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Stable and complete overcoming of MDR1/P-glycoprotein-mediated multidrug resistance in human gastric carcinoma cells by RNA interference

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Multidrug resistance (MDR) is the major cause of failure of effective chemotherapeutic treatment of disseminated neoplasms. The ''classical'' MDR phenotype of human malignancies is mediated by drug extrusion by the adenosine triphosphate binding cassette (ABC)-transporter P-glycoprotein (MDR1/P-gp). For stable reversal of ''classical'' MDR by RNA interference (RNAi) technology, an H1-RNA gene promoter-driven expression vector encoding anti-MDR1/P-gp short hairpin RNA (shRNA) molecules was constructed. By introduction of anti-MDR1/P-gp shRNA expression vectors into the extremely high drug-resistant human gastric carcinoma cell line EPG85-257RDB, the MDR phenotype was completely reversed. The reversal of MDR was accompanied by a complete suppression of MDR1/P-gp expression on mRNA and protein level, and by a considerable increased intracellular anthracyline accumulation in the anti-MDR1/P-gp shRNA-treated cells. The data indicate that stable shRNA-mediated RNAi can be tremendously effective in reversing MDR1/P-gp-mediated MDR and is therefore a promising strategy for overcoming MDR by gene therapeutic applications.
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Drug resistance is the major reason why antineoplastic drug treatment modalities of human malignancies may fail. Human cancer cells can exhibit a cross-resistant phenotype against several unrelated antineoplastic drugs that differ widely with respect to molecular structure and target specificity. This phenomenon has been termed multidrug resistance (MDR).[1] The "classical" MDR phenotype is characterized by a typical cross-resistance pattern against natural-product anticancer agents, such as vinca alkaloids, anthracyclines, or taxanes, and the reversibility by the calcium channel inhibitor verapamil and cyclosporin A derivatives. The underlying mechanism conferring this MDR phenotype is the cellular overproduction of the MDR1 gene encoded 170-kDa, membrane-spanning P-glycoprotein (MDR1/P-gp, P-170, PGY1, MDR1, ABCB1),[2] member of the superfamily of ABC (adenosine triphosphate binding cassette)-transporters.[3] An inhibition of MDR1/P-gp-mediated drug extrusion results in a resensitization of tumor cells to treatment with antineoplastic agents, and therewith may allow a successful drug treatment of the multidrug-resistant cancer cells.

Low molecular weight pharmacologically active compounds, designated as MDR modulators or chemosensitizers, may circumvent the "classical" MDR phenotype by inhibiting the efflux pump activity of MDR1/P-gp.[4–6] An obstacle in applying classical MDR modulators arises from their commonly occurring intrinsic toxicity at doses necessary to be active, for example, heart failure, hypotension, hyperbilirubinemia, and immunosuppression by cyclosporin A. Moreover, improved so-called second-generation MDR modulators were demonstrated to induce enhanced activity of liver enzymes of the family of cytochrome *P*450 mixed-function oxidases resulting in an increased pharmacokinetic turn over of the applied anticancer drugs. Additionally, tumor cells can acquire resistance against the applied chemosensitizers, a so-called tertiary resistance. Consequently, it is necessary to develop alternative, less toxic and more efficient strategies to overcome MDR. Such an alternative procedure to circumvent MDR1/P-gp-mediated MDR in cancer cells is to prevent the biosynthesis of MDR1/P-gp by selectively blocking the expression the MDR1/P-gp-specific MDR1 mRNA by gene therapeutic technologies. This approach is aimed at increasing the efficiency and specificity of chemosensitization of multidrug-resistant cancer cells while at the same time reducing toxicity and undesirable side effects. Thus, in previous studies, antisense oligonucleotides (OND),[7,8] hammerhead ribozymes[9,10] and, recently, chemically synthesized small interfering RNAs (siRNAs)[11,12] were shown to modulate MDR1/P-gp-dependent MDR by decreasing the expression level of the MDR1/P-gp encoding mRNA.

