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QUARTERLY

Antisense hairpin loop oligonucleotides as inhibitors of expression of multidrug resistance-associated protein 1: Their stability in fetal calf serum and human plasma✪

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Multidrug resistance-associated protein (MRP1) is a transmembrane pump protein responsible for the efflux of chemotherapeutic drugs, an important cause of anticancer treatment failure. Trying to circumvent MRP-mediated resistance we designed and synthesized hairpin loops forming antisense oligodeoxyribonucleotides (ODNs), both phosphodiesters (PO-ODNs) and their phosphorothioate analogues (PS-ODNs), to reduce the protein expression by targeting its mRNA in a sequence specific manner. Melting temperature measurements as well as polyacrylamide gel electrophoresis supported the preferential formation of a secondary structure, which was expected to protect ODNs against 3′-exonuclease degradation. ODNs and PS-ODNs designed in this work were successfully tested as antisense inhibitors of the expression of MRP1 in the leukaemia HL60/ADR cell line. Foreseeing the necessity to perform clinical studies with such ODNs we investigated their stability against the 3′-exonuclease activity of fetal calf serum and human plasma. Under the conditions, corresponding to physiological ones, we observed high stability of hairpin loop forming ODNs, especially those containing longer (e.g. 7 base pair) stems. Comparative studies on the stability of chemically unmodified hairpin loop forming ODNs and their PS-counterparts indicated that endonuclease activity did not play any important role

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Abbreviations: FCS, fetal calf serum; MRP, multidrug resistance-associated protein; ODNs, oligodeoxyribonucleotides; PO-ODNs, phosphodiester ODNs analogue(s); PS-ODNs, phosphorothioate ODNs analogue(s); PNAs, peptide nucleic acids.

in the process of their nucleolytic degradation. Our studies provide strong evidence for high stability of chemically unmodified hairpin loop ODNs, making them an attractive alternative to phosphorothioate analogues commonly used in antisense strategy.

Antisense oligonucleotides (ODNs) are short synthetic fragments of single stranded DNA that are complementary to mRNA sequences encoding "unwanted" proteins. They act as selective inhibitors of the biosynthesis of a target protein due to RNase H-mediated degradation of the mRNA strand in the region of mRNA/DNA heteroduplex [1]. Therefore, they might serve as potential therapeutic agents in the treatment of AIDS, cancer and other diseases [2–5]. However, the administration of antisense oligonucleotides is associated with many problems concerning cellular uptake, intracellular distribution, sequence specificity or resistance to cleavage by intracellular and extracellular nucleases [6]. To solve the latter problem, some chemical modifications either of an oligonucleotide as a whole or only of its ends [7] have been introduced. Among them are phosphate group modifications including phosphorothioates [8, 9] and methylphosphonates [10], sugar moiety modifications (mostly in 2′-O-position) [11], or totally changed oligomer backbone as in peptide nucleic acids (PNAs) [12]. Another approach to the problem involved circularization of oligonucleotides by formation of covalent linkage between 5′- and 3′-ends of the molecule [13, 14]. Moreover, there were described the so called "self stabilising" structures in which the resistance to exonucleases was achieved by formation of hairpin or hairpin loop structures using self-complementary ends of oligonucleotide [15–20].

One of the rationally selected targets for the antisense strategy is a multidrug resistance-associated protein 1 (MRP1), as this transporter, together with P-glycoprotein, is responsible for the multidrug resistance typically observed in many forms of cancers. Its overexpression has been detected in drug- selected and non-selected cell lines derived from small cell lung cancer and non-small cell lung cancer, colon, breast and ovarian carcinomas, neuroblastomas as well as chronic and acute leukaemias, lymphomas and multiple myelomas [21, 22]. This overexpression is thought to be a major cause for the frequently observed failure of chemotherapy. One of the pharmacological approaches to circumvent the drug resistance employs chemosensitizers that enhance the cytotoxicity of antineoplastic agents [23]; the antisense strategy could be the other one.

Our preliminary studies on the application of antisense phosphorothioate ODNs to HL60/ADR cells overexpressing MRP1 resulted in selection of three most active sequences [24, 25]. In this paper we report the results obtained for these sequences flanked by additional fragments complementary to each other, enabling formation of an intramolecular hairpin loop-like structure. We describe the stability of such anti-MRP1 constructs and their properties as MRP1 expression inhibitors.

MATERIALS AND METHODS

Chemicals and media. All reagents were of analytical grade. T4 polynucleotide kinase was obtained from Amersham (Little Chalfont, U.K.). Fetal calf serum (FCS) and RPMI 1640 medium were obtained from GIBCO-BRL (Uxbridge, U.K.). Acrylamide, *N,N′*-methylene-bisacrylamide, ammonium persulfate, urea and TEMED were purchased from Serva (Heidelberg, Germany).

Bromophenol blue, xylene cyanol, and Stains-all reagent were obtained from Sigma. [γ-^{32}P]ATP was synthesized and purified in our laboratory from [^{32}P] orthophosphoric acid obtained from ICN Biomedicals, Inc. (Irvine, California, U.S.A.).

Synthesis of oligonucleotides. Oligodeoxyribonucleotides PO-1C, PO-2C, PO-3C and PO-1C2 (sequences shown in Table 1) were

synthesized by the phosphoramidite method [26] using an ABI 394 DNA synthesizer. In the case of phosphorothioates PS-1, PS-2, PS-3, PS-4, PS-4 (sense) and PO/PS-1C, PO/PS-2C, PO/PS-3C, the modified phosphoramidite method of synthesis [27] was used involving sulfurization with S-TETRA [28]. All ODNs were purified by RP HPLC followed by preparative electrophoresis in a 20% polyacrylamide gel containing 7 M urea.

