-gp expression by siRNA enhanced the intracellular accumulation of and restored the sensitivity to compounds transported by P-gp; however, the reversal of MDR1 expression in these studies was temporary and the original level of MDR1 was restored a few days after transfection [15,16]. In a recent report, development of stable RNAi-MDR1 cells was reported and these cells were stable for at least 30–35 days after the initial transfection [17]. In the present study, we demonstrate that stable, sequence specific silencing of human MDR1 gene expression and P-gp translation can be induced by endogenous expression of RNA hairpins and that this vector-based RNAi suppression is stable over several cell sub-cultivations. In addition, the RNAi-MDR1 clones kept their low MDR1 expression levels when re-cultured after long-term freezing. This modified Caco-2 cell line will allow us to investigate the importance of and to characterize efflux proteins other than P-gp and to study the gene function of MDR1 and other ABC-transporter proteins. This modified Caco-2 cell line also has the potential to improve the preclinical selection of new candidate drugs.

Materials and methods

Chemicals. GeneSuppressor plasmid-based siRNA system (IMG-800) for gene knockdown was from IMGENEX (San Diego, CA, USA). Lipofectamine 2000, DMEM, glutamine, MEM, OPTI-MEM, fetal bovine serum (FBS), and trypsin were from Invitrogen AB (Groningen, NL). RNeasy mini kit for isolation of total RNA was obtained from Qiagen (USA). Reagents for real-time PCR (TaqMan assay) including primers and probes were supplied by Applied Biosystems (CA, USA). Mouse anti-human P-gp monoclonal antibody (C494) was from Calbiochem (San Diego, CA, USA) and mouse anti-human actin monoclonal antibody was purchased from Chemicon International (CA, USA). Sheep anti-mouse IgG, ECL plus Western blotting detection, and Hyperfilm ECL were obtained from Amersham Biosciences (Buckinghamshire, UK). All other chemicals used were of highest available quality.

Cell culture. Human colon adenocarcinoma (Caco-2) cells obtained from American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM (high glucose, without sodium pyruvate, with phenol red) medium containing 10% heat inactivated FBS, glutamine (2 mM), and non-essential amino acids (0.01 mM). Cells were incubated at 37 °C in a 10% CO_2/air incubator with 90% humidity. Medium was changed every second day and the cells were sub-cultivated once a week.

Design of siRNAs and cloning of siRNA hairpin loops. Four 21-nt siRNA (pgpI, II, III, and IV) duplexes from four different parts of the human MDR1 gene (GenBank Accession No. NM_000927) were designed using the siRNA Target Finder and Design Tool available at http://www.ambion.com, and commercially obtained from Proligo Genset (Paris, France) or Qiagen-Xeragon (MD, USA). The siRNA sequences and the siRNA duplexes are listed in Table 1. The sequences of the chemically synthesized siRNA duplexes (pgpI and pgpIV) that were found to significantly downregulate the MDR1 gene and the corresponding P-gp in transient transfected Caco-2 cells were cloned into the pSuppressorNeo (pSupNeo) vector of the GeneSuppressor System (Imgenex, CA, USA). The oligonucleotides were designed to produce hairpin RNAs identical to the oligonucleotide siRNA duplex sequences and the sense strands are as follows: pgpI 5′-TGC GAC AGG AGA TAG GCT Gga gta ctg CAG CCT ATC TCC TGT CGC ATT TTT-3′ and pgpIV 5′-GGC CTA ATG CCG AAC ACA Tga gta ctg ATG TGT TCG GCA TTA GGC CTT TTT-3′. The pSupNeo vector contained compatible restriction sites to ensure cloning into the correct site of the linearized vector and the oligonucleotides were annealed and cloned into the vector according to the manufacturer's recommendations (www.imgenex.com).