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Currently, the use of siRNAs as a means for specific inhibition of a gene of interest is under extensive investigation.[13] These between 21 and 25 nucleotides (nt) long, double-stranded RNA (dsRNA) molecules can direct degradation of eukaryotic mRNAs in a sequence-specific manner. This ubiquitous mechanism of gene regulation in plants and animals was designated as RNA interference (RNAi).[14] Physiologically, RNAi is initiated by the dsRNA-specific RNAse III enzyme Dicer that is responsible for the processing of long dsRNA into siRNA. These siRNAs are incorporated into a protein complex that recognizes and cleaves its target mRNAs.[15] Introduction of dsRNA into mammalian cells does not result in efficient Dicer-mediated generation of siRNA and therefore does not induce RNAi.[16] The requirement for Dicer in maturation of siRNAs can be bypassed by introducing synthetic 21-nt siRNA duplexes that inhibit expression of transfected and endogenous genes in a variety of mammalian cells.[17] However, a major disadvantage of this approach is the transient gene expression-inhibiting effect of chemically synthesized siRNAs. Hence, the development of more effective and stable gene-silencing RNAi-mediating systems is of huge interest.

Thus, for stable long-termed RNAi effects, expression vectors have been developed that produce siRNA-like transcripts, that is, synthesis of short hairpin RNAs (shRNA).[18] In these vectors, an RNA polymerase III-specific H1-RNA promoter is used. Physiologically, this promoter drives the expression of a gene encoding H1-RNA, the RNA component of the human RNase P. The H1-RNA promoter has a defined start of transcription and a termination signal consisting of five consecutive thymidines (T5). Therewith, it can be used to direct the synthesis of small RNA molecules of interest lacking a poly-adenosin tail. Cleavage of the RNA transcript at the termination site is after the second uridine. Thus, the H1-RNA promoter produced small RNA is similar to the ends of chemically synthesized siRNAs containing two 3′ overhanging thymidines or uridines. The sequence of interest consists of a 19-nt sequence homologous to the target mRNA, linked with a 3–9-nt spacer sequence to the reverse complement of the same 19-nt target-specific sequence. The synthesized RNA transcript folds back to its complementary strand to form a 19-base pair shRNA molecule resembling a corresponding siRNA molecule.

In this study, an shRNA-expressing vector (psiRNA/MDR-A) was constructed to direct the synthesis of shRNAs directed against the MDR1/P-gp-specific transcript. In previous experiments using chemically synthesized siRNA molecules (MDR-A) directed against the identical target sequence of psiRNA/MDR-A, the exciting gene silencing efficacy of MDR-A could be already demonstrated in a transient manner.[11]

Materials and Methods

Cell lines and cell culture

Establishment and cell culture of the human gastric carcinoma cell line EPG85-257P was described in detail previously.[19] The "classical" multidrug-resistant, MDR1/P-gp-positive derivative EPG85-257RDB was established by *in vitro* exposure to the anthracycline daunorubicin (Farmitalia Carlo Erba, Freiburg, Germany). In order to ensure maintenance of the MDR phenotype, medium for the MDR1/P-gp-expressing cell line EPG85-257RDB was supplemented with 4.2 μM (2.5 μg/ml) daunorubicin. The anthracycline-containing, supplemented Leibovitz L 15 medium[19] was replaced routinely twice a week.

Cytotoxicity assay for cell survival

Drug resistance was determined using a cell proliferation assay based on sulforhodamine B (SRB), a protein-binding reagent,[20] as described previously.[11,21,22] Briefly, in each experiment 1250 cells per well were seeded in 96-well plates and daunorubicin was added in dilution series in triplicate after 48 hours. Following 4 days exposure to daunorubicin, cell proliferation was stopped and the plates were stained with SRB. To determine the IC_{50}-values, the absorbance difference of control cells without drug was set to be 100%. A dose–response curve was plotted by using the Prism software (GraphPad Software, Inc.; San Diego, CA), and IC_{50}-values were calculated from multiple, at least three independent experiments for each cell line.

