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Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi)

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Abstract For reversal of MDR1 gene-dependent multidrug resistance (MDR), two small interfering RNA (siRNA) constructs were designed to inhibit MDR1 expression by RNA interference. SiRNA duplexes were used to treat human pancreatic carcinoma (EPP85-181RDB) and gastric carcinoma (EPG85-257RDB) cells. In both cellular systems, siRNAs could specifically inhibit MDR1 expression up to 91% at the mRNA and protein levels. Resistance against daunorubicin was decreased to 89% (EPP85-181RDB) or 58% (EPG85-257RDB). The data indicate that this approach may be applicable to cancer patients as a specific means to reverse tumors with a P-glycoprotein-dependent MDR phenotype back to a drug-sensitive one.

Key words: P-glycoprotein; MDR1; Gastric cancer; Pancreatic cancer; Gene therapy

1. Introduction

Resistance to antineoplastic drugs is the major reason why chemotherapy-based treatment modalities of malignant tumors may fail. Human cancer cells can exhibit a cross-resistant phenotype against several unrelated 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 (P-gp, P-170, PGY1, MDR1, ABCB1) [2], a member of the superfamily of ABC (ATP binding cassette) transporters [3]. A disruption of P-gp-mediated drug extrusion results in a re-sensitization of tumor cells to treatment with antineoplastic agents, and thus may allow a successful drug treatment of the multidrug-resistant cancer cells [4].

Pharmacologically active compounds, designated MDR modulators or chemosensitizers, may circumvent the 'classical' MDR phenotype by inhibiting the efflux pump activity of P-gp [5]. One obstacle in applying MDR modulators arises from their commonly occurring intrinsic toxicity at doses necessary to be active, e.g. heart failure, hypotension, hyperbilirubinemia, and immunosuppression by cyclosporin A. Additionally, tumor cells can develop resistance against the applied chemosensitizers, 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 P-gp-mediated MDR in cancer cells is to prevent the biosynthesis of P-gp by selectively blocking the expression the P-gp-specific MDR1 mRNA. 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 were shown to modulate P-gp-dependent MDR [6,7]. Moreover, hammerhead ribozymes were designed and successfully applied to decrease the expression level of the P-gp-encoding MDR1 mRNA [8,9].

A novel means for specific inhibition of a gene of interest is the use of small interfering RNA (siRNA). These 21–25 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 RNA interference (RNAi) [10]. 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 [11]. Introduction of dsRNA into mammalian cells does not result in efficient Dicer-mediated generation of siRNA and therefore does not induce RNAi [12]. 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 [13].

In this study, two 21-nt siRNA duplexes against two regions of the P-gp-encoding mRNA were designed for disruption of P-gp-mediated drug extrusion in a specific manner and re-sensitization of gastrointestinal tumor cells to treatment with the antineoplastic agent daunorubicin. The first siRNA molecule (MDR-A) was constructed according the recommendations by others ([13,14], http://www.mpibpc.gwdg.de/abteilungen/100/105/), the second siRNA (MDR-B) was designed to be homologous to a well-assessable ribozyme cleavage site analyzed in detail previously [8,15]. The biological activities of both siRNA constructs were examined.

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2. Materials and methods

2.1. Cell lines and cell culture

Establishment and cell culture of the human gastric carcinoma cell line EPG85-257P [16], and of the human pancreatic carcinoma cell line EPP85-181P [17] was described in detail previously. In both cases, 'classical' multidrug-resistant, P-gp-positive derivatives, EPG85-257RDB [18] and EPP85-181RDB [17], were established by in vitro exposure to daunorubicin (Farmitalia Carlo Erba, Freiburg, Germany). In order to ensure maintenance of the 'classical' MDR phenotype, cell culture medium for the P-gp-expressing lines EPG85-257RDB and EPP85-181RDB was supplemented with 5.09 μM (2.5 μg/ml) daunorubicin.

2.2. Cytotoxicity assay for cell survival

Chemoresistance was tested using a proliferation assay based on sulforhodamine B (SRB), a protein-binding reagent [19], as described previously [17,20]. Briefly, in each experiment 1500 cells per well were seeded in 96-well plates and daunorubicin was added in dilution series in triplicate wells. After 4 days, incubation was terminated by replacing the medium with 10% trichloroacetic acid, followed by incubation at 4°C for 1 h. Subsequently, the plates were washed five times with water and stained by adding 100 μl 0.4% SRB (Sigma, St. Louis, MO, USA) in 1% acetic acid for 10 min at room temperature. Unbound dye was eliminated by washing the plates five times with 1% acetic acid. After air-drying and re-solubilization of the protein-bound dye in 10 mM Tris–HCl (pH 8.0), absorbance was read at 562 nm against a background at 690 nm. To determine the IC_{50} values, the absorbance difference of control cells without drug was set at 1. A dose–response curve was plotted using the Prism software (GraphPad Software, San Diego, CA, USA), and IC_{50} values were calculated from multiple (at least three) independent experiments for each cell line. Cytotoxicity analyses were performed 48 h after siRNA treatment of MDR cells. Survival data of siRNA-treated cells were evaluated for statistical significance by the two-sided *t*-test.