Transfection of Caco-2 cells. Caco-2 cells (75,000 cells/well) were plated in a 12-well plate using media conditions as described above and incubated for 24 h. Cells were transfected with chemically synthesized siRNA (0.5 μg/well) or hairpin-producing plasmid (0.5 μg/well) using Lipofectamine 2000 (LF2000) and serum-free medium (OPTI-MEM) according to the manufacturer's recommendations. All transfections were done in triplicate and cells were harvested 48 h post-transfection. Optimal amount of LF2000 and cell number were determined prior to transfection using green fluorescent protein (GFP) expressing plasmids. Transfection efficiency was typically 60–80% for synthetic

Table 1
Sequences of siRNA oligos

SiRNA duplex	Target sequence	Sense strand (5′ → 3′)	Antisense strand (5′ → 3′)	Position in gene sequence
PgpI	AATGCGACAGGAGATAGGCTG	UGCGACAGGAGAUAGGCUGtt	CAGCCUAUCUCCUGUCGCAtt	889–909
PgpII	AAGATCTTGAAGGGCCTGAAC	GAUCUUGAAGGGCCUGAACtt	GUUCAGGCCCUUCAAGAUCtt	1643–1663
PgpIII	AAGCGAAGCAGTGGTTCAGGT	GCGAAGCAGUGGUUCAGGUtt	ACCUGAACCACUGCUUCGCtt	2113–2132
PgpIV	AAGGCCTAATGCCGAACACAT	GGCCUAAUGCCGAACACAUtt	AUGUGUUCGGCAUUAGGCCtt	3492–3512

siRNA and about 20% for vector-based hairpin siRNA estimated by fluorescence microscopy. Cells were also transfected with a siRNA targeting human GAPDH as a positive control and with siRNA oligos containing no homology with any human gene as a negative control.

Establishment of stable RNAi Caco-2 cell lines. Caco-2 cells were transfected with vector-based hairpin siRNAas described above. Forty-eight hours post-transfection cells were transferred to 10 cm cell plates and grown in medium containing G418 (400 μg/ml), until distinct clones could be isolated. Single clones were transferred to 96-well plates and grown until confluent and transferred to flasks. Thereafter, cells were cultivated until confluent, harvested, and frozen.

Western blotting. Membrane protein extracts were prepared by sonication of cells in PBS (containing 1% SDS) followed by centrifugation. Total protein content was determined in the extracts and 10 μg of sample was separated on SDS–PAGE. Separated proteins were transferred to a nitrocellulose membrane and membranes were incubated with mouse anti-human P-gp monoclonal antibodies C494 (1:500 in PBS/Tween). To assure equivalent protein loading, the membranes were simultaneously incubated with mouse anti-human actin monoclonal antibodies (1:3000 in PBS/Tween). Protein–antibody complexes were detected by chemoluminescence (ECL Plus, Amersham Biosciences).

Real-time quantitative PCR primers and probes. Primers and probes for human MDR1 (GenBank NM_000927) and GAPDH (GenBank NM_002046) were designed using the Primer 3 program (Biological Workbench; http://workbench.sdsc.edu/). The sequences for MDR1 probe were 5′-AAC TTT GGC TGC CAT CAT CC-3′, forward primer 5′-CAA TGG AGG AGC AAA GAA GA-3′, and reverse primer 5′-CAA ACA CCA GCA TCA TGA GA-3′, and the sequences for GAPDH probe were 5′-GGA TAT TGT TGC CAT CAA TGA CCC C-3′, forward primer 5′-GGC TGC TTT TAA CTC TGG TAA AGT-3′, and reverse primer 5′-AAC CAT GTA GTT GAG GTC AAT GAA-3′. Primers and probe for MDR1 amplification were designed such that their target sequence was 5′ end (upstream) of the RNAi target sequence. Primers and probe for MRP1, MRP2, BCRP, and villin were as previously described [18]. Prior to quantification the optimal concentrations of primer pairs and probes were determined as previously described [19].