Thermal denaturation studies. The experiments were performed with ODNs PO-1C, PO-2C and PO-3C and their chimeric counterparts with phosphorothioate core, PO/PS-1C, PO/PS-2C and PO/PS-3C. The concentration of ODNs was determined spectrophotometrically by UV absorbance at 260 nm in water, using extinction coefficients of 348×10^3, 325×10^3 and 244×10^3 $M^{-1}cm^{-1}$ (per strand) for PO-1C, PO-2C and PO-3C, as well as for PO/PS-1C, PO/PS-2C and PO/PS-3C, respectively [29]. The samples were lyophilized and redissolved in 10 mM Tris/Cl buffer, pH 7.0, containing 10 mM $MgCl_2$ and 100 mM NaCl. Melting profiles were recorded for the following oligonucleotide concentrations: PO-1C (0.57, 1.44, 2.88 μM), PO-2C (0.62, 1.54, 3.08 μM) and PO-3C (0.82, 2.05, 4.1 μM) in an 1 cm path length cell with a UV/VIS 916 Spectrophotometer equipped with a Peltier thermocell (GBC, Dandenong, Australia). Melting profiles for heteroduplex were recorded for equimolar amounts of ODN and RNA (1.3 μM of each strand). Absorbance (260 nm) was monitored while temperature was raised at a rate of 0.3°C/min. Melting temperatures (t_m) were determined by computer fit of the first derivative of absorbance.

Non-denaturing gel electrophoresis. Each oligonucleotide (typically 1.5 nmol) was dissolved in 10 μl of phosphate-buffered saline, pH 7.4 ($NaCl/P_i$) diluted with H_2O (1:1, v/v) and incubated for 10 min at 37°C. One microlitre of loading dye (50% glycerol, 0.03% bromophenol blue, 0.03% xylene cyanol) was added to the samples prior to gel electrophoresis. Electrophoresis was run on 20% polyacrylamide gel at 4°C in 50 mM sodium phosphate (pH 7.4) at 100 V. The gel was stained with Stains-all dye.

Plasma. Plasma was isolated from human blood containing 0.38% sodium citrate by centrifugation at $16000 \times g$ for 5 min. Plasma aliquots were carefully withdrawn, leaving the cell pellet untouched.

Oligonucleotide labelling. All ODNs were 5′-end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. A solution (20 μl) containing 10 mM Tris/Cl (pH 8.5), 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 25 μM oligonucleotide (0.5 nmol), 1 μl (10 μCi) of [γ-^{32}P]ATP, and T4 polynucleotide kinase (10 units) was incubated for 4 h at 37°C.

Assay for nucleolytic activity of human plasma and FCS. The samples of each oligonucleotide, at a concentration of 5 μM, were incubated in $NaCl/P_i$ buffer containing either 50% human plasma or RPMI 1640 medium supplemented with fetal calf serum (FCS, final concentration 10%). After various time intervals (usually after 4 and 8 h) 10 μl aliquots were withdrawn, and the enzymatic reaction was stopped by heating for 5 min at 95°C. Then 100 μl of water was added to each denatured sample. After vigorous shaking the protein precipitates were spun down and the aqueous solutions were lyophilized in a SpeedVac rotary evaporator. The resultant samples were dissolved in formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol (10 μl) and analyzed by means of PAGE under denaturing conditions (20% polyacrylamide, 7 M urea). The radioactive products were visualized by autoradiography. The autoradiograms were scanned using LKB Ultrascan XL densitometer. Reference sample for human plasma was 5′-^{32}P-labelled ODN dissolved in $NaCl/Pi:H_2O$ (1:1, v/v) incubated at 37°C for 8 h. Reference sample for FCS-containing RPMI 1640 medium with 5′-^{32}P-labelled ODN was incubated as above.

Effects of Mg^{2+} on time course of degradation of oligonucleotides in human plasma. $MgCl_2$ was added to 50% human

plasma (the final concentration 10 mM in a total volume of 40 μl) and incubated for 2 h at 37°C. Then, 5′-labelled ODNs PO-1C, PO-2C, PO-3C, PO/PS-1C, PO/PS-2C and PO/PS-3C (final concentration 5 μM) were added and the mixtures were incubated for various time periods (2, 4, 8 h) under the same conditions. The 5′-labelled ODNs were recovered from the protein precipitate and analyzed as described previously. The reference sample of NaCl/P_i/H_2O (1 : 1, v/v) was preincubated without Mg^{2+} (for all 5′-^{32}P-labelled ODNs) at 37°C for 2 h. Then, the ODNs were separately added and incubation was continued at 37°C for 8 h.

Cell cultures. The human promyelocytic cell lines HL60 and HL60/ADR were routinely maintained in RPMI 1640 medium supplemented with 20% heat inactivated fetal calf serum, penicillin and streptomycin in 90–95% humidified atmosphere of 5% CO_2 at 37°C. Cultures of HL60/ADR cells were additionally supplemented with 200 nM doxorubicin. Before addition of ODNs, cells were seeded into wells of 24-well tissue culture dishes at a concentration of 3×10^5/ml and were washed with a fresh portion of RPMI 1640 medium. ODNs were added at a concentration 0.5 μM. Routinely, the cells were treated twice with each ODN. Thus, the cells were incubated with ODNs at 37°C for 4 h, washed once with RPMI 1640 medium and then incubated again for 48 h in complete serum-containing medium. Then, the cells were treated again with ODN for 4 h in serum-free medium, washed as before, and cultured for another 24 h. After that the cells were harvested, washed 3 times with NaCl/P_i buffer and subjected to further analysis.

Measurement of MRP1 mRNA levels in HL60 and HL60/ADR cells. The mRNA isolation procedure was applied according to QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) procedure. The obtained RNA was analyzed by means of RT-PCR with random hexanucleotide primers followed by PCR with primers for GAPDH (GAPDH1: 5′-CAT CAT CTC TGC CCC CTC TG and GAPDH2: 5’-CCT GCT TCA CCA CCT TCT TG); and for MRP1 (PP1: 5′-TCT GTT TGC TGC CCT GTT TG and PP2: 5′-ACC TTT TCT CCC CCA TTG AT). Linear PS-M4 and PS-M4-(sense) were used as controls. The products of reactions were analyzed on 6% polyacrylamide gel stained with ethidium bromide solution. The gels were scanned using Enhanced Laser Densitometer (UltraScan XL, LKB Bromma, Sweden) and MRP mRNA level was expressed as percentage of the values obtained for GAPDH as the reference.