2.3. siRNA design

Two different P-gp-specific siRNA duplexes were designed to be homologous to the P-gp-encoding MDR1 mRNA consensus sequence (GenBank accession number NM_000927). Both 21-nt siRNAs contained 3′-dTdT extensions and were commercially obtained from Dharmacon (Lafayette, CO, USA). The target sequence of the first siRNA molecule, MDR-A (5′-AAG AAG GAA AAG AAA CCA ACU-3′), homologous to nt 503–523 of the MDR1 mRNA sequence NM_000927, was chosen according the recommendations by others ([13,14], http://www.mpibpc.gwdg.de/abteilungen/100/105/). The second siRNA, MDR-B (5′-AAA AUG UUG UCU GGA CAA GCA-3′), homologous to nt 3050–3070, was constructed against the well-assessable hammerhead ribozyme cleavage GUC site at nt 3058–3060 that was analyzed in detail previously [8,15].

2.4. Transfection with siRNAs

For transfection of MDR-A and MDR-B, either dsRNA or as control single-stranded oligonucleotide molecules were handled according the recommended procedure ([13,14], http://www.mpibpc.gwdg.de/abteilungen/100/105/) by using OligofectAMINE Reagent (Gibco BRL, Gaithersburg, MD, USA) for forming liposomes. Before starting transfection experiments with MDR-A and MDR-B, a dsRNA sequence for silencing the lamin A/C mRNA was used for optimization of the RNAi application as recommended ([13,14], http://www.mpibpc.gwdg.de/abteilungen/100/105/).

2.5. Northern blot analysis

The amount of the P-gp-encoding MDR1 mRNA was determined by Northern blot analysis applying standard procedures as described previously [17,20]. In brief, total cellular RNA was prepared using a RNeasy Mini Kit (Qiagen). 10 μg RNA was separated on 1% agarose-formaldehyde gels and transferred onto a Hybond-N^+ membrane (Amersham, Aylesbury, UK). Blots were hybridized with 25 ng of a 557-bp MDR1 cDNA fragment labeled with [^{32}P]dCTP by random primed labeling (Amersham). The hybridization probe was generated by reverse transcription polymerase chain reaction (RT-PCR) using a cDNA template obtained by reverse transcription of total RNA prepared from the cell line EPP85-181RDB using the SuperScript Preamplification System (Gibco BRL). Sequence-specific oligonucleotide primers were designed after alignment with the P-gp-encoding nucleic acids sequence NM_000927. Oligodeoxynucleotide primers used for amplification were PGP-fw: 5′-GCC CTT GGA ATT ATT TCT TT-3′ and PGP-rev: 5′-TGG GTG AAG GAA AAT GTA AT-3′. Subsequently, the PCR product was cloned into the pCR2.1 vector using an Original TA Cloning Kit (Invitrogen, San Diego, CA, USA). The identity of the PCR product was confirmed by dye terminator sequencing using an ABI-373 sequencer (Perkin Elmer). As control for equal RNA loading the membranes were stripped and rehybridized with a fructose-bisphosphate aldolase (aldolase)-encoding cDNA probe, prepared as described in Section 2.6.

2.6. Quantitative RT-PCR

For quantitative mRNA expression analysis, real-time RT-PCR was carried out with total RNA (see above) prepared 48 h after siRNA treatment using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). Oligodeoxynucleotide primers used for amplification were PGP-fw and PGP-rev (see above) and as control Ald-fw: 5′-ATC CTG GCT GCA GAT GAG TC-3′, Ald-rev: 5′-GCC CTT GTC TAC CTT GAT GC-3′ yielding an expected amplification product of 249 bp specific for a housekeeping gene encoding aldolase (GenBank accession number NM_000034). Likewise, the identity of the aldolase-specific PCR product was confirmed by sequencing. Reverse transcription was performed with SuperScript II enzyme (Gibco BRL) using arbitrary hexamers. Amplification products were detected on-line via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics). Cycling conditions for P-gp (aldolase) were as follows: initial enzyme activation at 95°C for 10 min, followed by 45 cycles at 94°C (95°C) for 15 s, 50°C (54°C) for 5 s and 72°C for 10 s. All cycling reactions were performed in the presence of 4 μM $MgCl_2$. Gene-specific fluorescence was measured at 84°C (86°C) and confirmed by melting curve analysis. For quantification, samples were normalized against the expression of the aldolase-encoding mRNA. RNA expression data of siRNA-treated cells were evaluated for statistical significance by the two-sided *t*-test.