Real-time reverse transcription polymerase chain reaction. Total RNA was isolated using the RNeasy mini kit (Qiagen). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using a TaqMan reverse transcription reagent (Applied Biosystems) and 200 ng of total RNA and an oligo(dT) primer in a 20 μl reaction. From this, 25 ng of RT-RNA was used for each TaqMan reaction. Each reaction (25 μl) contained the optimized probe (150 nM) and primer (300 nM) concentrations as well as the TaqMan universal PCR master mix. Expression levels of the target mRNAs in samples were quantified by comparison with a reference standard curve derived from known amounts of each target gene [19]. The standards ranged between 250 and 25,000,000 copies for all targets. All samples were run in duplicate. Amplification and detection of samples and standards were performed with the ABI 7700 sequence detection system (Applied Biosystems).

Permeability studies. Permeability assays were performed as previously described [20,21] using an automated setup. 0.5×10^{-6} cells were plated on 12 mm diameter polycarbonate membranes, 0.45 μm pore diameter (Transwell, Corning, Costar), and assays were performed 14–21 days after plating. Monolayer integrity was monitored using transepithelial electrical resistance (TEER) and [^{14}C]mannitol permeability, and ^{3}H-labelled P-gp modulators were used to estimate permeability in RNAi clones and wt Caco-2 cells. The following P-gp modulators were used: cyclosporine (0.17 μM), digoxin (0.04 μM), verapamil (0.02 μM), vinblastine (0.14 μM), and vincristin (0.17 μM). The apparent permeability constant (Papp) was calculated as previously reported [21] and the efflux ratio (B → A/A → B) between basolateral to apical (B → A) and apical to basolateral (A → B) transport was used as a measure for active transport. The efflux ratio for each test compound in stable RNAi clones was compared to those of wt Caco-2 cells and used as an estimate for functional changes in efflux due to MDR1 knockdown.

Results

To identify an efficient RNA target sequence for generation of stable vector-based RNAi clones, chemically synthesized siRNA constructs designed against four different parts of the MDR1 mRNA sequence were tested in transient transfections for their ability to downregulate expression levels of MDR1 mRNA and the corresponding P-gp in Caco-2 cells. At present, there is no absolute reliable way to predict the perfect target sequence for RNAi therefore the different siRNA oligos (pgpI, pgpII, pgpIII, and pgpIV) were designed according to published recommendations [22]. Among the four oligonucleotides two (pgpI and pgpIV) were shown to significantly reduce mRNA and protein levels of the MDR1 gene. Western blot analysis of extracted proteins showed decreased levels (up to 90%) of P-gp after transient transfection with siRNAs pgpI and pgpIV, whereas cells transfected with pgpII and pgpIII did not show significant decrease in protein levels (Figs. 1A and B). Additionally cells were transfected with a siRNA targeting human GAPDH gene expression as a positive control of RNAi, and with a siRNA having no homology with any human gene as a negative control (both available from Ambion). Results from real-time PCR showed significant decrease (50%) in GAPDH gene expression 48 h post-transfection using GAPDH siRNA, while cells transfected with the control siRNA showed no significant reduction in GAPDH expression levels (Fig. 2).

Based on results from transient transfections using chemical siRNAs, two target sequences (pgpI and pgpIV) were selected for vector-based RNAi experiments. The oligonucleotides containing the replicate

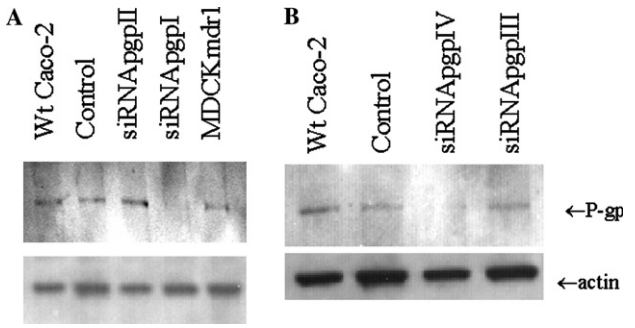

Fig. 1. (A) Western blot of cell extracts from Caco-2 cells transiently transfected with siRNA synthetic oligos. (A) and (B) represent two different representative experiments. Ten micrograms of protein was loaded in each well and membranes were probed with mouse anti-human P-gp antibodies (C494). To assure equal load of protein on the SDS–PAGE blots was simultaneously probed with mouse anti-human actin antibodies.