RNase H reaction with MRP1 RNA/ODN duplex. Target MRP1 RNA (5′-CUC CUU CCU CAG CAU CUU CCU UU) was synthesized by a phosphoroamidite method using an ABI 394 DNA synthesizer and purified by HPLC [semipreparative PRP1 (Hamilton) column, gradient 0–40% CH_3CN in 0.1 M TEAB]. The reaction mixture contained 145 pmol of 5′-[^{32}P]mRNA, 140 pmol of PO-1C2(35) and 0.5 U of RNase H. Buffer conditions for RNase H digestion were as follows: 4 mM Hepes, pH 8.0, 10 mM KCl, 2 mM $MgCl_2$ and 0.2 mM dithiothreitol. PO-1C2(35) and mRNA matrix were labelled by standard procedure with T4 polynucleotide kinase and [γ-^{32}P]ATP. After incubation at 37°C for 2 h the whole reaction mixture (10 μl) was placed on 20% nondenaturing polyacrylamide gel (TB, 10 mM $MgCl_2$) and electrophoresed. Product of the reaction was identified by means of autoradiography. The reaction mixture without enzyme and 5′-[^{32}P]-PO-1C2(35) was used as a control.

Measurement of MRP1 protein levels in HL60 and HL60/ADR cells. After a double treatment with oligonucleotide (with PS-4-(sense) used as a control) the cells were washed with NaCl/P_i buffer and protein concentration was determined according to Lowry *et al.* [30]. Aliquots containing 100 μg of protein were sonicated and 30 μl portions of Laemmli solubilizing buffer (final concentration: 100 mM Tris/HCl, 2% sodium dodecyl sulfate, 10% sucrose, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) were added,

and kept at –20°C until separation. Before electrophoresis, the aliquots were incubated at 37°C for 30 min to achieve full solubilization of membrane proteins. Polyacrylamide gel electrophoresis was performed in the discontinuous buffer system according to Laemmli [31] in 8% polyacrylamide using BioRad Mini Protean II apparatus. After electrophoresis, proteins were transferred onto Immobilon-P (Millipore) by electroblotting in the Towbin buffer (25 mM Tris/HCl, 193 mM glycine, 20% methanol, pH 8.3) for 16 h at 100 mA. Immobilon was blocked with 5% non-fat milk in Tween-TBS (20 mM Tris/HCl, 150 mM NaCl, 0.05% Tween, pH 7.6) and incubated for 16 h at 4°C with anti-human MRP monoclonal antibodies (clone MRP r1, rat, Ig62a from Kamina Biomedical Company, specific to the amino-terminus of MRP1, 1:1000). The excess of antibodies was removed by 3 washes with Tween-TBS and the blots were incubated with horseradish peroxidase-conjugated anti-rat antibodies (Sigma, 1:2000) for 90 min at room temperature. The excess of the second antibody was removed as above and protein bands were visualized by chemiluminescence (ECL Kit, Amersham) and exposure to X-ray film. The concentration of MRP1 was estimated densitometrically (Desaga CD-60).

Chemosensitivity test. HL60 and HL60/ADR cells were seeded at a concentration of 300 000/ml in serum-free RPMI 1640 medium and oligonucleotides (0.5 μM) were added to HL60/ADR cell cultures. In control experiment only serum-free medium was added. The cells were incubated for 4 h with oligonucleotides, washed once with complete medium, and incubated again for 48 h. The cells were

Table 1. Sequences of ODNs used in experiments

Group	Name	Sequence 5′-3′	Length (bases)
I	PS-1	d[**AsAsAsGsGsAsAsGsAsTsGsCsTsGsAsGsGsAsAsG**]	20
	PS-2	d[**AsTsGsGsAsGsAsAsGsGsTsGsAsTsGsCsCsAsTsT**]	20
	PS-3	d[**CsCsGsGsAsAsTsTsCsCsAsCsTsCsGsGsCsCsCsA**]	20
	PS-4	d[**TsCsCsAsGsCsTsCsCsTsCsAsTsGsGsGsAsGsAsG**]	20
	PS-4 (sense)	d[**CsTsCsTsCsCsCsAsTsGsAsGsGsAsCsCsTsGsGsA**]	20
II	PO-1C	d[GTGCC**AAAGGAAGATGCTGAGGAAGG**GGGCA]	31
	PO-2C	d[GGTCC**ATGGAGAAGGTGATGCCATT**GGACC]	30
	PO-3C	d[TGGG**CCGGAATTCCACTCGGCCCA**]	24
III	PO/PS-1C	d[GTGCC**AsAsAsGsGsAsAsGsAsTsGsCsTsGsAsGsGsAsAsG**GGGCAC]	31
	PO/PS-2C	d[GGTCC**ATsGsGsAsGsAsAsGsGsTsGsAsTsGsCsCsAsTT**GGACC]	30
	PO/PS-3C	d[TGGG**CCGGsAsAsTsTsCsCsAsCsTCGGCCCA**]	24
IV	PO-1C2 (27)	d[GCG**AAAGGAAGATGCTGAGGAAGG**CGC]	27
	PO-1C2 (29)	d[TGCG**AAAGGAAGATGCTGAGGAAGG**CGCA]	29
	PO-1C2 (31)	d[GTGCG**AAAGGAAGATGCTGAGGAAGG**CGCAC]	31
	PO-1C2 (33)	d[AGTGCG**AAAGGAAGATGCTGAGGAAGG**CGCACT]	33
	PO-1C2 (35)	d[CAGTGCG**AAAGGAAGATGCTGAGGAAGG**CGCACTG]	35
	PO-1C2 (31) Mism	d[GTGCC**AAAGGcAGATGCTGAGtAAGG**GGCAC]	31

Underlined letters indicate complementary stem-forming ends. Bold letters indicate regions complementary with target mRNA; letter "s" within the sequence means internucleotide phosphorothioate bond. Lower case letters indicate mismatched bases.

then washed with serum-free medium and incubated for 4 h with another portion of oligonucleotides (0.5 μM). After the second treatment, cells were resuspended in complete medium, dispensed in to 96-well plates, and doxorubicin was added at a concentration of 200 nM, 1 μM, 2 μM and 4 μM. Following 48-h incubation, cell viability was determined using the MTT method [32]. Doxorubicin cytotoxicity was expressed as a percentage of the absorbance values obtained for cells cultured without doxorubicin.