2.7. Western blot analysis

For detection of P-gp, membrane protein extracts were prepared as described previously [17,20]. Samples of 10 μg of membrane proteins each were diluted with sample buffer and separated on 4% stacking and 7.5% resolving sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were transferred to a 0.2 μm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany). To avoid unspecific binding, the filters were incubated in 5% skim milk, 0.05% Tween 20 in TBS overnight. Subsequently, filters were incubated with mouse monoclonal antibodies (mAbs) C219 (Alexis, San Diego, CA, USA) directed against human P-gp diluted in 1% skim milk in 1×TBST (20 mM Tris–HCl; 137 mM NaCl; 0.05% Tween 20; pH 7.5) (1:100) for 2 h and, afterwards, with peroxidase-conjugated mouse anti-rabbit IgG (1:10 000) (Sigma, St. Louis, MO, USA; #A-1949). As control for equivalent protein loading, the filters were simultaneously incubated with a mouse mAb directed against actin (Chemicon, Temecula, CA, USA; #MAB 1501R) diluted 1:3000. The protein–antibody complexes were visualized by chemoluminescence (ECL system, Amersham, Buckinghamshire, UK).

3. Results

3.1. Decrease of the P-gp-encoding mRNA expression using siRNAs

Two different siRNA constructs, MDR-A and MDR-B, were used to decrease the expression of the P-gp-encoding MDR1 mRNA in the human multidrug-resistant gastric and pancreatic carcinoma cell lines EPG85-257RDB and EPP85-181RDB, respectively. Northern blot analyses demonstrated that both siRNAs MDR-A (Fig. 1B) and MDR-B (Fig. 1D) decreased the MDR1 mRNA expression level in the multidrug-resistant gastric carcinoma cell line EPG85-257RDB in a time-dependent manner. Using both siRNAs, after 1 day of siRNA transfection only a weak MDR1 mRNA-specific signal could be detected. Following 3 and 5 days the MDR1