shRNA design and construction of an shRNA expression system

Sequence of the used shRNA was chosen to be homologous to a chemically synthesized anti-MDR1/P-gp siRNA (anti-MDR-A) demonstrated for its gene-silencing activity previously.[11] The anti-MDR-A target sequence (5′-AAG AAG GAA AAG AAA CCA ACU-3′), corresponds to nt 503–523 of the MDR1/P-gp encoding mRNA sequence (GenBank Accession Number: NM_000927). As shown in Figure 1a, two homologous single-stranded DNA (ssDNA) molecules were chemically synthesized. Annealing of the ssDNA molecules was performed by incubation of 2.5 μM of each complementary ssDNA oligonucleotide in 0.15 M NaCl in a total volume of 20 μl. The annealing mixture was incubated at 80°C for 2 minutes followed by cooling to 35°C. The annealed dsDNA, consisting of the anti-MDR-A sense sequence, a 5-nt 5′-CCACC-3′ spacer sequence, the anti-MDR-A antisense sequence, and *Bbs*I specific 5′-overhangs, was cloned into the *Bbs*I restriction site of the expression vector psiRNA-hH1zeo (InvivoGen, San Diego, CA), to generate psiRNA/MDR-A (Fig 1b). The construction of the psiRNA/MDR-B expression vector was performed in the same way using the anti-MDR-B target sequence (5′-AAA AUG UUG UCU GGA CAA GCA-3′) homologous to nt 3050–3070 of the MDR1/P-gp transcript. The correct insertion of the specific shRNA-encoding DNA molecules was confirmed by sequencing. The sequencing reactions were carried out with AmpliTaq™ DNA Polymerase FS dye terminator cycle sequencing chemistry using the "ABI PRISM™ BigDye™ Terminator Sequencing Ready Reaction Kit"

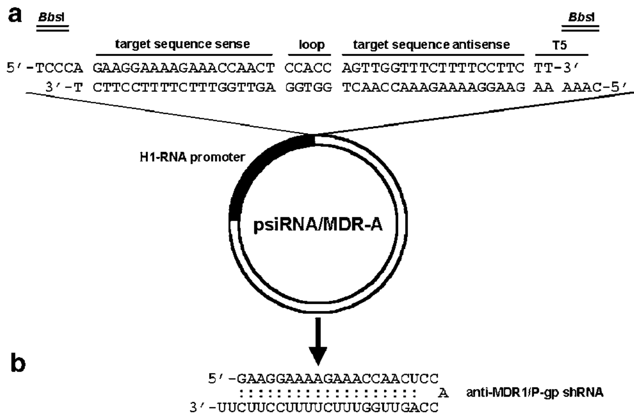

Figure 1 Construction of an anti-MDR1/P-gp shRNA expression system: (**a**) sequence of the chemically synthesized shRNA-encoding DNA oligonucleotides and schematic structure of the anti-MDR1/P-gp shRNA expression vector psiRNA/MDR-A. The shRNA-encoding DNA consists of *Bbs*I-specific 5′- and 3′-assymetric cohesive overhangs that are not compatible, of the target sense and antisense sequence separated by a loop structure, and of the termination site composed by five thymidines (5T). (**b**) Predicted secondary structure of the MDR-A anti-MDR1/P-gp shRNA encoded by psiRNA/MDR-A.

(Perkin Elmer, Foster City, CA) using an automated ABI 377 sequencer (Perkin Elmer).

Plasmid transfection selection of stable tranfected cell clones

The "classical" multidrug-resistant gastric carcinoma cell line EPG85-257RDB was transfected with 2 μg of expression vector DNA, psiRNA/MDR-A, psiRNA/MDR-B, or as a control with psiRNA-hH1zeo containing an active β-galactosidase (LacZ) producing EM7-lacZ α-peptide cassette instead of the shRNA-specific sequence. Each experiment was performed in 50–60% confluent six wells using 10 μl of the transfection reagent SuperFect (Qiagen, Hilden, Germany). The transfected cells were selected in zeocin-containing (400 μg/ml) cell culture medium. After 3 weeks, visible clones were picked in 96 wells, expanded in 12 wells, and finally transferred to regular cell cultur flasks. Various psiRNA/MDR-A-, psiRNA/MDR-B-, and psiRNA-H1zeo control vector-transfected clones derived from EPG85-257RDB cells were isolated and expanded. All these clones were prescreened for gene-silencing activity by Northern blot analyses using an MDR1/P-gp- specific cDNA probe. Representative clones were chosen and used for the detailed characterization of the gene-silencing effects of the shRNA expression vector construct.