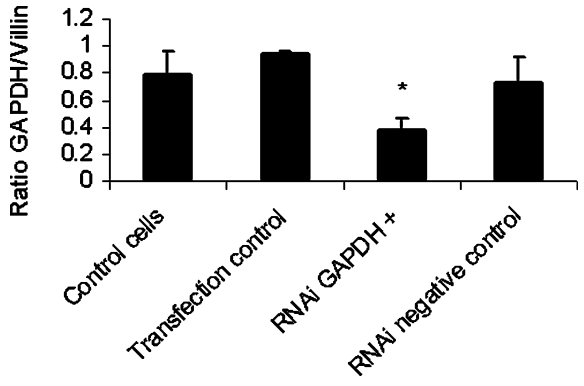

Fig. 2. Expression levels of GAPDH measured by real-time PCR and normalized by villin. Total RNA isolated from Caco-2 cells after transient transfection with synthetic siRNA targeting human GAPDH and control siRNA having no homology with any human gene (kit available form Ambion). *$p < 0.1$ compared with wt Caco-2 cells using two-sided distribution (Student's t test).

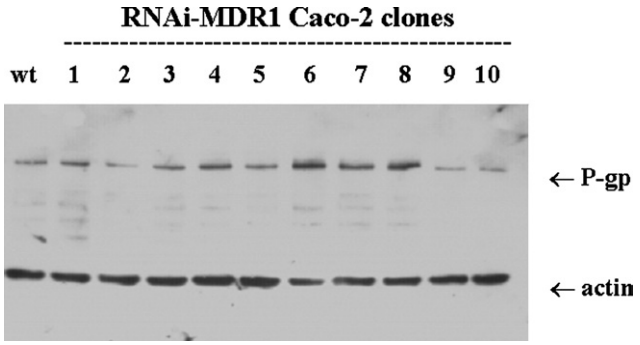

Fig. 3. Western blot of stable RNAi Caco-2 clones. Wt: control Caco-2 cells; lanes 1–10: different stable RNAi Caco-2 clones. The Western blot is probed with mouse anti-human P-gp (C494) and mouse anti-human actin antibodies. Ten micrograms of protein was loaded in each well on the gel.

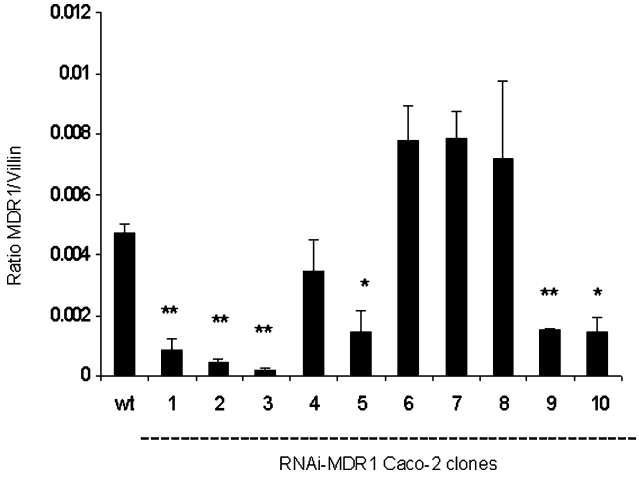

Fig. 4. Expression levels of MDR1 in stable RNAi Caco-2 clones. Levels are quantified by real-time PCR and normalized by villin. Wt: wt Caco-2 cells; 1–10: represent different RNAi stable clones randomly selected. All clones were collected 60–70 days after the initial transfection. *$p < 0.05$; **$p < 0.01$ compared with wt Caco-2 cells using two-sided distribution (Student's t test).

