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Reversal of multiple drug resistance *in vitro* by phosphorothioate oligonucleotides and ribozymes

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In the present study we investigated the effectiveness of 14, 15 and 18 nucleotide antisense phosphorothioate oligonucleotides (S-ODNs) directed to four different regions of the published *mdr-1* gene sequence to reduce the level of *mdr-1* gene product (p170, P-glycoprotein) and its function in the over-expressing cell lines LoVoDx^R, S180Dx^R and KBCh^{R}8-5. The highest efficiency in reduction of multiple drug resistance was obtained at a concentration of 2 μM. In proliferation assays a growth reduction of 50% was observed after exposure of doxorubicin-resistant cells to S-ODN3. p170 protein expression of the resistant cell line LoVoDx^R was reduced to the level of the sensitive cell line LoVo as shown by Western blot analysis. S-ODN3 reduced the ID_{50} of the two human cell lines up to 60% (LoVoDx^R) and 21% (KBCh^{R}8-5), respectively, but showed no effect in the murine cell line S180Dx^R. In contrast, S-ODN1 was most effective in the murine system (67% reduction of the ID_{50}), less effective in LoVoDx^R (34%) and exhibited no effect in cell line KBCh^{R}8-5. Based on the results with the antisense oligonucleotides, a ribozyme directed against the mRNA target region of S-ODN3 was designed. This ribozyme was able to reduce the *mdr-1* mRNA in total RNA preparations from cell line LoVoDx^R up to 80% after an incubation time of 6 h in the presence of 10 mM $MgCl_2$ at pH 7.5. A modified ribozyme was investigated in cell culture and reduced chemo-resistance of the resistant cell line LoVoDx^R and *ex vivo* cultured blasts of acute myeloid leukemia patients up to 50%.

Key words: Antisense oligonucleotides, multiple drug resistance, P-glycoprotein, ribozymes.

Introduction

Although substantial progress has been achieved in the chemotherapy of malignant disorders within the last 15–20 years, most solid tumors are still not curable by currently available cytostatic agents and ultimately prove to be resistant. Major efforts have therefore been undertaken to elucidate the underlying mechanisms of the phenomenon of drug resistance.[1,2] During these studies the multiple drug resistance (MDR) phenotype has gained special attention, at least in experimental cell systems, since it is characterized by cross-resistance to a variety of structurally and functionally unrelated drugs such as anthracyclines, antibiotics, vinca alcaloids and epipodophyllotoxins.[1–4] The underlying mechanism appears to be mediated through the over-expression or gene amplification[5] of the *mdr-1* gene, resulting in an increased concentration of its gene product, the p170 glycoprotein (P-gp), which then results in an increased energy-dependent drug efflux.[6,7] Therefore, the reversal of MDR by antagonists of the P-gp drug transport has achieved great interest. So far, the chemomodulation of the MDR system has been investigated by the use of the calcium antagonists verapamil,[8–11] and trifluoperazine,[12] non-toxic derivatives of cancerostatic drugs such as *N*-acetyl-daunorubicin[13] or immunosuppressive agents such as cyclosporin A.[11] Lack of specificity (calcium antagonists) or toxic effects (cyclosporin A) result in a limited clinical application of these chemomodulators.[14] Therefore, more specfic alternatives such as molecular approaches may improve attempts for the reversal of MDR. A more specific interference with p170 expression which may be achieved by modern antisense technology[15] may be a more promising approach. First results have been reported recently by several groups,[3,4,16–18] although a successful suppression of p170 production was not achieved unambiguously. The current study complements these efforts but furthermore extends the antisense method to the use of catalytic RNAs (the ribozymes) which, due to their catalytic activity, might have an advantage compared with antisense oligonucleotides. In addition, four antisense oligonucleotides directed against different target regions of the *mdr-1* mRNA were developed and applied to compare their efficiency in the down-regulation of p170 expression in

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cell culture systems. Antisense oligonucleotides directed to a specific sequence of a selected gene may hybridize to the mature mRNA and thereby block the synthesis of a selected protein by translation arrest.[19,20] A second mechanism reported for some systems is the degradation of mRNA–oligonucleotide hybrids by a cellular RNase,[21] resulting in a decreased steady state level of the targeted mRNA and thereby reduced protein synthesis.

Materials and methods

Cell lines and culture conditions

Cell lines LoVo H67P, LoVo doxorubicin (Dx) resistant (^{R}), KB 3-1, KBCh^{R}8-5, S180 and S180Dx^R were used throughout this study. LoVo H67P and LoVoDx^R (see Rivoltini *et al.*[22] for characterization of these cell lines) were kindly provided by Dr Rivoltini (Milan), cell lines S180 and S180Dx^R by Dr Volm (DKFZ, Germany), and cell lines KB 3-1 and KBCh^{R}8-5 were obtained from the ATTC. Cells were grown in Clicks/RPMI medium (Gibco) containing 100 U/ml penicillin, 100 mg/ml streptomycin, supplemented with 10% fetal calf serum (FCS). The MDR-resistant cell lines were transfered alternatingly in medium with and without doxorubicin at a concentration of 5 μg/ml (S180Dx^R) or 1 μg/ml (LoVoDx^R and KBCh^{R}8-5). For the antisense experiments, cells were harvested and washed three times with the above-mentioned medium without FCS. Cells were adjusted to 5×10^4/ml and seeded in a final volume of 100 μl/well in microtiter plates. Phosphorothioate antisense oligonucleotides (S-ODNs) were added to the desired final concentration (0.2, 2 or 5 μM) in a total volume of 10 μl medium without FCS. Only a single dose of S-ODNs or the respective controls was applied. After an additional incubation of 12 h, FCS was added to a final concentration of 10%. After 72 h, cells were subjected to the different assays (e.g. proliferation assay, Western blotting or functional assay).