Northern blot analysis for MDR1/P-gp mRNA detection

Expression of the MDR1/P-gp encoding mRNA was determined by Northern blot analysis using a standard protocol as described previously.[11,21,22] In brief, 10 μg of total cellular RNA were size-fractionated on a 1% agarose–formaldehyde gel and transferred onto a Hybond-N$^+$ membrane (Amersham, Aylesbury, UK). Blots were hybridized with a [^{32}P]dCTP-labeled MDR1/P-gp-specific cDNA fragment. As control for equal RNA loading, the membranes were stripped and rehybridized with a fructose-bisphosphate aldolase (aldolase A) encoding cDNA probe.

Western blot analysis for MDR1/P-gp protein detection

Cellular content of the MDR1/P-gp transporter molecule was detected by Western blot analysis using membrane protein extracts as described previously.[11,21,22] In brief, samples of 20 μg membrane proteins were separated on 4% stacking and 7.5% resolving SDS-polyacrylamide (PA) gel. Proteins were transferred to a 0.2 μm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany) and incubated with the mouse mAb C219 (Alexis, San Diego, CA) directed against human MDR1/P-gp (dilution 1:100) and, afterwards, with peroxidase-conjugated mouse anti-rabbit IgG (dilution 1:10,000) (Sigma, St Louis, MO; # A-1949). As control for equivalent protein loading, the filters were simultaneously incubated with a mouse mAb-directed against actin (chemicon, Temecula, CA; # MAB 1501R) (dilution 1:3000). The protein–antibody complexes were visualized by chemiluminescence (ECL system, Amersham, Aylesbury, UK).

Drug accumulation assay

Measurement of cellular anthracycline accumulation was performed by flow cytometry as described previously.[11,21,22] In brief, 2.5 $\times$ 10^5 cells were seeded in six-well plates. After 48 hours, cells were incubated with 1 μM (580 ng/ml) daunorubicin for 60 minutes at 37°C. Cells were washed with ice-cold PBS, trypsinized and resuspended in 500 μl PBS. Intracellular fluorescence of daunorubicin was determined using a flow cytometer instrument (Calibur 750; Becton-Dickinson, San Jose, CA). The cells were excited at 480 nm and emission was collected at 550 nm. A minimum of 10^4 cells was analyzed for each sample. Data of at least three independent experiments in duplicate were used to build a geometric mean.

Statistical analysis

In cell proliferation assays and anthracycline accumulation studies, levels of statistical significance were evaluated by calculation of the two-tail *P*-values by performing the unpaired *t*-test using the Prism software (GraphPad Software).

Results

Construction of an anti-MDR1/P-gp shRNA expression system

In order to use the phenomenon of RNAi for MDR1/P-gp-specific gene silencing in classical multidrug-resistant gastric carcinoma-derived cell lines, an H1-RNA promoter-driven expression vector that directs the synthesis of anti-MDR1/P-gp shRNA was constructed. Chemically

synthesized sense and antisense ssDNA oligonucleotides encoding the MDR-A (Fig 1a) or MDR-B shRNA were annealed and cloned directed into the assymetric, not compatible *Bbs*I site of the psiRNA-hH1zeo vector to generate psiRNA/MDR-A (Fig 1b) or psiRNA/MDR-B.

Decrease of the MDR1/P-gp-specific mRNA and protein expression by shRNAs

Altogether 24 MDR-A shRNA and 26 MDR-B shRNA anti-MDR1/P-gp shRNA-transfected zeozin-resistant clones derived from the gastric carcinoma cell line EPG85-257RDB were isolated and expanded. Moreover, 12 control clones were isolated. These clones were transfected with a psiRNA-H1zeo expression vector containing an active β-galactosidase (LacZ) producing EM7-lacZ α-peptide cassette instead of a shRNA encoding sequence. All these clones were prescreened by Northern blot experiments concerning the putative gene-silencing activity of the anti-MDR1/P-gp shRNA construct.