sequences (hairpin loops) were cloned into the pSupNeo vector. All clones were sequence verified and transient transfections using the vector constructs (pgpI-pSupNeo or pgpIV-pSupNeo) resulted in downregulation of MDR1 mRNA levels (data not shown). However, these results were difficult to reproduce due to low transfection efficiency and high cytotoxicity of the LF2000-pSupNeo complex. To establish stable clones, Caco-2 cells were transfected with the pgpI-pSupNeo or the pgpIV-pSupNeo construct containing a neomycin resistance gene and subjected to G418 selection. Protein and total RNA were isolated from a number of clones and analysed by Western blotting and real-time PCR, respectively. Western blots show that several clones have reduced levels of P-gp compared to control cells (Fig. 3), and these results were in agreement with data obtained by real-time PCR showing that several RNAi clones have reduced MDR1 mRNA levels by as much as 96% of that of wt Caco-2 MDR1 levels (Fig. 4). All clones were analysed 60–70 days after the initial transfection. To investigate whether the established stable clones would continue to suppress MDR1 expression through several sub-cultivations, several clones, randomly selected, were cultured over at least six passages. The cells were sub-cultivated and cell samples were collected once a week. RNA was isolated and results from real-time PCR show that MDR1 expression levels are significantly reduced over the time period in all clones analysed (Fig. 5).

Based on results from real-time PCR, four different stable clones with significantly reduced transcription levels of MDR1were selected for cellular permeability studies across RNAi clone monolayers. Using five P-gp modulators, permeability data were obtained from RNAi clone monolayers and compared to that of wt Caco-2. Three out of the four selected clones did not

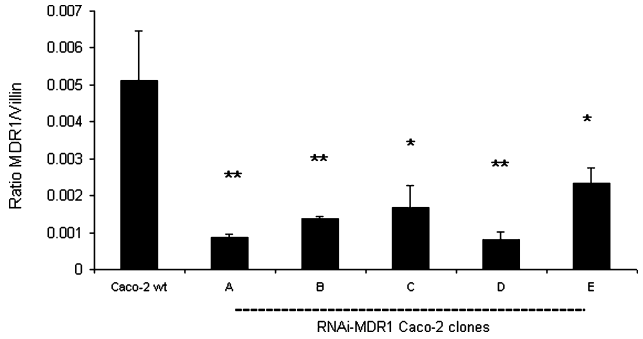

Fig. 5. Expression levels of MDR1 in stable RNAi clones analysed by real-time PCR and normalized by villin. The clones were cultured for up to six weeks, and sub-cultivated and sampled once a week. Bars represent an average transcription level over the time period and A–E represent different RNAi clones. *$p < 0.05$; **$p < 0.01$ compared with wt Caco-2 cells using two-sided distribution (Student's t test).

give functional data. One clone did not develop tight cell monolayers and two of the clones did not show reduced efflux ratios compared to wt Caco-2 cells (data not shown). However, results from one RNAi clone show significant difference in permeability (B → A/A → B ratio) of cyclosporine A, digoxin, vinblastine, and vincristine in the RNAi clone compared to those of wt Caco-2 cells. Cyclosporine A, digoxin, vinblastine, and vincristine showed 58%, 61%, 91%, and 78%, respectively, lower efflux ratio than wt Caco-2 cells. On the contrary, the permeability of verapamil was unaffected (5%) by the reduced MDR1/P-pg levels in the RNAi-MDR1 Caco-2 clone (Table 2). Monolayer integrity measured by the TEER values was within the normal range for the RNAi clone, and [^{14}C]mannitol permeability was low, indicating that the clone was able to form tight non-leaky monolayers. RNA samples from the RNAi clone were also analysed by real-time PCR. Cell samples were collected from five consecutive sub-cultivations and expression levels of MDR1, multidrug resistant protein 1 (MRP1), multidrug resistant protein 2 (MRP2), and breast cancer resistant protein (BCRP) were analysed. Results show that the expression level of MDR1 remains low over the time period (96.4% reduction). Expression levels of MRP1 and MRP2 were not significantly changed in the RNAi clone compared to wt Caco-2. On the contrary, BCRP showed reduced levels of expression in the RNAi clone. Results are summarized in Table 3.

