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Multidrug resistance-associated protein — reduction of expression in human leukaemia cells by antisense phosphorothioate oligonucleotides★✪

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Multidrug resistance-associated protein (MRP1) causes cellular drug resistance in several cancer cell lines. In this paper we show that antisense oligonucleotides decrease MRP1 expression in human leukaemia cells. We investigated biological activity of a series of 12 linear phosphorothioate oligonucleotides, complementary to several regions of MRP1 mRNA. The oligonucleotides were administered to leukaemia HL60/ADR cells overexpressing MRP1 protein. Then, the level of MRP1 mRNA was determined by means of semiquantitative RT-PCR and the protein level by reaction with specific monoclonal antibodies. Some of the investigated antisense oligonucleotides decrease the expression level of the MRP1 protein by 46% and its mRNA level by 76%.

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Abbreviations: MRP, multidrug resistance-associated protein; NaCl/P_i buffer, phosphate-buffered saline; ODN, oligonucleotide; PS-ODN, phosphorothioate oligonucleotide.

Multidrug resistance-associated protein (MRP1) was identified as a transmembrane protein belonging to the ABC transporters superfamily [1, 2]. It was recognised as a M_r 190 000 protein possessing an ATP-binding cassette, which functioned as an outward drug pump. As substrates MRP1 accepts anthracyclines, vinca alkaloids and epipodophyllotoxins which are removed from cells as conjugates of glutathione or cotransported with glutathione [3–5]. It was detected in several cancer cell lines, however, its level was particularly high in several leukaemias such as chronic lymphocytic leukaemia, promegalocytic leukaemia, acute myelocytic a lymphocytic leukaemia, and multiple myeloma [6–8]. MRP1 overexpression causes cellular drug resistance, which is the reason of failure of numerous chemotherapy protocols. Both verpamil (a calcium channel blocker) and cyclosporin A (an immunosuppresive cyclic peptide) are ineffective as MRP1 reversing agents, therefore other reagents able to decrease MRP1 expression in cancer cells are under investigation including antisense oligonucleotides (ODNs) [9]. Phosphorothioate derivatives are the most commonly used in antisense inhibition experiments as they are resistant to 3′-exonuclease degradation, form a very stable duplex with complementary mRNA encoding the desired protein, and activate RNase H [10–12]. In the case of reduction of the multidrug resistance phenomenon they were successfully used to inhibit the expression of glycoprotein P (PGP, M_r 170 000) in several cell lines and the MRP1 protein in human lung cancer H69AR cells [9, 13]. In this paper we present the results of an investigation in which a series of antisense phosphorothioate oligonucleotides (PS-ODNs) was employed. They were complementary to MRP1 mRNA sequences encoding different parts of the protein including the transmembrane and nucleotide-binding domains. Antisense ODNs were administered to the leukaemia HL60/ADR cell line which was previously described as overexpresing MRP1 at a level about 70 times higher then the parental HL60 cells [14]. The potential inhibitory properties of the investigated ODNs were evaluated by determination of MRP1 mRNA and MRP1 protein levels.

MATERIALS AND METHODS

Oligonucleotides synthesis. Oligonucleotide sequences were selected based on the MRP1 mRNA secondary structure analysis using computer programs from the Genetic Computer Group. Oligodeoxyribonucleotide phosphorothioates (sequences are shown in the Table 1) were synthesised by the modified phosphoramidite method using an ABI 394 DNA Synthesiser with S-tetra sulfurization [15, 16]. All ODNs were purified by RP-HPLC followed by preparative electrophoresis on a 20% polyacrylamide gel containing 7 M urea.