In two of the MDR-A anti-MDR1/Pgp shRNA-treated clones derived from the multidrug-resistant gastric carcinoma cell line EPG85-257RDB, a complete downregulation of the MDR1/P-gp-specific mRNA could be observed. More than 50% of the clones (13 of 24 clones) showed an intermediate decrease of the MDR1/P-gp-specific mRNA expression level, whereas in nine clones no modulation in the MDR1/P-gp mRNA level could be detected. In none of the MDR-B anti-MDR1/P-gp shRNA-treated clones a significant downregulation of the MDR1/P-gp mRNA transcript could be detected (data not shown). None of the control clones derived from the cell line EPG85-257RDB exhibited any alterations in MDR1/P-gp mRNA expression level. Figure 2a demonstrates the complete MDR-A anti-MDR1/P-gp shRNA-dependent inhibition of the MDR1/P-gp-specific mRNA expression in two clones (EPG85-257RDB/shRNA clone 1 and 2). No decrease in MDR1/P-gp mRNA expression could be detected in the representative control clone or in the classical multidrug-resistant cell line EPG85-257RDB that was cultivated in the absence of any drug. The corresponding drug-sensitive parental gastric carcinoma cell line EPG85-257P was completely negative for expression of the MDR1/P-gp mRNA.

As shown in Figure 2b, Western blot experiments also demonstrated a complete disappearance of the MDR1/P-gp-specific protein signal in the anti-MDR1/P-gp-treated cells, whereas none of the controls showed any modulation of MDR1/P-gp protein synthesis. As expected, parental EPG85-257P cells did not show any MDR1/P-gp-specific signal.

Increase of cellular anthracycline accumulation by shRNAs

The relative cellular accumulation of the anthracycline daunorubicin in nonresistant, parental cells, in "classical" multidrug-resistant cells, and in anti-MDR1/Pgp shRNA-treated cells was examined by flow cytometry. As shown in Figure 3, the drug-sensitive gastric carcinoma cell line EPG85-257P exhibited a considerable accumulation of the anthracyline, that is, 75-fold increased drug accumulation in comparison to the MDR1/Pg-p-overexpressing MDR variant EPG85-257RDB. In the MDR cell line EPG85-257RDB and the control clone EPG85-257RDB/vec, merely a negligible drug accumulation could be measured. In contrast, by treating EPG85-257RDB cells with the anti-MDR1/P-gp shRNA expression construct, the drug accumulation could be enhanced to 38% of the drug accumulation of drug-sensitive cells in EPG85-257RDB/shRNA clone 1, and to 74% in EPG85-257RDB/shRNA clone 2.

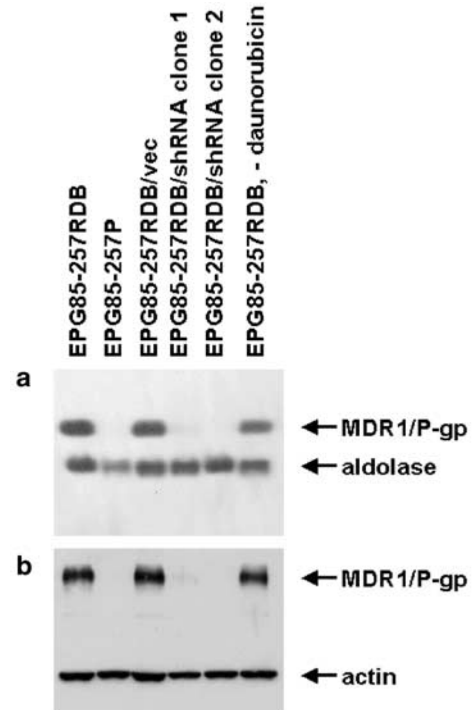

Figure 2 Characterization of shRNA-mediated decrease of MDR1/P-gp mRNA expression and cellular MDR1/P-gp content in human gastric carcinoma cells. (**a**) Northern blot analysis depicting MDR1/P-gp mRNA expression during silencing using the MDR-A shRNA expression vector construct. As control, the blots were probed using an aldolase-specific cDNA. (**b**) Western blot analysis of cell membranous MDR1/P-gp content following MDR-A shRNA expression. As control for equivalent protein loading, Western blot membranes were simultaneously incubated with an actin-specific mAb. EPG85-257RDB, multidrug-resistant gastric carcinoma cells; EPG85-257P, drug-sensitive gastric carcinoma cells. For control of stable MDR1/P-gp expression, EPG85-257RDB cells were also cultivated in drug-free medium for the identical time period than the anti-MDR1/P-gp shRNA-transfected clones.