Proliferation assay

The effect of antisense and control S-ODNs on cell proliferation was evaluated as a decrease of [^{3}H]thymidine incorporation. After an incubation time of 72 h in the presence of the respective S-ODN, cells were pulsed with 0.2 μC/well of [^{3}H]thymidine for 6 h or, in some cases, for 12 h (after a preincubation for 4 h in the presence of doxorubicin). Microtiter plates were stored at −20°C to disrupt cell structure, thawed and subsequently harvested; radioactivity was measured in a beta scintillation counter.

Determination of P-gp

After cells had been incubated for 72 h in the presence of the different S-ODNs they were harvested and resuspended in a buffer containing 20 mM Tris, pH 7.5; 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride. Protein concentration in cell extracts was determined according to the Lowry method (Sigma) following the manufacturer's instructions. Discontinuous SDS gels[23] were run at 100 V for 2–3 h, loaded with 20 μg/lane of total protein. Because of the small assay volumes and corresponding low number of cells of the antisense experiments, membrane preparations of the cells were not performed. Protein transfer to nitrocellulose membranes was carried out for 2 h at 70 mA by use of a semidry blotting equipment (Phase, Lubeck, Germany). After protein transfer, additional binding sites on the nitrocellulose membrane were blocked either for 12 h at 4°C or for 1 h at room temperature under agitation in TBS-T buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% dry milk powder. After washing, the membranes were incubated for 12 h at 4°C with monoclonal antibody (mAb) C219 (Isotopen Diagnostik CIS, Dreieich, Germany) diluted in TBS. Subsequent detection of the murine primary antibody was performed using a mouse antibody detection kit (Amersham) according to the manufacturer's instructions.

In vitro drug assays

Modulation of doxorubicin cytotoxicity by S-ODNs was monitored by a colorimetric assay utilizing the tetrazolium salt MTT as described.[24] Briefly, cells were incubated in the presence of the respective S-ODN and doxorubicin at different concentrations (0–100 μg/ml) in microtiter plates. After an incubation period of 72 h, the tetrazolium salt (5 mg/ml stock solution) was added at 25 μl/well; after 2 h of incubation at 37°C, 100 μl of the extraction buffer (20% w/v SDS, 50% DMF, 50% H_2O, pH 4.7 [2.5% of 80% acetic acid, 2.5% 1 N HCl]) was added. Absorption was measured after 12 h of incubation at 37°C using a Dynatech 96-well multiscanner at 570 nm. Medium without cells subjected to the same treatment was used as a blank. Data are expressed as the

percentage of survival of cells without doxorubicin calculated from the absorbance corrected for background absorbance. A similar approach was used for the determination of chemomodulation of MDR by verapamil or tamoxifen. Instead of the S-ODNs, these drugs were added to the cells (verapamil: 1 and 10 μM; tamoxifen: 0.05, 0.5 and 5 μg/ml) to evaluate the drug-induced reduction of the ID_{50} value for doxorubicin.

Ribozyme *in vitro* assays

Ribozymes were generated by *in vitro* transcription from the T7 (ribozyme 2) or the SP6 (ribozyme 1) promoter using DNA fragments generated with two oligonucleotides (allowing for a tail-to-tail hybridization) after filling up in a Taq polymerase reaction and subsequent *in vitro* transcription using *in vitro* transcription kits (Ampliscribe T7 or SP6; Biozym, Germany). Oligonucleotides were as follows:

(1) Ribozyme 1: oligonucleotide 1 5′-GAT CAA CTT TCG GCC TCA CGG CCT CAT CAG GTA GGA G-3′; oligonucleotide 2 5′-TTA GGA TTT AGG TGA CAC TAT AGA ATA CTC CTA CCT G-3′ with 10 overlapping nucleotides yielding a tail-to-tail hybridization.
(2) Ribozyme 2: oligonucleotide 1 5′-CGT AGG AGT GTT TCG GCC TAA CGG CCT CAT-3′; oligonucleotide 2 5′-AAA TTA ATA CGA CTC ACT ATA GGG AGA TTG TGA TCC ACG CTG ATG AGG CCG TTA-3′ with 12 overlapping nucleotides.

Substrates for the ribozyme investigations were either total cellular RNA or short (300–360 nucleotides) *in vitro* transcription products of a polymerase chain reaction (PCR)-generated part of the *mdr-1* gene. Total RNA was isolated according to Chirgwin *et al.*[25] After cDNA synthesis from total RNA (LoVoDx^R) according to manufacturer's instructions (Superscript; Gibco BRL), PCR products were generated from bp 2181 to 2582 in the first round of PCR and from bp 2311 to 2582 in the nested second PCR of the *mdr-1* mRNA sequence. PCR conditions were as follows: first and last step delay 5 min; 92°C, 1 min; 58°C, 1 min; 72°C, 30 s; 25 cycles with the following primers;

(1) First · PCR: oligonucleotide 1: 5′-AGG CAT TTA CTT CAA ACT TGT C-3′; oligonucleotide 2: 5′-TAA TGG CAC AAA ATA CAC CAA C-3′.
(2) Second PCR: oligonucleotide 3 (containing the T7 promoter sequence): 5′-CTA TAA TAA CGA CTA ACT ATA GGG AGA GAC AGC AGG AAA TGA AGT TGA A-3′.