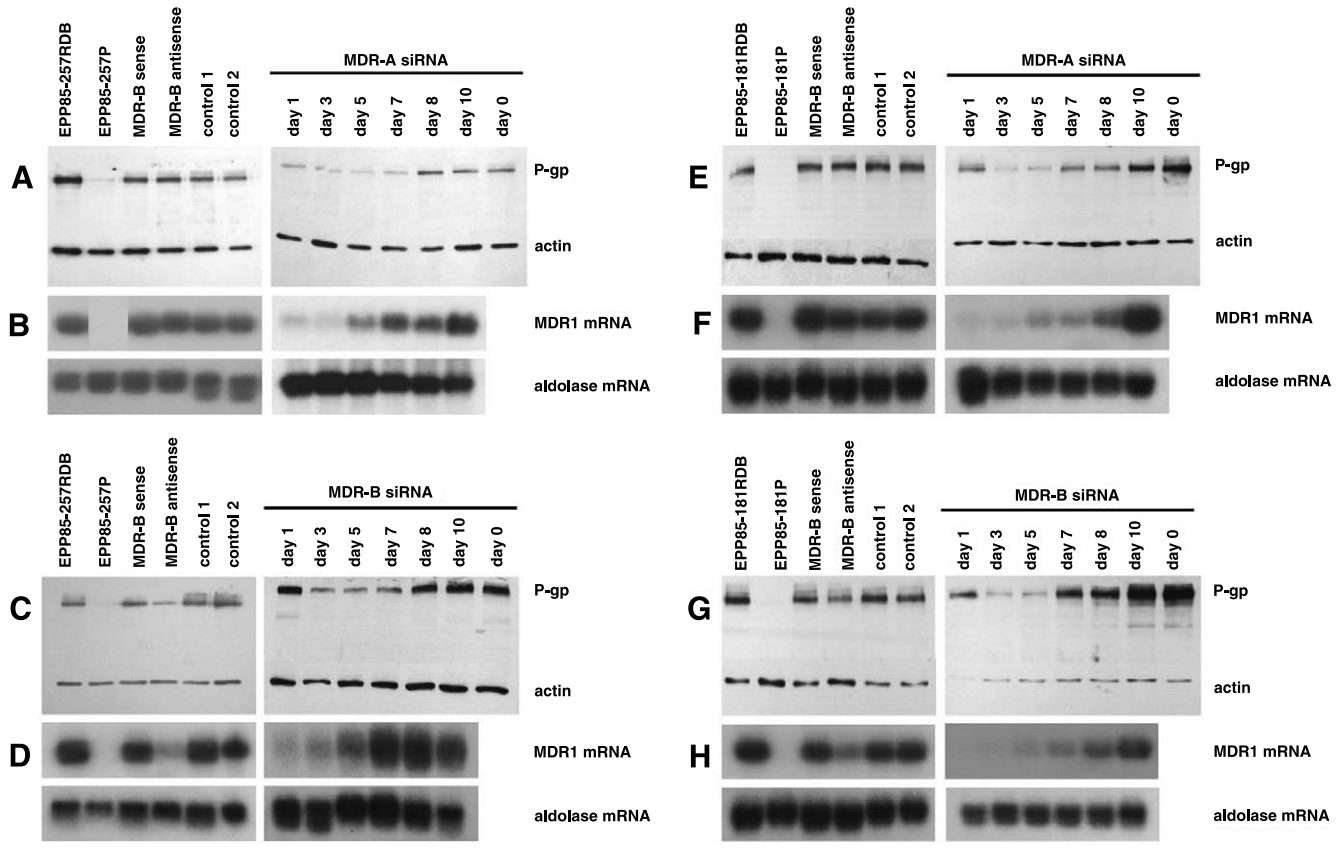

Fig. 1. Characterization of siRNA-mediated decrease of MDR1 mRNA expression and cellular P-gp content in human gastric carcinoma and pancreatic carcinoma cells. A: Western blot analysis of cell membranous P-gp content following MDR-A siRNA exposure in gastric carcinoma cells. B: Northern blot analysis depicting MDR1 mRNA expression during silencing using the MDR-A siRNA construct in gastric carcinoma cells. C: Western blot following MDR-B exposure in gastric carcinoma cells. D: Northern blot following MDR-B treatment in gastric carcinoma cells. E: Western blot following MDR-A exposure in pancreatic carcinoma cells. F: Northern blot using MDR-A in pancreatic carcinoma cells. G: Western blot following MDR-B exposure in pancreatic carcinoma cells. H: Northern blot after MDR-B treatment in pancreatic carcinoma cells. In each case, Northern blot membranes were stripped and reprobed for aldolase-encoding mRNA as loading control. 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; EPP85-181RDB, multidrug-resistant pancreatic carcinoma cells; EPP85-181P, drug-sensitive pancreatic carcinoma cells; MDR-A siRNA, double-stranded biologically active siRNA; MDR-A sense, single-stranded MDR-A RNA in sense orientation; MDR-A antisense, single-stranded MDR-A RNA in antisense orientation; MDR-B siRNA, double-stranded biologically active siRNA; MDR-B sense, single-stranded MDR-B RNA in sense orientation; MDR-B antisense, single-stranded MDR-B RNA in antisense orientation; control 1, medium; control 2, medium with transfection reagent.

mRNA expression level tardily increases, reaching the original mRNA expression value after 7 days.

In the case of multidrug-resistant pancreatic carcinoma cells, Northern blot experiments revealed that the gene-silencing effects of both siRNA molecules were much more pronounced than in the gastric carcinoma model. By treatment with MDR-A (Fig. 1F) or MDR-B (Fig. 1H), the expression level of the MDR1 mRNA was reduced to the point at which it could no longer be detected up to 3 days after siRNA transfection. After that time period the MDR1 mRNA expression started to increase and reached the original mRNA expression level after 10 days.

Using the conventional designed siRNA molecule MDR-A, none of the controls, MDR-A-specific single-stranded sense or antisense RNA oligonucleotides, medium in the absence of any supplements, and medium with transfection reagent, showed any effects on the MDR1 mRNA expression level after 48 h in Northern blot experiments (Fig. 1B,F). In contrast to this observation, the second single-stranded antisense MDR-B RNA oligonucleotide directed against a well-assessable hammerhead ribozyme cleavage side showed slight gene-silencing effects on MDR1 mRNA expression in multidrug-resistant gastric carcinoma as well as in pancreatic carcinoma cells (Fig. 1D,H).

