

RNAi induced inhibition of *MRP1* expression and reversal of drug resistance in human promyelocytic HL60 cell line

Masoud Golalipour^{1,2}, Frouzandeh Mahjoubi^{1*}, Mohammad Hossein Sanati^{1*}

¹Department of Genetics, Natinal Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14965-161, Tehran, I.R. Iran ²Department of Molecular genetics, Faculty of Science, Tarbiat Modares University, P.O. Box 14115-331, Tehran, I.R. Iran

Abstract

Multidrug resistance (MDR) is a complex phenomenon in which many different genes regulating drug transport, cellular repair, detoxification and drug metabolism are involved. Nevertheless, in most drug resistant cell lines and cancer patients up-regulation of ABC-transporter genes such as MDR associated Protein (MRP1) gene could be at the basis of the drug resistance phenotype. We aimed to decrease MRP1 expression at the mRNA level to modulate drug resistance phenotype in the methotrexate-resistant HL60 cell line. We designed a small interfering RNA (siRNA) molecule against MRP1 and applied it to HL60 cell line in a 0 to 72 hours time range. siRNA could specifically inhibit gene expression by 80% of the initial mRNA level with in 36 to 48 hours. The siRNA-treated cells demonstrated 100-fold reduction in methotrexate (MTX) resistance compared to untreated cells. The data indicate that this approach may be applicable to the study of MRP1 expression and development of future strategies to reverse the MRP1 dependent drug-resistance phenotype in tumors back to a drug-sensitive one.

Keywords: Multidrug resistance; protein 1; siRNA; HL60.

INTRODUCTION

A significant problem encountered in the treatment of cancer patients is that, very often cancer cells evolve resistance to antineoplastic agents. Although new drugs and treatment protocols have improved the disease prognosis in leukemia patients, in many cases initially responsive tumors ultimately relapse and develop resistance to the drugs (Buchner, 1997; Sonneveld, 2000). Drug resistance is believed to arise from multi-

ple mechanisms such as increased drug efflux or decreased influx by transporters, activation of detoxifying systems, activation of DNA repair systems and evasion from apoptosis) which may act singly or simultaneously (Gottesman *et al.*, 2002). Although several different mechanisms contribute to drug resistance, *MRP1* has been reported to be the major transporter responsible for non-*MDR1* mediated multidrug resistance in cancers (Cole *et al.*, 1992; Loe *et al.*, 1996; Marie *et al.*, 1996). The *MRP1* gene is a member of the ABC-transporter superfamily of membrane drug transporters, located on chromosome 16 p13. Its protein product has been shown to transport chemicals conjugated to sulfate, glutathione or glucuronate and various other organic anions and confers *in vitro* and *in vivo* resistance to a wide range of anticancer drugs (Cole and Deeley, 1998; Lockhart *et al.*, 2003).

There are many pharmacologically active compounds, designated as chemosensitizers, which circumvent the classical MDR phenotype by inhibiting the efflux pumps (Liscovitch and Lavie, 2002). One obstacle in applying chemosensitizers arises from toxicity of these drugs (e.g. heart failure, hypotension, hyperbilirubinemia and immunosuppression) at appropriate doses needed to be effective. In addition, cells can develop tertiary resistance against the chemosensitizers (Seiden *et al.*, 2002). Therefore, it is necessary to develop less toxic strategies for reversing the MDR phenotype. Blocking the expression of ABC-transporter's mRNAs, increases the efficiency and specificity of the chemosensitization of MDR cancer cells while at the same time reducing the effective dose of drug and its toxic side effects on normal cells.

A novel mean for specific inhibition of a gene at the post-transcriptional level is the use of siRNA,

*Correspondence to: Mohammad Hossein Sanati, Ph.D.

and Frouzandeh Mahjoubi, Ph.D.

Tel: +98 21 44580346; Fax: +98 21 44580350

E-mail: m-sanati@nrcgeb.ac.ir and frouz@nrcgeb.ac.ir

which is a double-stranded RNA molecule that induces sequence-specific degradation of homologous single-stranded RNA. RNA interference has been described in several eukaryotic organisms such as *Trypanosoma brucei*, *Caenorhabditis elegans*, *Drosophila*, *Planaria*, plants, zebrafish, mice and humans (Kim, 2005). siRNA can be effective in lowering the amount of targeted mRNA and thus inhibiting its subsequent expression to protein. The silencing effect is highly specific because one nucleotide mismatch between the target mRNA and the central region of the siRNA is sufficient to prevent silencing (Ryther *et al.*, 2005; Verma and Dey, 2004). In this study, we aimed to design a siRNA duplex for disruption of *MRP1* gene expression in a human promyelocytic cell line and evaluate re-sensitization of the cell line to treatment with methotrexate. Our data confirmed the effectiveness of siRNA to inhibit *MRP1* expression and the subsequent reversal of drug resistance.