Ribozymes and target (either *in vitro* transcribed products or total RNA) were incubated for varying times ranging from 0 to 6 h in the following buffer: 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA. Reactions were stopped by the addition of loading buffer (98% formamide, 0.01% bromphenolblue, 0.01% xylene cyanol, 10 mM EDTA, pH 8.0) and analyzed on PAGE gels (6%). Gels were silver-stained according to Budowle *et al.*[26] Total RNA was run on formaldehyde agarose gels [1%, MOPS buffer (40 mM MOPS, pH 7.0, 5 mM Na-acetate, 0.9 mM EDTA, 2.2 M formaldehyde)], blotted on nitrocellulose membranes and hybridized with a *mdr-1* probe (nucleotides 2310–2602) labeled with digoxigenin (DIG) UTP (Boehringer, Mannheim, Germany). Hybridization was performed in the presence of 50% formamide, 5 × SSC, 5 × Denhardt's solution, 25 mM $NaPO_4$ buffer, pH 6.5, 1% (w/w) SDS at 65°C for 12 h. After three washing steps (0.1 × SSC, 0.1% (w/w) SDS, 45°C), *mdr-1* mRNA was detected with the DIG detection kit from Boehringer Mannheim and AMPPD from Tropix (Serva, Heidelberg, Germany), and quantified with a densitometer (Molecular Dynamics, Münster, Germany).

Drugs and oligonucleotides

Doxorubicin, MTT, verapamil and tamoxifen were obtained from Sigma (München, Germany). Oligonucleotides (Figure 1A) were either synthesized by two of us (WH and K-HS) or by Ribonetics (Göttingen, Germany). P-gp antibody C219 from Isotopen (Diagnostik CIS, Dreieich, Germany) was used.

Results

Effect of S-ODNs on proliferation and level of P-gp

In the present study four different target regions were used to study the effect of S-ODNs in modulating *mdr-1* gene expression and P-gp function. Target regions have been sequenced in the cell lines used in this study to confirm the sequence in this part of the gene (data not shown). The first target region is located in intron −1 between the upstream and downstream promoter, the second target region spans the AUG codon, the third target region is

(A)

AS		CONTROL	
S-ODN1	5′ GTC TTT CTT CAT CAT GCT	S-ODN1	5′ TGC CAT TTC TAC TTT GTC
S-ODN2	5′ ATC CAT CCC GAC CTC	S-ODN2	5′ TCA CCC GAT CCC TAC
S-ODN3	5′ CCT ACG AGT TGA TC	S-ODN3	5′ GCA CGT GAT TGA CT
S-ODN4	5′ GAG TAA CAG TGT TAG TTG	S-ODN4	5′ CTG AGA GAC ATT ATT TGG

(B)

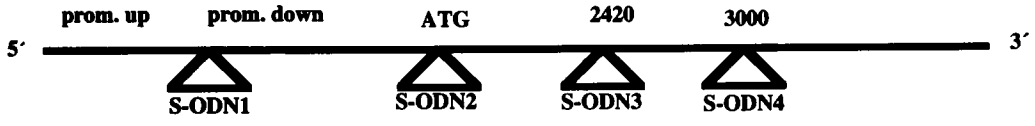


Figure 1. (A) Sequences of the antisense and control oligonucleotides used throughout this study. The oligonucleotides were all-phosphorothioate oligonucleotides. (B) Position of the oligonucleotides in relation to the *mdr-1* mRNA.

positioned in the middle of the *mdr-1* mRNA (at nucleotide positions 2420–2434) and the fourth region was selected from nucleotides 2990 to 3007 (Figure 1B). The four S-ODNs varied in length from 14 to 18 nucleotides, they were all-phosphorothioate S-ODNs, and had a GC content between 40 and 60%. The effect of S-ODN2 and S-ODN3 on the *mdr-1* over-expressing cell line LoVoDx^R (resistance factor 50) was investigated at an S-ODN concentration of 2 μM, applied at a single dose. After 72 h of incubation, a [^{3}H]thymidine incorporation assay was run in the presence of doxorubicin showing a reduction of 50% in the assays with S-ODN3 (Figure 2A). In contrast, S-ODN2 had almost no effect (approximately 10%) on the amount of [^{3}H]thymidine incorporation (Figure 2B). Control assays were run in the absence of doxorubicin to evaluate any unspecific toxicity of the oligonucleotides. In the absence of doxorubicin, no effect of the antisense oligonucleotide on [^{3}H]thymidine incorporation could be found. It is apparent that the oligonucleotides themselves are not toxic to a significant extent.

For a more specific evaluation of the S-ODN effect on *mdr-1* expression, Western blots were performed using mAb C-219 (Figure 3). Whereas S-ODN2 and S-ODN4 were not able to reduce P-gp expression compared with untreated LoVoDx^R cells (data not shown), S-ODN3 reduced the *mdr-1* protein down to the P-gp level of the sensitive cells. S-ODN1 showed a reduction in the *mdr-1* gene pro-

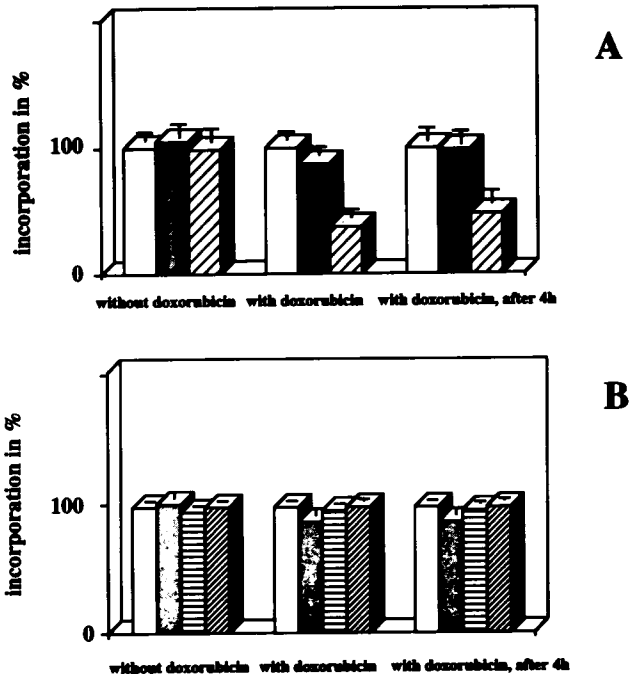


Figure 2. Reduction of [^{3}H]thymidine incorporation by antisense oligonucleotides directed against *mdr*-1 mRNA sequences (A) S-ODN3 (□, control oligonucleotide; ◻, random sequence; ■, S-ODN3) and (B) S-ODN2 (□, control oligonucleotide; ▨, S-ODN2; ■, sense sequence; ■, random sequence) in comparison to the respective control sequences. The proliferation assay was performed as outlined under Materials and methods. The resulting values are expressed as the percentage ± SD (n = 6).