Cell cultures. The human promyelocytic cell lines HL60 and HL60/ADR [14] kindly provided by Dr. M. Center (Division of Biology, Kansas State University, U.S.A.) were routinely maintained in RPMI 1640 medium containing 200 nM doxorubicin in the case of HL60/ADR cells supplemented with 20% heat inactivated fetal calf serum, penicillin and streptomycin in 90–95% humidified atmosphere of 5% CO_2 at 37°C. Cells were seeded into wells of 24-well tissue culture dishes at a concentration of 3×10^5/ml. Before the addition of oligonucleotides the cells were washed with a fresh portion of RPMI 1640 medium. ODNs were added at a concentration of 0.5 μM. Routinely, the cells were treated with each ODN twice. Thus, the cells were incubated with an oligonucleotide at 37°C for 4 h, washed once with RPMI 1640 medium and then incubated for 48 h in complete medium. Then, the cells were treated again with the oligonucleotide for 4 h, washed as before and cultured for further 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/ADR cells. HL60 and HL60/ADR cells were seeded into wells of 24-well tissue culture dishes. ODNs (final concentration 0.5 μM) were added in two doses as described above and after the second treatment the incubation was continued at 37°C for 24 h. The cells were collected, washed 3 times with NaCl/P_i buffer and mRNA was isolated according to the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) procedure. The mRNA 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). The reaction products were analyzed on 6% polyacrylamide gels stained with ethidium bromide. The pictures of gels were scanned densitometerically (UltraScan XL, Enhanced Laser Densitometer, LKB Bromma, Sweden) and the MRP1 mRNA level was calculated as the percentage of the density values obtained for GAPDH.

Measurement of MRP1 protein levels in HL60/ADR cells. After the double treatment with ODN cells were washed with NaCl/P_i buffer and protein concentration was determined according to Lowry methods [17]. Aliquots containing 100 μg of protein were sonicated and 30 μl portions of Laemmli solubilizing buffer (final concentration 100 mM Tris/HCl, 2% SDS, 10% sucrose, 5% β-mercaptoethanol, 0.01% Bromphenol 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 [18] in 8% polyacrylamide using a 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. The membrane Immobilon-P 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 monoclonal rat antibodies specific to the amino-terminus of human MRP1 (Kamina Biomedical Company, 1:2 000). The excess of antibodies was re-

Table 1. Oligonucleotide sequences used in experiments on MRP1 expression inhibition

Oligonucleotides	Sequence (5′–3′)	Size	MRP1 mRNA target
PS-M1	CCCGAAAACAAAACAGGGTC	20	Coding region
PS-M3	ATGGAGAAGGTGATGCCATT	20	Transmembrane region
PS-M4	TCCAGCTCCTCATGGGAGAG	20	Coding region
PS-M5	AAGACACGAAGGCTGTCTGG	20	Transmembrane region
PS-M6	AGGACCAGAGGTCACTGCCC	20	Coding region
PS-M7	GGGCTACCAGCCTGAAAGTG	20	Transmembrane region
PS-CYT1	GTCGGGGTTGCTGGTATTCC	20	Cytoplasmic
PS-CYT2	CCAGTTCAAGTACGTGGTGA	20	Cytoplasmic
PS-NBD1	TTTCCGCAGCCCACCTGGCC	20	Nucleotide binding domain
PS-NBD2	CGACTTCCCAGCTCCCGTCC	20	Nucleotide binding domain
PS-TMR10	AAAGGAAGATGCTGAGGAAGG	21	Transmembrane region
PS-M17	CTCTCCCATGAGGAGCTGGA	20	MRP4 sense

moved by 3 washes with Tween-TBS and the blots were incubated with horseradish peroxidase-conjugated anti-rat antibodies (Sigma, 1:2 000) for 90 min at room temp. The excess of the second antibody was removed as above and protein bands were visualized using the Enhanced Chemiluminescence detection (ECL) system (Amersham) and exposure to X-ray film. The concentration of MRP1 was estimated densitometrically in a Desaga CD-60 densitometer. Results are presented as mean ± S.D. of 3–6 experiments.