Reversal of the multidrug-resistant phenotype by shRNAs

The shRNA-mediated reversal of the multidrug-resistant phenotype in gastric carcinoma cells was assessed by comparison of the anthracycline-specific IC_{50}-values determined by a cell proliferation assay. By comparing

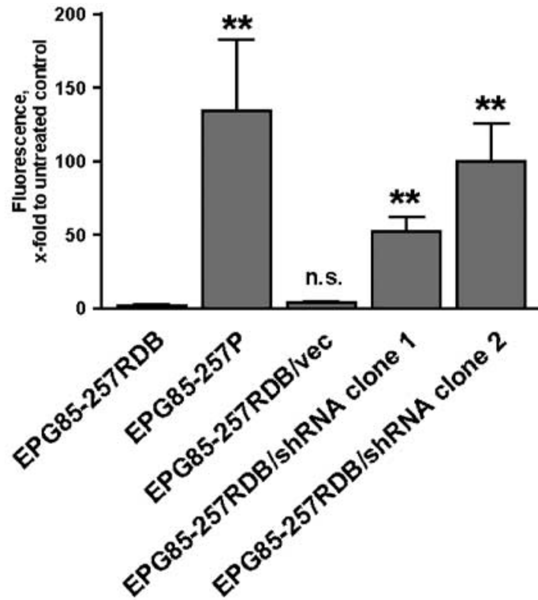

Figure 3 Daunorubicin accumulation in MDR-A anti-MDR1/P-gp shRNA-expressing gastric carcinoma cells. EPG85-257RDB, multidrug-resistant gastric carcinoma cells; EPG85-257P, drug-sensitive gastric carcinoma cells; EPG85-257RDB/vec, EPG85-257RDB transfected with the control vector psiRNA-H1zeo; EPG85-257RDB/shRNA clone 1 and 2, EPG85-257RDB transfected with the anti-MDR1/P-gp shRNA expression vector psiRNA/MDR-A. (n.s., not significant; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ in comparison to the untreated multidrug-resistant cell line EPG85-257RDB).

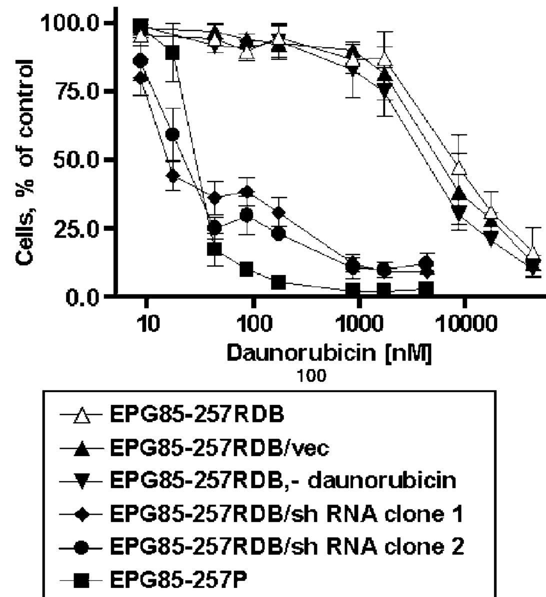

Figure 4 Reversal of daunorubicin resistance by anti-MDR1/P-gp shRNAs as measured by a monolayer proliferation assay to assess daunorubicin-specific IC_{50} values. EPG85-257RDB, multidrug-resistant gastric carcinoma cells; EPG85-257P, drug-sensitive gastric carcinoma cells; EPG85-257RDB/vec, EPG85-257RDB transfected with the control vector psiRNA-H1zeo; EPG85-257RDB/shRNA clones 1 and 2, EPG85-257RDB transfected with the anti-MDR1/P-gp shRNA expression vector psiRNA/MDR-A. For control of stable MDR1/P-gp expression, EPG85-257RDB cells were also cultivated in drug-free medium for the identical time period than the anti-MDR1/P-gp shRNA-transfected clones Epg85-257RDB,-daunorubicin. EPG85-257RDB/vec and (Epg85-257RDB,-daunorubicin) did not show significant alterations in IC_{50} values, whereas the drug-sensitive cell line EPG85-257P ($P < .05$), EPG85-257RDB/shRNA clones 1 ($P < .05$), and EPG85-257RDB/shRNA clones 2 ($P < .05$) showed significant lower IC_{50} values in comparison to the untreated multidrug-resistant cell line EPG85-257RDB.