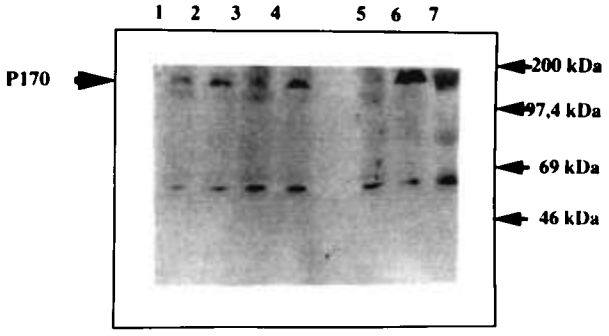


Figure 3. Western blot analysis of protein extracts from cell line LoVo using mAb C219 (see Materials and methods). Lane 1, doxorubicin-resistant cell line after treatment with S-ODN1; lane 2, doxorubicin-resistant cell line after treatment with control oligonucleotide; lane 3, doxorubicin-resistant cell line after treatment with S-ODN3; lane 4, doxorubicin-resistant cell line after treatment with control oligonucleotide; lane 5, doxorubicin-sensitive parental cell line; lane 6, doxorubicin-resistant cell line; lane 7, molecular weight marker. Cells were harvested after incubation in the presence of the respective oligonucleotides. Cell extracts were analyzed for protein content. SDS gels were run using 20 μg protein/lane. The amounts of total protein were identical in each pair of lanes as checked by staining with Ponceau S and which can also be seen from an unspecifically stained protein at around 69 kDa.

duct (around 75%), although the reduction was not as pronounced as with S-ODN3. These findings were confirmed in a time-course experiment in which extracts of S-ODN3-treated LoVoDx^R cells have been analyzed at time zero, and after 24, 48 and 72 h, respectively. The time schedule was chosen according to the half-life time of P-gp which is in the range of 20 h. After 24 h, however, the p170 amount remained unchanged, after 48 h a reduction was apparent and after 72 h the p170 level of the doxorubicin resistant cells was in the range of the sensitive parental cell line. Even after 144 h the reduction of P-gp was still in the same range after this single S-ODN3 application (analyzed by densitometry, data not shown).

Increase of drug sensitivity by S-ODNs

The evaluation of *mdr-1* product function by testing the chemosensitivity of the cells gives more valid information about the modulation of *mdr-1* expression and primary function of S-ODNs. Therefore, we used a functional chemosensitivity assay (MTT dye reduction) to determine the effect of S-ODN3 and S-ODN1 on three *mdr-1* over-expressing cell lines [S180Dx^R (resistance factor 150), KBCh^{R}8-5 (resistance factor 8) and LoVoDx^R]. The results are shown in Table 1. S-ODN3 was most effective in cell line LoVoDx^R (62% reduction of ID_{50} in the presence of doxorubicin), whereas the addition of S-ODN1 to the cells reduced the ID_{50} only in the range of 20%. In cell line KBCh^{R}8-5, S-ODN1 did not change the ID_{50} in the presence of doxorubicin. Addition of S-ODN3, however, decreased the *mdr-1* expression related ID_{50} by about 30%. In the murine sarcoma cell line S180Dx^R, S-ODN3, the most effective S-ODN in the two human cell lines, did not alter the doxorubicin related ID_{50} values. However, S-ODN1 reduced the ID_{50} in the range of 60%.

For the evaluation of the optimal S-ODN concentration for reduction of *mdr-1* expression, three different concentrations (0.2, 2 and 5 μM) were tested and compared to the respective concentration of the control oligonucleotide. At a concentration of 0.2 μM there was no alteration of the ID_{50} in the presence of doxorubicin, whereas at a concentration of 2 or 5 μM the ID_{50} was decreased up to 60% (Figure 4). Application of 2 μM S-ODN3 resulted in the most efficient down-regulation of p170 function, at 5 μM the effect was slightly smaller. Therefore the lower concentration, which is also

Table 1. Reduction of cell viability (ID_{50}) by S-ODN3 and S-ODN1, and the respective control sequence in the doxorubicin resistant cell lines LoVoDx^R, KBCh^{R}8-5 (human) and S180Dx^R (murine)

Cell line	Doxorubicin ID_{50} (μg/ml)			
	without oligonucleotide	oligonucleotide		
		control	S-ODN3	S-ODN1
LoVoDx^R	5 (100 ± 2%)	4.5 (90 ± 1%)	1.7 (37.8 ± 1%)	3.3 (66 ± 1)
KBCh^{R}8-5	1.9 (100 ± 1%)	1.9 (100 ± 3%)	1.5 (78.9 ± 3%)	1.9 (100 ± 3%)
S180Dx^R	63 (100 ± 2%)	66 (104 ± 5%)	66 (104 ± 3%)	21 ((33 ± 2%)

The reduction of the ID_{50} values for doxorubicin was measured using the MTT dye reduction assay (see Materials and methods). Viability in the absence of oligonucleotides was set to 100%. The respective doxorubicin sensitive parental cell lines (LoVo, KB3-1 and S180) were not affected by the addition of any of the oligonucleotides (data not shown). Resulting values are expressed as the percentage ± SD (n = 6).