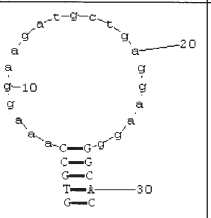
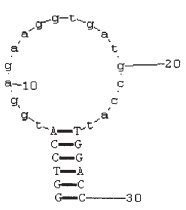
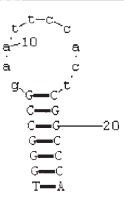
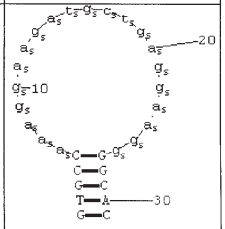
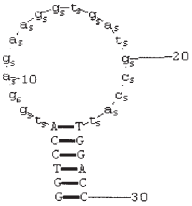
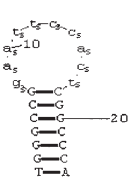
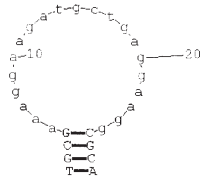
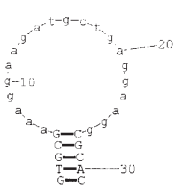
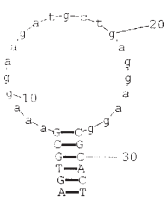
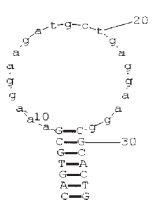
RESULTS

Description of ODNs

Oligonucleotide sequences used in this work were selected after analysis of MRP1 mRNA secondary structure using the computer program obtained from Genetic Computer Group. The MFOLD program, which is an adaptation of the mfold package by Zucker & Jaeger *et al.* [33] that has been modified to work with the Wisconsin Package [33, 34] was applied. Default parameters were used to predict secondary structure of MRP1 mRNA at 37°C and the structure with minimum free energy was selected for further analysis. Antisense ODNs were designed to be complementary to selected single stranded regions of target mRNA (Table 1).

Four series of oligonucleotide constructs were synthesized: linear PS-ODNs containing only all-phosphorothioate core sequence (I); chemically unmodified PO-ODNs with secondary hairpin-loop structure (II); chimeric PO/PS-ODNs containing phosphorothioates in the central region (loop) but with unmodified self complementary flanks (III); unmodified hairpin-loop ODNs differing in length of the stem but with identical central loop region (IV). Hairpin loop structures of oligonucleo-

Table 2. Computer drawn structures of the hairpin loop forming oligonucleotides used in this work

PO-1C (II)	PO-2C (II)	PO-3C (II)	PO/PS-1C (III)
			
PO/PS-2C (III)	**PO/PS-3C (III)**	**PO-1C2(29) (IV)**	**PO-1C2(31) (IV)**
			
PO-1C2(33) (IV)	**PO-1C2(35) (IV)**		
			

tides presented in Table 2 were generated by RNAstructure 3.5 software [35]. The length of ODNs ranged from 22 to 35 nucleotides. They possessed two structural domains: a double stranded stem domain containing 5 (PO-1C, PO/PS-1C), 6 (PO-2C, PO/PS-2C) or 7 (PO-3C, PO/PS-3C) base pairs and the single stranded loop domain of varying length (Table 2). Group I represented a control set of ODNs, which are not able to form a hairpin-loop structure. Groups II and III constituted the basic set we used in search for the most active antisense constructs. The main difference between them concerns the chemical modification of the loop. Phosphorothioate linkages (mixture of diastereomers) in PO/PS-1C, PO/PS-2C and PO/PS-3C (group III) were introduced into the loop region for the protection against possible endonucleolytic degradation. As a control in degradation studies, linear phosphorothioate PS-4 and PS-4(sense) were used. Group IV was derived from PO-1C oligomer, the member of group II that gave the best results in MRP1 inhibition tests. These constructs had the same sequence of the loop as PO-1C and stems of different length (Table 2).

Formation of hairpin loop structure

The preference of ODNs to form a hairpin loop structure was proved by melting temperature measurement and by electrophoresis experiments. The effect of oligonucleotide concentration on the melting temperatures was used to assign their preferred structure [36]. Thermal denaturation profiles for PO-1C, PO-2C and PO-3C appeared to be biphasic, suggesting the presence of two structures in the solution (Fig. 1). The lower temperature transitions corresponded to the melting of an intermolecular duplex as judged from its dependency on oligomer concentration, whereas those at higher temperature corresponded to the concentration independent dissociation of the intramolecular hairpin structure (54, 63 and 82°C for PO-1C, PO-2C and PO-3C, respectively). Contrary to PO-1C, PO-2C and PO-3C, their phosphorothioate analogues PO/PS-1C, PO/PS-2C and PO/PS-3C were found to form exclusively intramolecular structures. Their melting curves are monophasic and concentration independent. Since their t_m values for intramolecular melting are very high (56, 59, and 82°C, respectively), we concluded that under physiological conditions all the investigated compounds exist predominantly as hairpin loop structures.

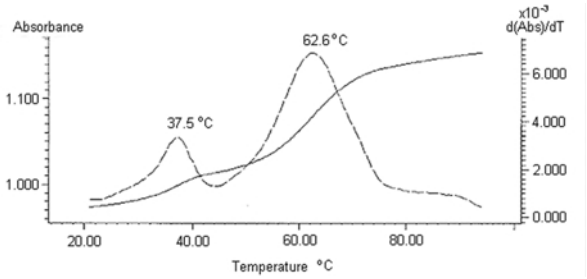


Figure 1. Melting temperature profile of PO-2C.