To obtain quantitative MDR1 mRNA expression values, quantitative real-time RT-PCR experiments were performed after 48 h of treatment with siRNA constructs or controls. These quantitative MDR1 mRNA expression values confirmed the data obtained by Northern blot analyzes, but revealed some more detailed information.

As shown in Fig. 2A, the multidrug-resistant gastric carcinoma cell line EPG85-257RDB exhibited a 933-fold overexpression of MDR1 mRNA when compared to the drug-sensitive, parental variant EPG85-257P. Treatment with MDR-A decreased the MDR1 mRNA level to 13%, treatment with MDR-B decreased it to 26% of the original MDR1 mRNA expression value, i.e. 87% and 74% gene-silencing activity in gastric carcinoma cells. In the case of MDR-A, the single-stranded MDR-A sense oligonucleotide and the single-stranded MDR-A antisense oligonucleotide showed no relevant effects on MDR1 mRNA expression. In contrast, both single-stranded MDR-B oligonucleotides distinctly decreased

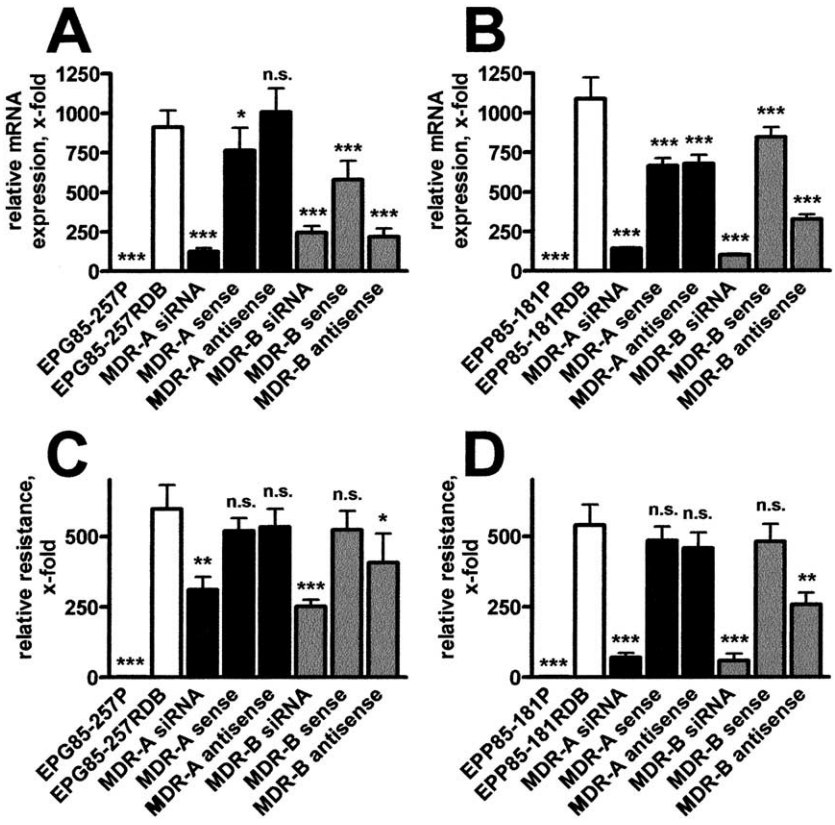

Fig. 2. Analysis of siRNA-mediated silencing of MDR1 mRNA expression determined by quantitative real-time RT-PCR and characterization of MDR reversal by depicting the resistance against daunorubicin as determined by IC_{50} values in human gastric carcinoma and pancreatic carcinoma cells. All experiments were performed 48 h after treatment with siRNA molecules. A: Relative MDR1 mRNA expression level normalized against aldolase mRNA expression in gastric carcinoma cells. The MDR1/aldolase expression ratio in drug-sensitive EPG85-257P cells was set at 1. B: Relative MDR1 mRNA expression level normalized against aldolase mRNA expression in pancreatic carcinoma cells. The MDR1/aldolase mRNA expression ratio in drug-sensitive EPP85-181P cells was set at 1. C: Relative resistance against daunorubicin in gastric carcinoma cells. Resistance value in drug-sensitive, parental EPG85-257P cells was set at 1. D: Relative resistance against daunorubicin in pancreatic carcinoma cells. Resistance value in drug-sensitive EPG85-257P cells was set at 1. EPG85-257RDB, multidrug-resistant gastric carcinoma cells; EPG85-257P, drug-sensitive gastric carcinoma cells; EPP85-181RDB, multidrug-resistant pancreatic carcinoma cells; EPP85-181P, drug-sensitive pancreatic carcinoma cells; MDR-A siRNA, double-stranded biologically active siRNA; MDR-A sense, single-stranded MDR-A RNA in sense orientation; MDR-A antisense, single-stranded MDR-A RNA in antisense orientation; MDR-B siRNA, double-stranded biologically active siRNA; MDR-B sense, single-stranded MDR-B RNA in sense orientation; MDR-B antisense, single-stranded MDR-B RNA in antisense orientation. The relative expression values are means of at least three independent experiments in triplicate, error bars show S.D. Treatment of multidrug-resistant gastrointestinal cancer cells statistically significantly decreased the MDR1 mRNA expression level and the resistance against daunorubicin: n.s., non-significant difference to the multidrug-resistant cell line; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