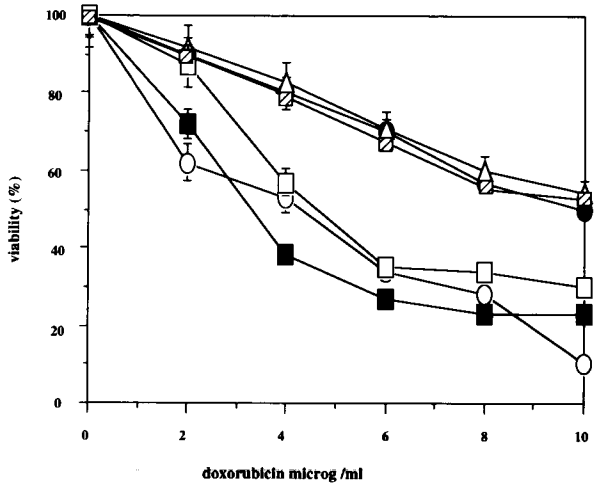


Figure 4. Concentration dependent reduction of drug resistance by S-ODN3. Cells were incubated in the presence of 0.2, 2 and 5 μM S-ODN3; after an incubation time of 72 h they were subjected to the MTT dye reduction assay. Data are expressed as mean ± SD (n = 6). No reduction was observed using the control oligonucleotides at the respective concentration. ○, LoVo; ●, LoVoDx^R; △, LoVoDx^R + AS 0.2 μM; ■, LoVoDx^R + AS 2 μM; □, LoVoDx^R + AS 5 μM; ▨, LoVoDx^R + control sequence at 0.2, 2 or 5 μM.

preferable especially in the context of possible therapeutic applications, was used for further investigations.

Comparison of S-ODN and verapamil/ tamoxifen induced effects on MDR modulation

In order to compare S-ODN related MDR modulation to known chemomodulators of MDR function, we tested the calcium antagonist verapamil at different concentrations under the same conditions as the S-ODNs. Verapamil, tested at 1 and 10 μM, showed an effect on doxorubicin resistance of cell line LoVoDx^R only at the relatively high concentration of 10 μM (ID_{50}:5 μg Dx/ml with verapamil, 7.5 μg Dx/ml without verapamil); there was no measurable effect at a concentration of 1 μM (Figure 5a). Reduction of ID_{50} in the MTT dye reduction assay at 10 μM verapamil was in the range of 40–50%. Tamoxifen was applied at 0.05, 0.5 and 5 μg/ml. There was an effect on the ID_{50} (2 mg Dx/ml with tamoxifen; 6.3 mg Dx/ml without tamoxifen) only at 0.5 μg/ml in the range of 65% (Figure 5b).

These findings demonstrate that the most effective S-ODN3 under the conditions tested so far was

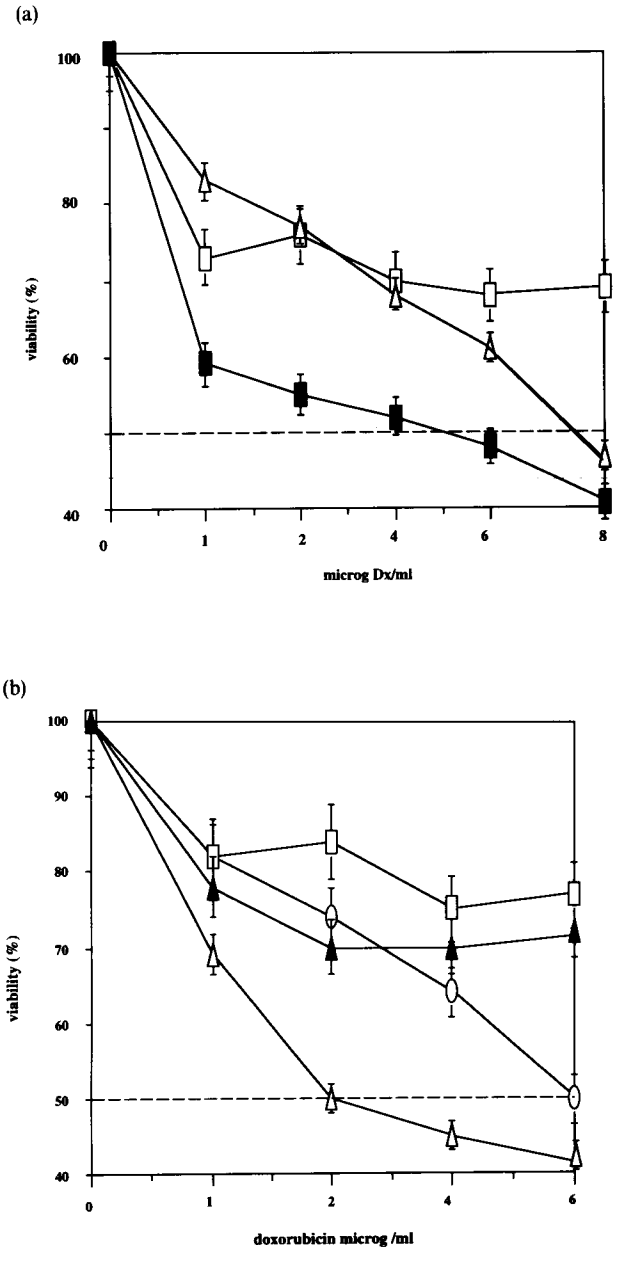


Figure 5. (a) Modulation of chemo-sensitivity by different concentrations of verapamil. Incubation was performed for 72 h in the presence of 1 and 10 mM of verapamil and increasing concentrations of doxorubicin (0–100 μg/ml) in 96-well microtiter plates. Cell viability was measured with the MTT assay. The resulting values are expressed as the percentages ± SD (n = 6). △, LoVoDx^R; □, LoVoDx^R + 1 μM verapamil; ■, LoVoDx^R + 10 μM verapamil. (b) Modulation of chemo-sensitivity by different concentrations of tamoxifen. Cells were incubated with 0.05, 0.5 and 5 mg/ml of tamoxifen in the presence of doxorubicin (0–100 μg/ml). Cell viability was measured using the MTT assay after 72 h. Error bars indicate percentages ± SD (n = 6). ○, LoVoDx^R; □, LoVoDx^R + 0.05 μg/ml tamoxifen; △, LoVoDx^R + 0.5 μg/ml tamoxifen; ▲, LoVoDx^R + 5 μg/ml tamoxifen.

as effective as the investigated chemo-sensitizers in reducing the ID_{50}.