Similar, S-shaped melting profiles were obtained for both PO-3C and PO-1C (not shown).

Hairpin loop formation by each ODN was also investigated by electrophoresis on 20% native polyacrylamide gel at 4°C. The hairpin structures of PO-1C, PO-2C and PO-3C migrated with higher electrophoretic mobility than their intermolecular duplex (PO-2C and PO-3C) and tetraplex (PO-1C) structures (Fig. 2). The content of hairpin loop structure was 97%, 78% and 55% for PO-1C, PO-2C and PO-3C, respectively, as determined by densitometric measurement. Contrary to PO-2C and PO-3C, their phosphorothioate analogues PO/PS-3C and PO/PS-2C were shown to form exclusively hairpin loop structures. In the case of PO/PS-1C the percentage of hairpin loop structure was the same as for PO-1C.

Effect of hairpin loop formation on nucleolytic degradation of ODNs and PO/PS-ODNs

The sensitivity of ODNs forming hairpin loop structures both unmodified (PO-1C,

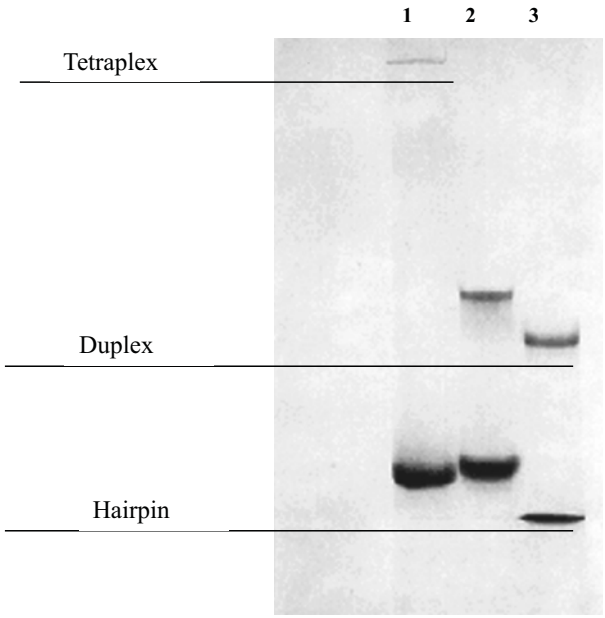


Figure 2. A hairpin, duplex and tetraplex structure in non-denaturing PAGE (20% gel).

Lane 1, PO-1C; Lane 2, PO-2C; Lane 3, PO-3C

PO-2C and PO-3C) and chimeric (PO/PS-1C, PO/PS-2C, PO/PS-3C) to 3′-exonuclease degradation was studied by incubating them with 50% human plasma (HP) and with 10% fetal calf serum (FCS). The obtained results indicated complete resistance of the oligomers to the 3′-exonuclease activity of human plasma. Thus, only intact hairpin loop structures were observed even after incubation for 8 h at 37°C. Since our preliminary studies indicated that the presence of Mg^{2+} increases the rate of hydrolysis of the unmodified oligomer d[T_{12}] by the 3′-exonuclease from human plasma (not shown), we decided to investigate the effect of magnesium ions on the rate of degradation of 5′-labelled ODNs (PO-1C, PO-2C and PO-3C) and chimeric PO/PS-ODNs (PO/PS-1C, PO/PS-2C, PO/PS-3C) in 50% human plasma. In the presence of Mg^{2+} (concentration 10 mM) intact PO-1C was detected in 50% human plasma even after 8 h incubation under identical conditions. At the same time point, PO-2C was degraded to the extent of 26%. The enzyme cleaved off only two nucleotides from the 3′-end (not shown). Surprisingly, oligomer PO-3C exhibited a somewhat different behavior. A small percentage of short oligomers originating from the 5′-end (dTGGGC, dTGGG, dTGG, dTG) was observed on 20% polyacrylamide denaturing gel after 8 h incubation (Fig. 3). This result indicated both endo- and exonucleolytic activity of human plasma. The endonuclease from human plasma was not very active and after 8 h incubation only 10% degradation of the oligomer PO-3C was detected. The endonucleolytic activity towards other oligonucleotides used in these studies was not observed (Table 3).

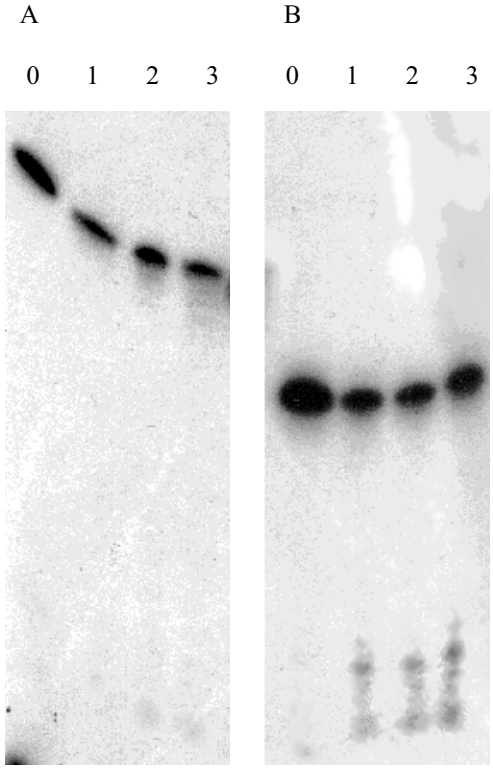


Figure 3. Stability of PO-1C (A) and PO-3C (B) in 50% human plasma containing Mg^{2+} ions.

The successive lanes in panels A and B correspond to the same incubation time. Lane 1, reference sample; lane 2, 2 h; lane 3, 4 h; lane 4, 8 h.