RESULTS AND DISCUSSION

In spite of the existence of elaborated methods of designing antisense ODNs, including those with secondary structure of mRNA modelling, it is not fully possible to predict the most sensitive target sequence. Instead, synthesising a set of antisense ODNs aiming at different parts of target mRNA and their experimental investigation in human cells makes proper selection possible. Therefore we designed a set of 11 oligonucleotides that were complementary to different coding regions of the MRP1 mRNA sequence: PS-TMR10 (transmembrane domain), PS-NBD1 and PS-NBD2 (nucleotide-binding domain), PS-CYT1 and PS-CYT2 (cytosolic), PS-M1, PS-M3, PS-M4, PS-M5, PS-M6 and PS-M7 (other coding). We also synthesised one ODN (PS-M17) which had the sequence complementary to PS-M4 as the sense control. Among different derivatives of ODNs described in the literature we decided to use PS-ODNs because they are known to exhibit good cellular uptake and high resistance to nuclease degradation [19, 20]. For screening the ODNs ability to decrease MRP1 expression we chose the leukaemia HL60/ADR cells. Considering the 19-h half-life of the mature protein reported by Stewart *et al.* [9] we used ODNs at a concentration of 0.5 μM in double doses with a 48 h interval. Cationic lipids routinely used in antisense strategy were also tested. However, in preliminary experiments we found that at a broad range of concentrations neither lipofectin nor cytofectin did reduce the MRP1 protein level inside HL60/ADR cells when used with ODNs (not shown). Each PS-ODN was screened for its ability to decrease both the MRP1 protein and MRP1 mRNA levels. Based on the level of MRP1 expression we concluded that among the screened PS-ODNs several possessed relatively good inhibitory activities. The following PS-ODNs: M3, M4, M7, NBD2 and TMR10 were able to decrease the protein level to about 60% of that in ODN-untreated cells. In a parallel experiment the sense control PS-M17 decreased neither the MRP1 protein nor mRNA concentration. Among the best MRP1 protein expression inhibitors PS-TMR10 decreased MRP1 mRNA level by about 76%. The others reduced this level by about 60% of control (Table 2).

There is only one example of antisense phosphorothioate ODNs administered to cell lines overexpressing MRP1 decreasing the expression level of this protein. Stewart *et al.* [9] described a set of PS-ODNs which were applied with lipofectin to H69AR cells cultured from human lung cancer. The authors obtained a reduction of MRP1 protein expression by about 49% and its mRNA by about 88%. Evidence was found that PS-ODNs recognised the complementary sequence of mRNA and were able to form a duplex stabilised by Watson-Crick bonds which in turn was recognised and cleaved by RNase H resulting in a decrease of MRP1 mRNA concentration. The results presented in this paper are the second example of specific inhibition of MRP1 by antisense PS-ODNs and the first one in leukaemia cells. A significant reduction of MRP1 expression together with the decreased level of MRP1 mRNA indicate that in the case of leukaemia cells PS-ODNs can be efficient as specific inhibitors of "unwanted" biosynthesis. Most probably a further decrease of MRP1 expression levels is still possible to achieve. The final result depends on several factors which are specific for the group of compounds

Table 2. Effect of phosphorothioate oligonucleotides on MRP1 protein and MRP1 mRNA levels in HL60/ADR cells

Oligonucleotides	MRP1 protein level as % of expression in untreated cells	MRP1 mRNA level as % of expression in untreated cells
PS-M1	63.4 ± 10.2	48.0 ± 9.4
PS-M3	58.4 ± 6.7	43.2 ± 5.2
PS-M4	54.1 ± 7.1	52.0 ± 7.1
PS-M5	63.5 ± 11.5	43.3 ± 7.4
PS-M6	62.7 ± 12.3	41.0 ± 11.2
PS-M7	55.5 ± 0.5	40.0 ± 9.8
PS-CYT1	85.0 ± 1.0	44.0 ± 14.0
PS-CYT2	64.8 ± 6.3	36.0 ± 8.3
PS-NBD1	85.5 ± 0.5	40.0 ± 11.2
PS-NBD2	61.0 ± 9.9	43.3 ± 6.4
PS-TMR10	61.0 ± 6.2	24.3 ± 10.1
PS-M17	97.0 ± 8.4	88.9 ±15.2

Oligonucleotides were used at 0.5 μM concentration. The values for MRP1 protein and MRP1 mRNA are presented as percentage of controls, not treated with ODN. Values of protein and mRNA levels are means ± S.D. of 3–6 and 3 experiments, respectively.

investigated and particular cell line such as ODN uptake, sub-cellular distribution, resistance to nuclease degradation, elimination time, specificity in target recognition, and others. Further chemical and structural modifications of ODNs as well as changing experimental conditions (use of other carriers) could lead to higher inhibitory activity.

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