the MDR1 mRNA expression, especially the MDR-B antisense oligonucleotide showed a similar gene-silencing activity like the complete siRNA construct.

The multidrug-resistant pancreatic carcinoma cell line EPP85-181RDB showed a 1088-fold MDR1 mRNA overexpression in comparison to the non-resistant counterpart EPP85-181P (Fig. 2B). In this model system, MDR-A decreased the MDR1 mRNA expression level likewise to 13%, MDR-B even to 9% of the initial value. In other words, in pancreatic carcinoma cells 87% and 91% gene-silencing activity could be demonstrated. Both single-stranded MDR-A control oligonucleotides showed similar slight effects on MDR1 mRNA expression, i.e. decrease to 61% (MDR-A sense) or 62% (MDR-A antisense). The single-stranded MDR-B sense oligonucleotide showed negligible activity, whereas a distinct MDR1 mRNA expression-inhibiting activity to 30% could be observed by applying the single-stranded MDR-B antisense oligonucleotide.

As determined by quantitative real-time RT-PCR, both drug-sensitive, parental cell lines showed a very low comparable basic MDR1 mRNA expression that could not be detected by the less sensitive Northern blot procedure. The lowest MDR1/aldolase mRNA expression ratio was measured in the drug-sensitive pancreatic carcinoma cell line EPP85-181P; this was set at 1. The parental gastric carcinoma cell line EPG85-257P showed a slightly increased MDR1 mRNA expression value of 1.8-fold when compared to the pancreatic carcinoma line EPP85-181P. Thus, relative MDR1 mRNA expression values were normalized against the pancreatic carcinoma cell line. As a consequence, both multidrug-resistant cancer cell lines showed MDR1 mRNA overexpression in a similar range, where a slightly enhanced value could be measured in the multidrug-resistant gastric carcinoma line, i.e. 1571-fold overexpression in gastric cancer cells instead of 1088-fold in pancreatic carcinoma-derived MDR cells.

3.2. Knockdown of P-gp on protein level by siRNAs

Western blot experiments demonstrated that both siRNA constructs decreased the cellular P-gp content in both multidrug-resistant gastrointestinal tumor cell models. This reduc-

tion of transmembrane transport protein concentration was time-slipped in relation to the decrease in MDR1 mRNA expression. In MDR gastric carcinoma cells as well as in pancreatic carcinoma MDR cells the peak of protein reduction was reached after 3–5 days using MDR-A siRNA (Fig. 1A,E) or MDR-B siRNA (Fig. 1C,G). As shown at the mRNA level, the biological activity of both siRNAs was more pronounced in the pancreatic carcinoma model EPP85-181RDB. The Western blot analyses showed that in none of the cases the cellular P-gp content could be reduced completely. As demonstrated at the mRNA level, single-stranded MDR-B antisense oligonucleotides also showed a biological activity at the protein level, whereas no effect could be observed by any other of the controls.

3.3. Reversal of the drug-resistant phenotype by siRNAs

The siRNA-mediated reversal of the multidrug-resistant phenotype was assessed by comparison of the IC_{50} values determined by a cell proliferation assay in siRNA-treated tumor cells and controls. Cytotoxicity experiments were performed 48 h after treatment with siRNA molecules. In both gastrointestinal tumor cell systems, MDR-B siRNA showed a slightly enhanced chemosensitizing activity compared to MDR-A (Fig. 2C,D). In the gastric carcinoma system, both RNAi constructs showed less chemosensitizing activity than in the pancreatic carcinoma model. MDR-A siRNA decreased the resistance factor from 595-fold to 310-fold (decrease to 52% of the initial value, i.e. 48% reversal; $P < 0.01$), MDR-B siRNA decreased the resistance against daunorubicin from 595-fold to 250-fold (decrease to 42% of the initial value, i.e. 58% reversal; $P < 0.001$) in EPG85-257RDB gastric carcinoma cells. The multidrug-resistant phenotype of the pancreatic carcinoma cell line EPP85-181RDB was reversed from 538-fold to 68-fold (decrease to 13% of the initial value, i.e. 87% reversal; $P < 0.001$) by MDR-A siRNA, and from 538-fold to 57-fold (decrease to 11% of the initial value, i.e. 89% reversal; $P < 0.001$) by MDR-B siRNA. The single-stranded MDR-B sense and both single-stranded MDR-A control oligonucleotides showed no MDR-modulating effects, whereas a distinct chemosensitizing activity of the MDR-B antisense oligonucleotide could be observed in both multidrug-resistant gastrointestinal tumor cell lines, i.e. 32% reversal in EPG85-257RDB ($P < 0.05$) and 52% reversal in EPP85-181RDB ($P < 0.01$).