Finally, a comparative incubation of all six ODNs in 10% fetal calf serum was performed. Hairpin loop forming ODNs, PO-3C and PO/PS-3C were digested to approximately 50% after 8 h incubation in 10% FCS. In this case the nuclease present in the medium had a preference to cleave off only the first nucleotide from the 3′-end. Unmodified ODNs PO-2C

Table 3. Melting temperatures, secondary structures and stability of ODNs in 10% FCS and 50% human plasma (HP).

Conditions are as indicated in Materials and Methods.

ODN	t_m (°C)	Hairpin loop structure (%)	Percentage of undegraded substrate after 8 h incubation in:		
			10% FCS	50% HP	
				in the absence of Mg^{2+}	in the presence of Mg^{2+} (10 mM)
PO-1C	54	97	90	100	100
PO-2C	62.5	78	70	100	74
PO-3C	82.5	55	50	100	90
PO/PS- 1C	56.0	97	54	100	100
PO/PS- 2C	59.0	100	50	100	100
PO/PS- 3C	81.5	100	48	100	100

and, in particular, PO-1C were rather highly resistant towards 10% FCS since 70% and 90% of molecules, respectively, remained intact after 1 h incubation. Quite surprisingly, their chimeric phosphorothioate counterparts PO/PS-2C and PO/PS-1C were degraded to the extent of 50% under identical conditions. It should be noted that prolonged incubation time (8 h) did not result in a high degree of degradation of these ODNs (Table 3).

Formation of RNA/DNA heteroduplex

The availability of the loop part for hybridization with complementary RNA was investigated by thermal denaturation as well as degradation by RNase H experiments. In both cases we used a synthetic 28-mer oligoribonucleotide as a matrix. Thermal dissociation studies were performed for a series of hairpin loop structures varying only in the length of the stem PO-1C2(29), PO-1C2(31), PO-1C2(33), PO-1C2(35). The data in Table 4 show that the stability of intramolecular duplex increased with the length of the stem. However, in the presence of complementary RNA the melting temperatures were the same for all structures indicating formation of identical heteroduplexes between the loop and RNA matrix. The sharp and monophasic shape of the appropriate melting profiles (not shown) suggests that the association of RNA caused dissociation of the intramolecular duplex. Another evidence for the existence of heteroduplex was provided by RNase H degradation pattern (Fig. 4).

A slow migrating band observed on lane 3 corresponds to the intact structure. In the presence of enzyme this species disappeared and a product of degradation can be seen (lane 1). The RNA matrix alone was not digested under the same conditions (lane 4).

Table 4. Melting temperatures of hairpin loop structures and their complexes with RNA

ODN	t_m (ODN) (°C)	t_m (ODN+RNA) (°C)
PO-1C2(29)	49	67
PO-1C2(31)	54	67
PO-1C2(33)	60	67
PO-1C2(35)	64	67

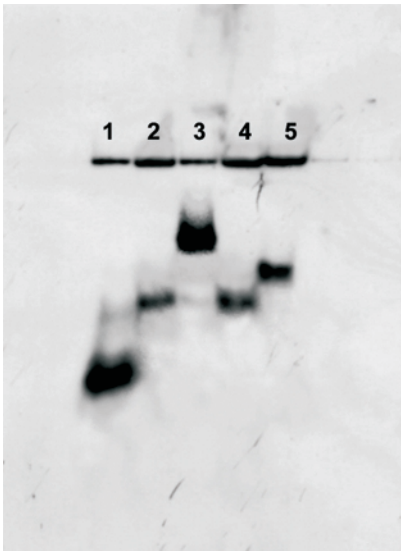


Figure 4. Electrophoretic analysis of ODN-RNA heteroduplex formation and its degradation by RNase H.

Lane 1, 5′-[^{32}P]mRNA + PO-1C2(35) + RNase H; Lane 2, 5′-[^{32}P]mRNA + RNase H; Lane 3, 5′-[^{32}P]mRNA + PO-1C2 (35); Lane 4, 5′-[^{32}P]mRNA; Lane 5, [^{32}P]PO-1C2 (35).

Reduction of MRP1 mRNA and MRP1 protein expression levels

The properties of ODNs as MRP1 expression inhibitors were tested in the drug selected HL60/ADR cell line which is known to overexpress that protein, whereas as control of MRP1 expression were chosen HL60 cells which produce this protein at a very low level. Considering the half-life of MRP1 (approximately 20 h) ODNs were administered into cells at 0.5 μM concentration in two doses at an interval of 48 h between treatments. Our preliminary experiments with Lipofectin, used to facilitate administration of antisense ODNs into HL60/ADR cells were discouraging, so in the present studies we did not use either this or any other uptake enhancer.

Then, concentrations of MRP1 protein and MRP1 mRNA were determined. The protein level was examined by means of Southern blotting followed by reaction with two antibodies. The first one recognized MRP1 in a specific manner, the second had an incorporated peroxidase which catalyzed the staining reaction. The MRP1 mRNA level was determined after isolation of whole mRNA from the cell culture by semi-quantitative RT-PCR. The GAPDH mRNA was used as an internal standard since this protein is known to be expressed in HL60 and HL60/ADR cells at a constant level. One of ODNs tested (PO-1C) was found to inhibit protein expression reproducibly and more strongly than other ODNs. After the administration of this ODN, MRP1 concentration decreased to 64% of that in untreated cells. The MRP1 mRNA level was decreased to about 31%. In the case of PO/PS-ODNs the best inhibitor was PO/PS-1C, which was found to reduce MRP1 expression level down to about 60%. Other PO/PS-ODNs were less effective MRP1 expression inhibitors (Fig. 5).