In vitro studies of ribozymes directed to *mdr-1* mRNA

In order to study the applicability of *mdr-1* directed ribozymes for an improved reduction of *mdr-1* expression by these catalytic compounds compared to S-ODNs, we designed two ribozyme sequences directed against the mRNA region which has been found to be the most effective one for reversal of MDR with S-ODNs (Figure 6). Ribozyme 1 covered exactly the same target region as S-ODN3 with a CUC target sequence which is sufficient for ribozyme mediated cleavage of RNA (Ludwig, personal communication). Ribozyme 2 was directed to the nearest region containing a GUC target site (nucleotide 2440). Both ribozymes were generated by *in vitro* transcription (see Materials and methods) and investigated in cell-free assay systems with total RNA from the doxorubicin-resistant cell line LoVoDx^R or *in vitro* generated mRNA fragments (nucleotides 2311–2582) of the *mdr-1* gene. Dependence of the cleavage reaction as a function of pH, $MgCl_2$ concentration and target : ribozyme ratio were investigated. There was an increase in ribozyme activity with increasing $MgCl_2$ concentrations (Figure 7) up to the highest concentration tested (20 mM $MgCl_2$). The pH optimum was at pH 7.5 (data not shown); the target : ribozyme ratio gave the most efficient substrate cleavage at a ratio of 2:1 (not shown). We observed a time-dependent decrease in *mdr-1* mRNA after incubation with ribozyme 1 or ribozyme 2 (Figure 8). Ribozyme 2 reduced the *mdr-1* mRNA amount in total RNA preparations from cell line LoVoDx^R up to 80% after an incubation time of 6 h in the presence of 10 mM $MgCl_2$ at pH 7.5 (Figure 9). Modified ribozymes (Figure 10) with an increased stability against ribonucleolytic attack have been investigated in the doxorubicin-resistant cell line LoVoDx^R and the parental-sensitive cell line. Two types of control ribozymes have been used, one missing the catalytic domain and a second disabled ribozyme with a G to A replacement at position 12 of the catalytic core. None of the ribozymes changed the chemo-resistance of the parental cell line nor did the controls with the resistant cell line. However, f-rilon and rilon 1 reduced chemo-resistance of the LoVoDx^R line up to 50 and 35%, respectively, using the MTT assay as described in Materials and methods (Table 2). Furthermore, chemo-resistance of *ex vivo* cultured blasts from acute myeloid leukemia (AML) patients could be reduced between 11 and 52%, respectively. Only in patients with a detectable expression of P-gp a reduction of drug resistance

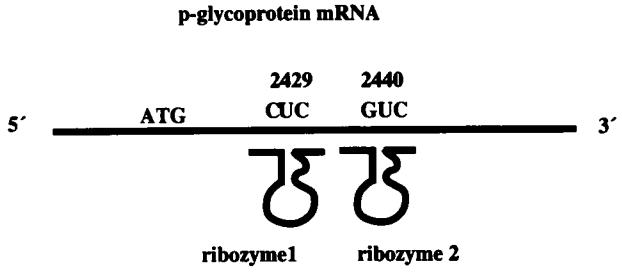


Figure 6. Position of ribozyme 1 and ribozyme 2 on the *mdr-1* mRNA. Ribozyme 1 is directed against a CUC target sequence, whereas ribozyme 2 is positioned at a GUC triplet (see Results).

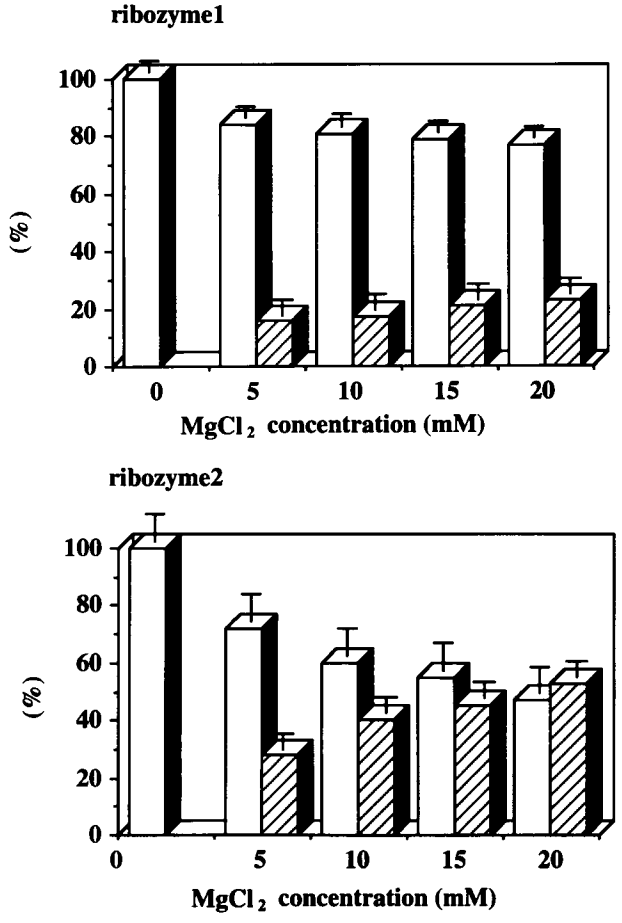


Figure 7. Effect of $MgCl_2$ concentration on substrate cleavage for ribozyme 1 and ribozyme 2. Ribozymes and substrate were incubated for 6 h in Tris–HCl buffer, pH 7.5. Substrate and products of the cleavage reaction were run on 6% PAGE gels, silver stained and quantified by densitometry. Data are expressed as percentages ± SD (n = 3). □, Substrate; ■, products.