4. Discussion

Experimental endeavors to overcome the obstacles in the use of conventional MDR modulators resulted in the development of antisense and antigene strategies. These included the application of oligonucleotides [6,7] and hammerhead ribozymes [8,9] directed against the P-gp-encoding MDR1 mRNA and against the transcripts of alternative genes encoding MDR-mediating factors, such as LRP [21], MRP1 [22], MRP2 [23], or BCRP [24,25]. In the present study, the modulation of the P-gp-mediated 'classical' MDR phenotype was demonstrated by an alternative, novel gene therapeutic means, i.e. by RNAi triggered by synthetic siRNA molecules homologous to the MDR1 mRNA. Both of the applied anti-MDR1 siRNA constructs, MDR-A designed according conventional recommendations ([13,14], http://www.mpibpc.gwdg.de/abteilungen/100/105/) and MDR-B constructed to be homologous to a well-assessable hammerhead ribozyme cleavage site [8,15], decreased the MDR1 mRNA expression level in a specific manner in two different multidrug-resistant cell systems derived from gastric carcinoma or pancreatic carcinoma. As a consequence, the cellular amount of P-gp was decreased and both multidrug-resistant gastrointestinal carcinoma cell lines became concomitantly sensitive to anthracycline treatment, a hallmark of the P-gp-mediated MDR phenotype.

Both siRNA constructs showed a more pronounced MDR1 gene-silencing activity in MDR pancreatic carcinoma cells at the mRNA as well as the protein level. Moreover, these effects were accompanied by a more effective MDR-reversing activity in the MDR pancreatic carcinoma cell model. These observations might be explained by the more elevated MDR1 mRNA expression level in the multidrug-resistant gastric carcinoma cell line EPG85-257RDB and/or by tissue-specific features, e.g. variable cellular siRNA molecule uptake or differences in the effectiveness of the cellular RNAi mechanisms leading to mRNA degradation in discriminative cell systems.

In both gastrointestinal MDR models, the chemosensitizing activity of the MDR-B siRNA construct was more pronounced. This effect might be explained by an additional antisense effect of the single-stranded MDR-B antisense oligonucleotide that could be observed in both cellular systems. The MDR-reversing effect of the single-stranded MDR-B antisense oligonucleotide was accompanied by a distinct biological effect at the mRNA (Northern blot, real-time RT-PCR) and protein (Western blot) levels. Real-time RT-PCR quantifying of the P-gp-specific mRNA even demonstrated a similar MDR1 gene-silencing effect of the single-stranded MDR-B antisense oligonucleotide like the complete double-stranded siRNA construct. This distinct putative antisense activity might be explained by the well-assessable secondary structure of the MDR1 mRNA in this region that was demonstrated by ribozyme cleavage experiments previously [15].

The more pronounced gene-silencing effects of the MDR-B siRNA construct are in contradiction to the observations made so far by others who concluded that compared to antisense or ribozyme technology, the secondary structure of siRNA target mRNAs does not appear to have a strong effect on gene silencing [14]. Thus, in future experiments the secondary structure of the target molecule should be taken into consideration. Moreover, the design of biologically active siRNA molecules can be improved by the elaborate experiences in the use of antisense oligonucleotides and hammerhead ribozymes obtained previously. Whether the more pronounced gene-silencing effect of MDR-B is indeed the result of an additional antisense effect or of a more effective RNAi effect at this RNA secondary structure remains open. However, in a potential therapeutic application the more biologically effective siRNA construct should be chosen.