However, Stewart *et al.* [37] found no direct correlation between the effect of antisense ODNs on MRP1 protein and mRNA level. This could be due to additional factors that might

Table 5. Drug sensitivity after treatment of HL60/ADR cells with an anti-MRP antisense oligonucleotide PO-1C2 [31]

Concentration of doxorubicin (μM)	Cell counts ± S.D.			
	HL60	HL60/ADR	HL60/ADR + PO-1C2(31) mism.	HL60/ADR + PO-1C2(31)
0.2	28.5 ± 4.5	95.7 ± 5.4	96.1 ± 1.2	85.0 ± 4.4
1	25.1 ± 7.9	93.1 ± 7.0	95.2 ± 4.8	81.2 ± 6.9
2	23.5 ± 1.5	87.8 ± 9.1	95.5 ± 1.5	76.0 ± 6.2
4	7.0 ± 3.2	72.5 ± 4.4	71.1 ± 6.2	61.8 ± 5.8

Cell viability was measured with MTT assay (see Material and Methods)

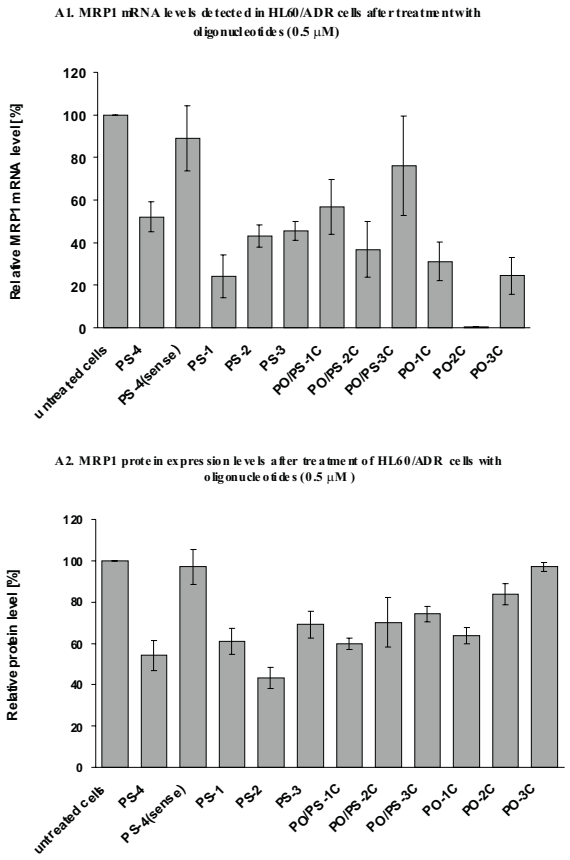


Figure 5. MRP1 mRNA (A1) levels detected in HL60/ADR cells after treatment with oligonucleotides group I, II and III (0.5 μM) and MRP1 protein (A2) expression levels after treatment of HL60/ADR cells with oligonucleotides group I, II and III (0.5 μM).

Error was calculated as standard deviation from a series of three experiments for each antisense oligonucleotide.

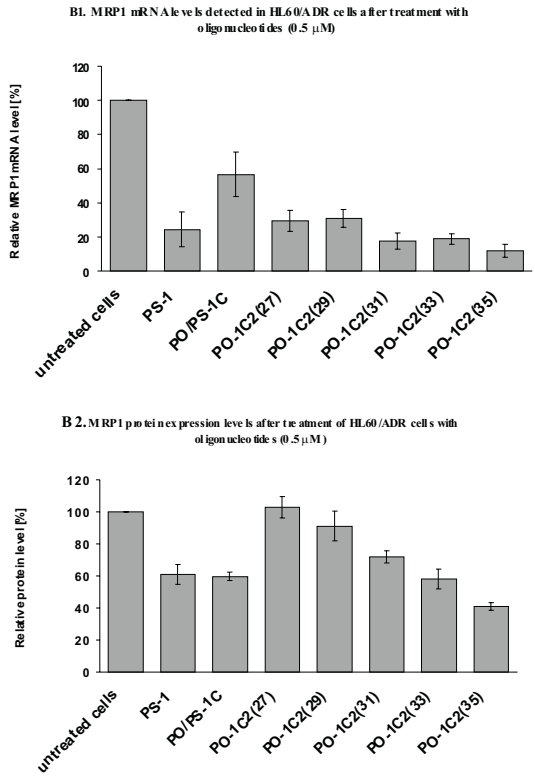


Figure 6. MRP1 mRNA levels (B1) detected in HL60/ADR cells after treatment with oligonucleotides group IV (0.5 μM) and MRP1 protein (B2) expression levels after treatment of HL60/ADR cells with oligonucleotides group IV (0.5 μM).

Error was calculated as standard deviation from a series of three experiments for each antisense oligonucleotide.

have had some influence on cellular uptake and other biological properties of ODNs, such as direct interactions with proteins.

After the most effective ODN sequence (PO-1C) was selected, a series of hairpin-loop forming ODNs was prepared (group IV) differing in the length of the stem (from 3 to 7 base pair, see Tables 1 and 2). These oligonucleotides were also tested in HL60/ADR cells as inhibitors of MRP1 biosynthesis. The oligonucleotide with the longest stem (PO-1C2(35)) gave a significant reduction of levels of MRP1 protein (to about 41% of original level) and mRNA (to about 12%) (Fig. 6).

Chemosensitivity test results

In order to get a better insight into the nature of the observed inhibitory activity of anti-MRP1 antisense oligonucleotides able to form hairpin loop structures, chemosensitivity tests were performed, including exposure of the oligonucleotide treated cells to doxorubicin. It was found that after 48 h exposure to doxorubicin, following double treatment of HL60/ADR cells with PO-1C2 [35], the relative cytotoxicity caused by doxorubicin (4 μM) was 61.8 ± 5.8% as compared

with 72.5 ± 4.4% in oligonucleotide untreated HL60/ADR cells (Table 5).

On the other hand, after successive exposure of parent HL60 cells to a broad concentration range of doxorubicin (200 nM–4 μM) the cells developed partial drug resistance. So, in spite of the fact that in the case of HL60/ADR cells which show 80–100-fold overexpression of MRP1 protein as compared to the parent HL60 cell line, only a partial sensitization to doxorubicin was observed, the studies described here provide strong evidence for the oligonucleotide induced decrease of MRP1 mRNA and protein level in these cells. Such reduction of MRP1 expression may be sufficient for chemosensitization of multidrug-resistant haematological malignancies of clinical origin showing a 2–40-fold overexpression of MRP1.