Table 3
ABC transporter gene expression

Target	RNAi clone	Wt Caco-2	% Reduction of wt Caco-2
MDR1	0.037	1.0	96**
BCRP	0.39	1.0	61*
MRP1	0.75	1.0	n.s.
MRP2	1.09	1.0	n.s.

Gene expression in wt Caco-2 is set to 1.0. Gene expression of target is normalized for villin. *$p < 0.05$; **$p < 0.01$ compared to wt Caco-2. n.s., not significant.

Discussion

In the present study, we use vector-based RNAi to establish a Caco-2 cell line with a stable suppression of the MDR1 gene and the corresponding P-gp. The use of RNAi to inhibit gene expression in mammalian cells is a promising new tool for the study of gene function and has the potential to increase our knowledge of MDR and ABC transporter protein function. Two MDR1 target sequences were identified by transient transfection of synthetic siRNA oligos (pgpI and pgpIV) and shown to significantly knock down P-gp in Caco-2 cells (Figs 1A and B). It is known that not all positive synthetic siRNAi oligos work in vector-based RNAi, therefore two different oligo hairpin sequences were designed, cloned into the pSupNeo vectors, and used to generate stable RNAi clones. The pSupNeo vector contains neomycin resistance allowing selection of only the clones containing the hairpin sequence. Many RNAi vector systems available do not contain a selective marker within the RNAi plasmid making the selection of positive RNAi clones more difficult [17,23].

Caco-2 cells stably transfected with the U6 (RNA pol III promoter) expression vector transcribing hairpin siRNAs gave the most effective RNAi knockdown of MDR1 expression levels. No difference in the reversal of MDR1 expression between RNAi clones derived from the two constructs (pgpI-pSupNeo and pgpIV-pSupNeo)

Table 2
Permeability studies

P-gp modulators		Caco-2 wt			RNAi-MRD1 stable clone			
Substance	μM	Papp A → B	Papp B → A	Efflux	Papp A → B	Papp B → A	Efflux	% Reduction
Cyclosporine A	0.17	0.45	6.98	15.3	0.59	3.76	6.4*	58
Digoxin	0.04	1.07	17.80	16.6	2.08	13.60	6.5*	61
Verapamil	0.02	10.51	22.57	2.1	9.75	19.30	2.0	5
Vinblastine	0.14	0.73	31.71	43.6	1.69	6.71	4.0**	91
Vincristine	0.17	0.13	5.26	39.7	0.17	1.53	8.8**	78

All substances are ^{3}H-labelled. Papp expressed as 10^{-6} cm/s. A → B: apical to basolateral direction; B → A: basolateral to apical direction. Data represent an average of three representative assays. *$p < 0.05$; **$p < 0.01$ compared to wt Caco-2.

was observed. Shown by real-time PCR, 6 out of 10 clones had up to 96% reduction in the MDR1 mRNA level compared to wt Caco-2 levels (Fig. 4). For unknown reasons, a few clones showed increased MDR1 transcription levels (Fig. 4; clones 6, 7, and 8) compared to wt Caco-2. During cloning there is no control of the number of vector copies that are integrated into the genome nor at what position the integration occurs, and this may contribute to the differences seen in downregulation of the target gene among the various clones. Real-time PCR analysis of neomycin resistant gene (NeoR) expression levels indicated that there might be a correlation between the number of NeoR copies and the number of MDR1 copies in the stable RNAi clones, a low number of NeoR correlated with a low number of MDR1 copies (data not shown). Hence, quantification of NeoR might possibly be used as an early screening tool for RNAi efficiency in stable clones.

The expression levels of MDR1 remained low for up to at least six passages (6 weeks culture) after selection of stable clones. In addition, RNAi-MDR1 clones frozen long-term (up to 6 months) at −150 °C kept their low levels of MDR1 when thawed and re-cultured. On the contrary, in transient transfection experiments using synthetic siRNA the downregulation of MDR1 and P-gp was only effective for 24–48 h, and the effectiveness of the RNAi was highly dependent on the transfection efficiency. Other reasons for the partial reduction of P-gp in transient transfections using synthetic siRNA could be a combined effect of the short duration of RNAi (24–48 h) and the long protein's half-life (14–17 h) of P-gp [24].