The 'classical' MDR phenotype could be reversed 48% by MDR-A and 58% by MDR-B in MDR gastric cancer cells, and 87% by MDR-A and 89% by MDR-B in the pancreatic carcinoma MDR cell line EPP85-181RDB. Thus, the chemosensitizing effects of these transiently added siRNA molecules are in the same range that could be found in cells stably transfected with expression vector constructs encoding hammerhead ribozymes directed against alternative MDR factors [8,9,25]. Thus, more pronounced MDR-modulating effects of both anti-MDR1 mRNA siRNA constructs could be expected by stable transfection of the multidrug-resistant cell lines us-

ing siRNA-containing expression vectors. Moreover, it is important to note that the MDR cell models used are extremely highly resistant and exhibit an enormously enhanced MDR1 mRNA expression level. In the clinical situation, a two-fold or three-fold increased resistance level is already sufficient to inhibit a successful antineoplastic drug-based cancer therapy. Thus, for the potential treatment of cancer patients, even a transient application of anti-MDR1 mRNA siRNA constructs might be an effective tool for the reversal of drug resistance.

However, for reversal of clinical MDR the use of synthetic siRNAs, as opposed to siRNAs expressed from an expression vector system such as recently described [26], possibly may have advantages. Chemically synthesized siRNAs obviate the need to address technical and ethical problems raised by the use of expression vectors, especially potential retrovirus- or adenovirus-based siRNA delivery systems. Furthermore, synthetic siRNAs are more suitable for combination therapies. It would be easier to use chemically synthesized siRNAs simultaneously in combination against the transcripts of various MDR-associated genes, e.g. alternative ABC transporter-encoding genes or genes encoding apoptosis- and cell cycle-regulating factors. Moreover, such a combination therapy could include siRNAs directed against additional targets, such as oncogenes or angiogenic factors.

The mechanism of RNAi-triggered mRNA destruction represents a novel and powerful tool for the application in gene therapy of cancer. Oncogene products and other malignancy-associated gene transcripts, e.g. drug resistance-mediating molecules, are the potential targets for such a gene therapeutic strategy. A prerequisite for a therapeutic application of siRNAs is that the targeted cancer cells contain a functional RNAi mechanism to bind to siRNAs and mediate mRNA degradation. So far only rare reports are available in which siRNAs were used for treatment of neoplastic cells, e.g. specific inhibition of the bcr-abl oncogene which causes chronic myeloid and bcr-abl-positive acute lymphoblastic leukemia [27], or the selective deletion of point-mutated p53 in cells expressing mutated and non-mutated p53, resulting in a restoration of the wild type protein function [28].

At the time of submission, this work presented the first evidence for silencing MDR-associated genes by siRNA in multidrug-resistant cancer cells. The data demonstrated the proof of principle that endogenous siRNAs can be applied to activate RNAi-mediated degradation of the MDR-associated MDR1 mRNA in targeted cancer cells. However, very recently siRNA-based suppression of MDR1 mRNA expression was reported in multidrug-resistant breast cancer and ovarian carcinoma cell lines [29]. That study applied a siRNA duplex similar to the MDR-A siRNA construct used in this study, i.e. the siRNA sequence targeted the MDR1 mRNA corresponding to nt 508–528 of the consensus sequence NM_000927 instead of nt 503–523 (MDR-A). Due to differences in the experimental procedures the effectiveness of both constructs cannot be compared directly. However, by applying a clonogenic assay instead of a proliferation assay, the anthracycline (doxorubicin) resistance-reversing activity of the nt 508–528 siRNA duplex was 80% in the case of 61-fold doxorubicin-resistant ovarian carcinoma cells, and 92% in the case of 300-fold doxorubicin-resistant breast cancer cells in comparison to a RNA oligonucleotide-free transfection reagent-containing control.

The utilized anti-MDR1 mRNA siRNA constructs used in this study not only appear to be effective laboratory tools for the investigation of MDR, but also have implications for the prevention, reversal of MDR, and prolonging disease-free survival in certain cases by gene therapeutic approaches. Although for clinical use additional problems have to be solved, e.g. delivery of siRNAs, the data presented here demonstrate that a new specific means to reverse the P-gp-dependent MDR phenotype is now available.

Additional problems for clinical P-gp inhibition by anti-MDR1 siRNAs may arise by the physiological expression of this ABC transporter in several epithelial and endothelial cells. For example, P-gp is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain [30] suggesting that P-gp plays an important role in the blood–brain barrier and thus is crucial for limiting the potential neurotoxicity of many anticancer drugs. Thus, in the clinical situation it may be necessary that the application of P-gp-inhibiting siRNAs should be restricted to P-gp-expressing 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, such as mutant adenoviral vectors efficiently and selectively replicating in multidrug-resistant cells [31].

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