DISCUSSION

The cell resistance to chemotherapy observed during leukaemia treatment remains the main cause of poor prognosis of malignancy. Two proteins have been shown to cause this effect in human tumor cells: the 170 kDa P-glycoprotein and the 190 kDa MRP1. Cells overexpressing these N-glycosylated phosphoproteins show decreased drug accumulation accompanied by increased drug efflux. The mechanism of multidrug resistance phenomenon is still not fully understood. Thus, the reasonable attempt to enhance the effectiveness of chemotherapy seems to be the reduction of expression of the protein responsible for this kind of activity.

Linear phosphorothioate analogs of ODNs are commonly used in the studies on antisense inhibition of protein expression and a considerable progress has been made in recent years towards their application in medicine. However, there are some unsolved problems like, for example, synthesis of these compounds as mixtures of diastereomers due to phosphorus chirality. This means that *de facto,* in antisense inhibition experiment an unseparated mixture of compounds is applied. Only in one case the application of stereodefined phosphorothioate oligonucleotides for antisense inhibition of protein expression was described [38]. In addition, phosphorothioate oligonucleotides have a tendency to bind directly to proteins [39] and some phosphorothioate sequences containing the CpG motif were found to possess unwanted immunostimulatory activity [40, 41].

Oligo(nucleoside phosphorothioate)s were introduced to medicinal chemistry mainly due to their resistance to nuclease degradation, as unmodified phosphodiester ODNs were very sensitive to nucleolytic hydrolysis. However, several years ago the so-called self-stabilized as well as circular ODNs were described as "DNA traps" [42] interfering with its replication (*via* triplex formation). Thus it was their secondary structures and not phosphorothioate modification which were proved to protect ODNs against degradation by 3′-exonucleases [43]. We have successfully combined that concept with antisense approach based on the formation of a duplex between MRP1 mRNA and ODN existing in the form of hairpin loop. In our hands, application of hairpin loop ODNs complementary to selected regions of the MRP1 mRNA sequence resulted in a remarkable reduction of the MRP1 expression level in the leukaemia HL60/ADR cell line. This was proved by determination of concentrations of MRP1 mRNA and MRP1 protein after treatment of leukaemia HL60/ADR cells with ODNs. Designing hairpin loop ODNs we intended to compare two groups of compounds-unmodified phosphodiester ODNs (II) and their chimeric phosphorothioate analogs PO/PS-ODNs (III), both able to form a secondary hairpin loop structure. Thus, one group of compounds (II) represented ODNs without any chemical modification, the other (group III) possessed the phosphorothioate modification only in the loop domain (chimeric PO/PS-ODNs). The formation of a secondary

hairpin loop structure by either group of compounds was proved by determination of t_m values and electrophoresis experiments. In both cases the presence of 55–100% of hairpin loop form was determined. Then, the comparative rate of degradation of phosphodiester ODNs and PO/PS-ODNs constructs was determined under conditions similar to those present in cell culture. It was observed that introduction of sulfur atoms into the loop region (PO/PS-ODNs) had no additional effect on the stability of hairpin loop oligonucleotides in 10% FCS. On the contrary, unmodified hairpin loop ODNs seemed to be more resistant to exonucleolytic degradation.

Each ODN was screened as antisense inhibitor targeting a desired part of MRP1 mRNA after a selection based on the computer analysis of MRP1 mRNA secondary structure of each target sequence. Some investigated ODNs showed remarkable inhibitory activity on the biosynthesis of MRP1 protein (Fig. 5; A2) and these were chosen to create group IV of antisense oligonucleotides PO-1C2(n). The antisense mechanism of inhibitory activity and ODN/RNA heteroduplex formation of investigated ODNs was proved in the experiment in which the heteroduplex composed from hairpin loop ODN and 5′[^{32}P]RNA was treated with RNase H, and degradation of only the RNA portion was observed (Fig. 3). To our knowledge ODNs able to form a secondary structure like hairpin loops have not been used before to circumvent MRP1-mediated resistance in leukaemia cells by the antisense approach. The reduction of MRP1 protein expression down to about 41% ODN-PO-1C2(35) seems to be significant by comparison with the results obtained by other authors with both MRP1 and PgP [4, 44, 45]. However, the results of chemosensitivity tests with doxorubicin were, surprisingly, rather negative for HL60/ADR cells. One of the possible explanations of this result was the extremely high overexpression level of MRP1 protein (80–100-fold) in these cells, much higher than the level of this protein in normal and transfected cells. On the other hand, drug resistant cell lines generated by transfection with MRP-complementary DNA, display some (but not all) characteristics of MRP-overexpressing cell lines produced by drug selection *in vitro* [46]. Comparison of the stability of ODNs and their antisense inhibitory activity let us to formulate an interesting conclusion on the relationship between ODNs structures and their biological activity: the phosphodiester oligonucleotide PO-1C and chimeric PS/PO constructs which exist almost exclusively in the form of a hairpin loop structure are better MRP1 expression inhibitors than other ODNs used in these studies, which exist in the form of a mixture of hairpin loop, linear and/or higher order structures with lower proportion of the hairpin loop. Thus, our results indicate that, under the experimental conditions applied, the resistance of hairpin loop forming ODNs to 3′-exonucleases is comparable with that of chemically modified oligonucleotides used commonly [47] at in antisense inhibition experiments (phosphorothioates). The unmodified hairpin loop oligonucleotide PO-1C2(35) with 7 bp stem is stable under experimental conditions and reduces MRP1 protein to about 41% and mRNA to about 12% of their original levels even in HL60/ADR cells, with their very high overexpression of MRP1 protein. Our results show that hairpin loop structure may be considered a serious alternative to phosphorothioates in designing new antisense inhibitors of protein expression.

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