Caco-2 cells differentiate spontaneously in vitro and form tight monolayers when cultured on permeable supports [21]. The passive permeability of compounds through Caco-2 cell monolayers correlates well with in vivo absorption in humans [25], and this has become an established in vitro method for estimation of absorption of orally administered drugs [21]. In the present work, the functional effect of the MDR1 RNAi on P-gp expression was determined by permeability studies measuring the compound's permeability through Caco-2 monolayers. The efflux ratio (B → A/A → B) was calculated for a number of P-gp modulators and found to be significantly reduced in the RNAi-MDR1 clones compared to wt Caco-2 cells (Table 2). The reduced efflux of cyclosporine A (58%), digoxin (61%), vinblastine (91%), and vincristine (78%) indicates the importance of P-gp in regulating the absorption of these compounds. Interestingly, the reduced efflux in the RNAi clone seems to be modulator specific. For example, P-gp seems to play a more important role in the efflux of vinblastine and vincristine than in the efflux of cyclosporine A. Verapamil is known to be an inhibitor as well as a substrate for P-gp; however, it acts as a substrate only at very low concentrations. Verapamil binds strongly to P-gp and may be used to inhibit P-gp to facilitate influx of other P-gp substrates [26]. In the present study, the reduced levels of P-gp did not affect the efflux of verapamil. The efflux of verapamil observed in wt Caco-2 cells is in accordance with a recent report [27]. This lack of change in permeability in spite of the reversal of P-gp expression could indicate that other transporters than P-gp contribute to the efflux of verapamil. Although there is a 96.4% reduction in MDR1 gene expression levels in the stable RNAi clone analysed, this does not completely abolish the drug sensitivity of the Caco-2 cell line, indicating that efflux transporters other than P-gp could be involved in the efflux mechanism of the compounds analysed.

ABC transporter proteins have their own unique substrate specificity but many compounds can be transported by two or more of these proteins [28]. Although P-gp is the most studied efflux transporter protein and known to affect absorption and distribution of many drugs, many other more recently discovered efflux proteins may influence the pharmacokinetics, tissue distribution, and pharmacodynamics of drugs [29]. Different Caco-2 sub-lines could express different levels of transporters proteins making comparison of studies among different laboratories difficult [18]. The wt Caco-2 cell line used in this study showed higher levels of BCRP and MRP2 compared to that of MDR1, indicating a more important role in the efflux of these transporters than previously suggested (data not shown). These differences could be due to the polyclonal origin of this cell line or possibly due to differences in culture conditions. The polyclonal origin of the Caco-2 cell line also increases the risk of variations in the cellular properties between different passages and cell batches. Comparison of expression levels of a number of efflux proteins in human jejunum and Caco-2 monolayers showed that transcript levels of 9 out of 10 ABC transporters studied correlated well between jejunum and Caco-2 cells. Furthermore, transcription levels of both BCRP and MRP2 in jejunum were 10-fold higher than the MDR1 levels, suggesting that the roles of these proteins in intestinal drug efflux have been underestimated [18].

The development of stable RNAi mammalian cell lines has the potential to expand the utility of RNAi. The modified Caco-2 cell line established in the present study is stable in culture over at least six weeks and in addition MDR1 levels remain low and stable during cycles of freezing and thawing, and represents an excellent model for studying the cellular function of MDR1 and other ABC transporters. Transcription levels of MRP1 and MRP2 are not affected by the downregulation of MDR1 expression levels, whereas the expression levels of BCRP were downregulated in the RNAi clones. Co-expression of MDR1 and BCRP gene has been reported previously [30], though further studies are needed to see whether the reversed levels of BCRP in the present study

are due to RNAi in general or a result of co-regulation between MDR1 and BCRP. In future studies, the modified Caco-2 cell line could be used to knock down other ABC transporter proteins, singly or simultaneously, allowing gene function and substrate specificity studies.

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