the IC_{50}-values, the "classical" multidrug-resistant gastric carcinoma cell line EPG85-257RDB exhibited a 345-fold higher resistance against daunorubicin than its parental counterpart EPG85-257P. As shown in Figure 4, treatment of EPG85-257RDB cells with the control vector or the cultivation of this cell line in the absence of anticancer agents have negligible influence on the IC_{50}-values. In contrast, treatment of the cell line EPG85-257RDB with the anti-MDR1/P-gp shRNA expression vector completely reversed the drug-resistant phenotype in both clones (EPG85-257RDB/shRNA clone 1 and 2) to a level that does not significantly differ from that measured in the drug-sensitive, parental cell line EPG85-257P.

Discussion

Transient application of chemically synthesized siRNAs is an elegant and convenient method for the specific downregulation of a given mRNA transcript of interest. However, this approach is restricted by low transfection efficiencies and short-term cellular persistence of the siRNA molecules. Further problems are the biological half-life times of the target transcript and its encoding protein. In the case of MDR1/P-gp, a recently published study using a human carcinoma cell line showed that the MDR1/P-gp-specific mRNA had a half-life of approximately 4 hours, and the corresponding transporter protein exhibited a half-life of approximately 16 hours.[23] Accordingly, the previous application of anti-MDR1/P-gp siRNAs in the "classical" multidrug-resistant cell line EPG85-257RDB resulted in a maximum decrease of MDR1/P-gp mRNA expression after 1–2 days and a maximum decrease of the transporter protein after 3–5 days.[11] After those minimum peak levels, the expression levels of the MDR1/P-gp encoding mRNA as well as the corresponding protein started to increase and reached the original mRNA expression levels after 7 days for the mRNA transcript and 10 days for the corresponding MDR-mediating ABC-transporter molecule. Thus, by transient application of the siRNA molecules used for construction of the expression vector used in this study, the MDR phenotype was merely decreased to 48–58% in EPG85-257RDB cells. Using a similar siRNA sequence, overlapping in 15 nucleotides with the MDR-A sequence, in alternative cell systems, likewise a complete reversal of the multidrug-resistant phenotype could not be achieved.[12] Since both studies used different experimental techniques, a proliferation assay[11] or a clonogenic assay,[12]

to determine the extent of the MDR modulation, the efficiencies of both siRNA sequences are not directly comparable.

For improving of RNAi-mediated gene-silencing some plasmid-based vectors have been developed that express siRNAs as inverted repeats that show similar potency to trigger RNAi. These siRNA-like molecules are commonly designated shRNAs. For production of shRNAs, these vectors were designed to drive the stable expression of shRNAs by strong RNA polymerase-II-dependent promoters like CMV or EF1α, or by RNA polymerse-III promoters like U6 or H1.[18,24–29] Since experiences with antisense and ribozyme technology indicate that RNA polymerase-III expression systems offer a great potency in stable expression of short RNA molecules *in vitro* as well as *in vivo*,[30] in this study an RNA polymerase-III-driven expression vector was applied. It was constructed on the basis of the first shRNA expression vector that was designed by Brummelkamp et al.[18] This H1-RNA promoter containing pSUPER expression vector was used to suppress the expression of the CDH1 gene, involved in activation of the anaphase-promoting complex (APC), in human MCF-7 breast carcinoma cells. Predicted shRNAs containing different loop structures directed against the CDH1 encoding mRNA transcript demonstrated that the size of the loop has a considerable influence on the gene-silencing activity. Brummelkamp et al[18] reported that a 9-nt loop showed more effects than a 7-nt loop, whereas a 5-nt loop structure showed only moderate effects. However, since alternative studies reported that very short loop structures including 3- and 5-nt loops did not show smaller shRNA-mediated gene-silencing effects than longer loops,[31,32] in this study a 5-nt loop structure was chosen for construction of the anti-MDR1/P-gp shRNA expression vector.