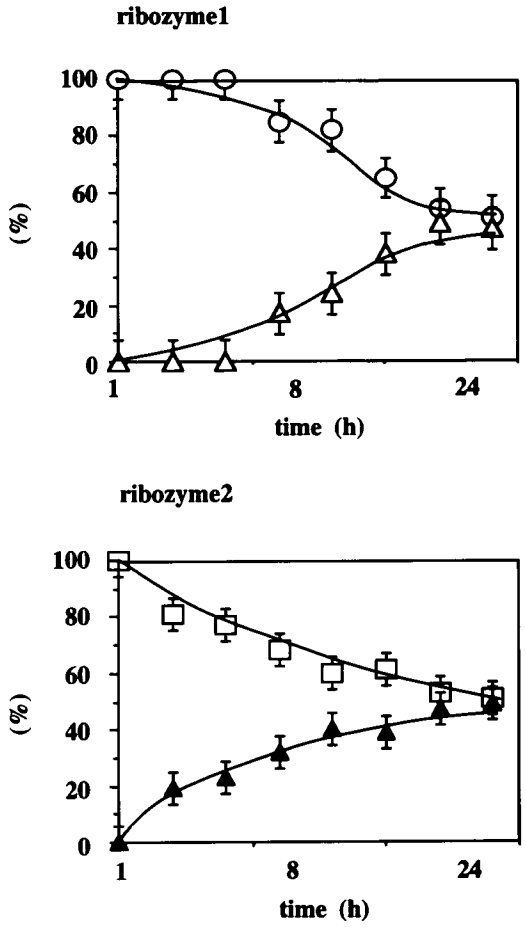


Figure 8. Time course of *mdr-1* mRNA degradation for ribozyme 1 and ribozyme 2. Ribozyme and substrate were incubated in Tris–HCl buffer, pH 7.5 at 10 mM $MgCl_2$ at a ratio of 1 : 1. Aliquots were withdrawn at the times indicated, the reaction was stopped and after 24 h all samples were analyzed on a PAGE gel (see Materials and methods). Data are expressed as percentages ± SD (n = 3). Circles and squares, substrate; triangles, products.

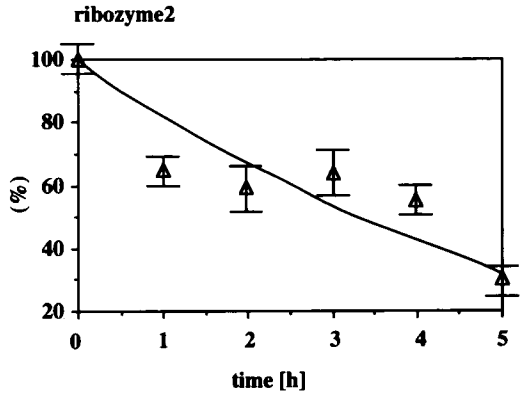


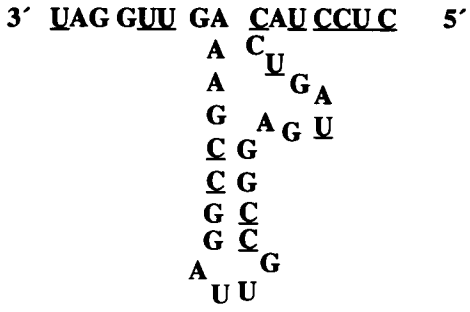
Figure 9. Ribozyme mediated cleavage of *mdr-1* in total RNA preparations, detected by Northern blot analysis and quantified by densitometry (see Materials and methods). Data are expressed as percentages ± SD (n = 3). △, Substrate.

could be found after incubation in the presence of rilon 1. Reduction of cell viability was different between *de novo* AMLs (11–27%) and relapsed AMLs (43–52%) but corresponded with P-gp expression.

Discussion

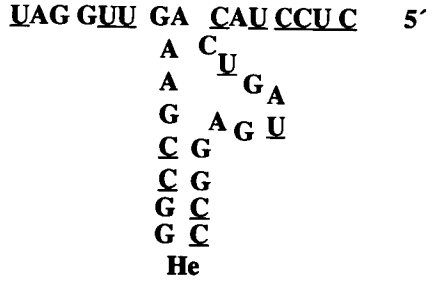
There are several hints arguing for an important role of *mdr-1* over-expression and elevated levels of the gene product (p170) for the development of drug resistance during cytotoxic chemotherapy of cancer patients.[1] Attempts for a chemomodulation of p170 function have been made not only in cell culture systems but also in cancer patients after therapy induced MDR resistance.[9,27] Due to toxic effects of some chemomodulators and undesired side effects (calcium antagonists), a more specific modulation of MDR is necessary which might also be used in combination with chemo-sensitizers for an improved reduction of drug resistance. In our experiments we could demonstrate a successful reduction of *mdr-1* expression by use of S-ODNs. Similar results have been reported only recently by Efferth and Volm[16] and Thierry *et al.*[4] We found that different target regions resulted in very different effects in MDR modulation, as was also reported by Jaroszewski *et al.*[3] and Thierry *et al.*[4] In the two human cell lines (LoVoDx^R and KBCh^{R}8-5), S-ODN3 directed against a region in the middle of the mRNA (nucleotides 2420–2434) showed the highest reduction in the *mdr-1* gene product (Figure 3) and was most efficient in the functional assay (Table 1). The accessibility of the target region for an antisense approach seems to be very important for the achievable reduction of gene expression caused by an antisense oligonucleotide, and may be the reason for the different effects of S-ODN1 and S-ODN3 in the two human cell lines, on one hand, and the murine cell line, on the other hand. This finding is also sustained by the fact that the PCR reactions amplifying the different target regions (with subsequent sequencing) used for our antisense approach (Figure 1B) gave best amplification results for the target region of S-ODN3. Accessibility of a chosen target region may also be the reason for the lack of *mdr-1* reduction with S-ODN2 and S-ODN4. S-ODN1, positioned 600 nucleotides upstream of the translation start codon, was only effective in one of the human cell lines (LoVoDx^R) but not in cell line KBCHh^{R}8-5. On the other hand, S-ODN1 was most