For better comparison of shRNA-mediated gene-silencing with siRNA-depending effects, the target sequences of the shRNAs were chosen to be homologous to the two MDR1/P-gp target sequences used for suppression of the MDR1/P-gp expression by chemically synthesized siRNAs previously.[11] The MDR1/P-gp-specific target sequences in that study were selected according the recommendations in the scientific literature[17,33] (MDR-A), or to be homologous to a well-assessable hammerhead ribozyme cleavage site analyzed in detail previously[9,34] (MDR-B). However, due to theoretically deliberations, there could be problems in using the second (MDR-B) shRNA sequence, whose correct insertion into the H1 RNA promoter-driven expression vector was verified by sequencing. The MDR-B-encoding sequence contains two thymidines at the 3′-terminus. Thus, the T5-termination signal could terminate the transcription of the MDR-B shRNA two nucleotides earlier as desired. This effect would result in a loss of the two 3′-overhanging uracils within the shRNA molecule, and therewith, in a diminished biological stability of the shRNA. Owing to the observation that transfection of multidrug-resistant EPG85-257RDB gastric carcinoma cells with an MDR-B encoding psiRNA-H1zeo plasmid-based shRNA expression vector did not result in dramatic downregulation of the MDR1/P-gp expression level (data not shown), this assumption was supported.

shRNA-mediated RNAi in multidrug-resistant EPG85-257RDB gastric carcinoma cells resulted in a considerable increase in accumulation of the classical MDR agent daunorubicin. Although the anthracycline accumulation did not reach the peak level of drug accumulation in the corresponding parental, drug-sensitive cell line EPG85-257P, the intracellular drug concentration in anti-MDR1/P-gp shRNA-treated cells was sufficient enough for the complete reversal of the MDR phenotype. The lower anthracycline accumulation in anti-MDR1/P-gp shRNA-treated cells might be the result of a possible residual pump activity mediated by remaining MDR1/P-gp molecules that could not be detected by the available protein detection techniques. However, the biological effects of the anti-MDR1/P-gp shRNA were sufficient that even in this extremely high drug-resistant cell line a complete reversal of the MDR phenotype could be achieved.

Since it was reported that withdrawal of anthracyclines from the cell culture medium of "classical" multidrug-resistant cancer cells resulted in loss of MDR1/P-gp expression,[35] an additional culture flask of the cell line EPG85-257RDB was cultivated in the absence of any antineoplastic agent for the same time period as anti-MDR1/P-gp shRNAs were used for stable transfection experiments. Expression analyses on mRNA and protein level as well as assessment of the IC_{50}-values demonstrated that withdrawal of daunorubicin had no influence on the multidrug-resistant phenotype in EPG85-257RDB cells.

Beside "delivery", the general obstacle of cancer gene therapy, additional problems for clinical MDR1/P-gp inhibition by anti-MDR1/P-gp shRNA expression vectors may arise by the physiological expression of this ABC-transporter in several epithelial and endothelial cells. For example, MDR1/P-gp is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain[36] suggesting that this efflux pump plays an important role in the blood–brain barrier and therewith is crucial for limiting the potential neurotoxicity of many anticancer drugs. Thus, in the clinical situation it may be necessary that the application of MDR1/P-gp-inhibiting shRNA expression vectors should be restricted to MDR1/P-gp-expressing multidrug-resistant cancer cells. One promising strategy for cancer cell-limited delivery of anti-MDR1 siRNAs would be the development of vector systems specific for multidrug-resistant cancer cells. Very recently, a "replication-defective" E1A-mutant adenoviral vector that efficiently and selectively replicates in "classical" multidrug-resistant cells has been described.[37] Such a virus may provide the basis for the development of novel vectors for the specific gene therapeutic treatment of multidrug-resistant cancer cells.

In conclusion, this study demonstrates that the shRNA-based RNAi approach can be tremendously effective in reversing MDR1/P-gp-mediated MDR, even in extremely high drug-resistant cell models. Thus, the RNAi technology has clinical implications for the prevention and the

reversal of MDR in human malignancies, and prolonging disease-free survival in certain cases by gene therapeutic approaches. However, it has to be taken into consideration that RNAi target sequences that showed very well gene-suppressing effects by using chemically synthesized siRNAs can exhibit an aberrantly effectiveness when using expression vector encoded shRNA directed against the same sequence. Thus, the siRNA- evaluated target sequences cannot be used in each case to design highly effective shRNA expression systems.

Acknowledgments

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