Model of the f-rilon



underlined nucleotides: 2′OH-group of the ribose is fluoro-substituted, phosphorothioate linkage at the 3′end

Model of rilon1



underlined nucleotides: 2′OH-group of the ribose is allyl-substituted, hexaethylene moiety at the 3′end and in stem II

Figure 10. Model of the modified ribozymes stabilized against ribonucleolytic attack by the addition of a phosphorothioate linkage or a hexaethylene glycol moiety at the 3′ end and fluoro- or allyl-substituted (2′OH group of the ribose) nucleotides at the underlined positions. Stem II of the hammerhead ribozyme 2 was replaced by a second hexaethylene glycol linker.

efficient in the murine sarcoma cell line S180Dx^R. These findings may argue against an over-estimation of a computer-based search for S-ODN target sequences. Computer calculatons, even dealing with more than the lowest energy plot of the mRNA secondary structure, so far have only given uncertain predictions for a suitable target sequence. The situation in cells may be very different from the thermodynamic considerations of stable loop structures covered by RNA binding proteins. In different cell lines the set of RNA binding proteins may be different, resulting in an altered accessibility of a selected target sequence for the same oligonucleotide in different cell lines. This might explain our

Table 2. Reduction of cell viability (IC_{50}) by modified ribozymes in the doxorubicin resistant cell line LoVoDx^R and *ex vivo* cultured blasts from AML patients as monitored in a chemo-sensitivity assay (MTT)

Cell line/ patient	Reduction of IC_{50} (Dx) in the MTT assay (%)	P170 expression detected by flow cytometry
LoVoDx^R [a]	35 ± 5	+
1	27 ± 3	+
2[b]	43 ± 7	+
3[b]	52 ± 10	+
4	NG[c]	–
5	11 ± 5	–[d]
6	—[e]	–[d]
7	—[e]	–[d]
8	12 ± 2	±
9	NG[c]	–

[a] f-rilon (fluoro-modified pyrimidine nucleotides) exhibited a reduction of 50 ± 3% for cell line LoVoDx^R and patient 1. MTT data were compared to a BrdU incorporation assay and were the same (37 and 30% reduction of BrdU incorporation, respectively) as in the chemosensitivity assay (BrdU incorporation assay was run 72 h after the addition of rilon1 as recommended by the manufacturer).
[b] Relapsed AML.
[c] No growth *ex vivo*.
[d] Not detectable.
[e] No difference with or without ribozyme.
Resulting values are expressed as the percentage ± SD (n = 6).

findings in human and murine cell lines using S-ODN1 and S-ODN3, although we had previously checked the target regions in our cell lines (data not shown). Moreover, the degree of resistance may also influence the efficiency of different antisense oligonucleotides. S-ODN3 showed a reduction of p170 expression in the two human cell lines (Figure 3), but to a different extent. The effect was more pronounced in the more resistant cell line LoVoDx^R. A comparable correlation was found previously;[28] *erb*B2 reduction by S-ODNs was more effective in the cell line with a higher *erb*B2 expression. More experiments in additional model systems have to be performed to corroborate these findings.

Whereas S-ODN3 reduced the p170 level in cell line LoVoDx^R nearly to the level of the doxorubicin-sensitive parental cell line, reduction in the functional assay was only in the range of 60%. This might also be due to the fact that development of MDR is not only associated with the over-expression of the *mdr-1* gene but also with different, yet not very well characterized, mechanisms.

Comparison of S-ODN-mediated MDR modulation with chemo-sensitizer modulated p170 function showed that S-ODN-mediated effects are in the same range or even higher than the MDR modulation by verapamil or tamoxifen. It has to be in-

vestigated if another form of S-ODN delivery (e.g. liposomal encapsulation, drug targeting) could improve the S-ODN-related effects. The work of Thierry *et al.*[4] demonstrates that liposomal encapsulation might improve cellular uptake of anti-*mdr* oligonucleotides in their cell system.

Another improvement of the reduction of *mdr* expression could be envisaged using catalytic active sequence specific molecules (ribozymes)[29–31] to reduce *mdr-1* mRNA steady state levels and thereby reducing *mdr-1* expression even at lower doses as compared to antisense oligonucleotides. In the present paper we demonstrate that ribozymes directed against two specific regions of the *mdr-1* mRNA, which were chosen according to the results of our antisense experiments, are able to reduce *mdr-1* mRNA levels in total RNA preparation in the range of 80% within 6 h (ribozyme 2, Figure 9). First experiments with stabilized ribozyme (Figure 10 and Table 2) in a resistant cell line and *ex vivo* cultured blasts from AML patients are very promising (reduction of ID_{50} in the range of 50%). The application of modified ribozymes to cell culture, the so-called interventional gene therapy, instead of retroviral delivery was chosen for two reasons: (i) retroviral delivery systems will result in stable modification of the genotype which might be undesired with regard to therapeutic applications and (ii) results of the effectiveness of a selected target sequence may be influenced by different copy numbers of the retroviral construct due to multiple infections. Therefore the copy number has to be evaluated before a comparison would be possible. Moreover, due to the potential role of the P-gp at the blood–brain barrier,[32] it will be very important to use a sophisticated fine tuning for the reduction of P-gp expression to avoid undesired side effects of p170 to down-modulation.

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