MATERIALS AND METHODS

siRNA design: The target sequence of the siRNA molecule, was 5' -CCA CAA CAG CAC CGC AGA ATT-3' homologous to nucleotides 2917 to 2935 of the *MRP1* mRNA sequence, and was chosen according to recommendations made by other researchers (Henschel *et al.*, 2004; Ding *et al.*, 2004; Silva *et al.*, 2003; Truss *et al.*, 2005; Yuan *et al.*, 2004). The oligonucleotide sequence was selected according to the *MRP1* mRNA secondary structure, GC content of 30-60%, absence of CCC or GGG sequence in siRNA, instability of the 5' end of the Antisense strand and partial internal instability in the cut site.

Cell culture and transfection: The human promyelocytic cell line HL60 (Rots *et al.*, 1999) was routinely maintained in RPMI 1640 medium containing 15% (v/v) heat inactivated fetal calf serum, 100 units/ml of penicillin and streptomycin in the presence of 5% CO₂ at 37°C (all materials from Invitrogen, Carlsbad, CA). Before the addition of siRNA, the cells were washed with fresh RPMI 1640 medium. The cells were then transfected using Metafectene reagent, according to the manufacturer's instruction (Biontex, Munich, Germany). In brief, cells were plated at a density of 5×10⁵ cells per each well of a microtiter plate in 5 ml of RPMI medium containing serum and antibiotics. The cells were then transfected 24 h later with siRNA: Metafectene complexes. To formulate siRNA: Metafectene complexes, eight tubes were filled with 300 ml of RPMI without serum and antibiotics. To the

first four tubes, 0, 3, 6 and 9 mg of siRNA and to the second four tubes 18 ml of Metafectene were added, so as to prepare 0, 1:6, 1:3 and 1:2 siRNA: Metafectene ratios, respectively. The contents of the tubes were mixed well, and then allowed to stand 20 min at room temperature to allow formation of siRNA: Metafectene complexes for transfection. After 24 to 72 h, the cells were harvested and analyzed.

Total RNA isolation and cDNA synthesis: Total RNA was isolated from cells using the TRIZOL reagent (Gibco BRL, Paisley, UK) in accordance with the manufacturer's protocol. Pelleted RNA was resuspended in Extragene E solution and stored at -80°C until use. One µg of total RNA from each sample was then used to synthesize first strand cDNA. The RNA was incubated for 1h at 42°C in a 20 µl of room temperature buffer containing 100 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, 20 units of RNasin, 1 mM of each dNTP and random hexamer primer (all from Promega). The resulting cDNA was diluted in diethyl procarbonate (DEPC) treated pure water and used in real time PCR.

Real time RT-PCR: The sequences of primers for assessment of *MRP1* expression were as follows: forward 5'-CGG AAA CCA TCC ACG ACC CTA ATC-3' and reverse 5'-ACC TCC TCA TTC GCA TCC ACC TGG-3'. The sequences of primers for the assessment of β-2-microglobulin (β2M) expression were forward 5'-CTA TCC AGC GTA CTC CAA AG -3' and reverse 5'-GAC AAG TCT GAA TGC TCC AC-3'. The primers were designed using Primer Premier 5.0 software and all primer sequences were checked by ASePCR program (<http://genome.Ewha.Ac.Kr/Ecgene/>) for absence of any false priming sites. The length of the amplicon was 294 bp for *MRP1* and 147 bp for β2M. For quantification of gene expression, we used the Lightcycler™ system (Roche Mannheim, Germany) and the Fast-Start DNA Master SYBR-Green I kit (Roche diagnostics, Mannheim, Germany). Each reaction mixture contained 0.6 µM of each primer, 2.5 mM MgCl₂ and 2 µl of Fast Start Master solution. A total of 18 µl of this reaction mixture was placed into glass capillaries and 2 µl of cDNA (produced from 1 µg total RNA) was added as template. A standard Lightcycler PCR program was established for each gene and PCR conditions were optimized with respect to primer and MgCl₂ concentrations and annealing temperatures. To ascertain that fluorescence signals were associated with specific products, melting curves for each reaction were analyzed and the PCR products were checked on 2% (w/v) agarose gel.

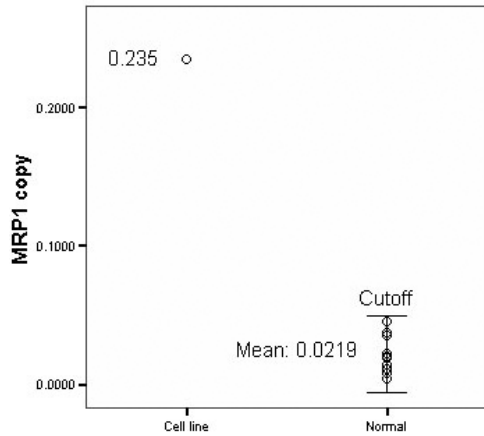


Figure 1. Expression of *MRP1* mRNA in peripheral blood of healthy individuals and HL60 cell line. The copy number of *MRP1* was expressed as the ratio of *MRP1* to β 2M copy number (each circle represents a sample). According to *MRP1* expression in healthy individual cutoff defined as mean \pm 2SD. HL60 resistant cell line showed *mrp1* expression level higher than cutoff.

Cytotoxicity assay for cell survival: MTX was purchased from Pharmacia and Upjohn S.P.A., Italy. HL60 cells were cultured in the presence of MTX (final concentrations ranging from 10^{-11} to 10^{-5} mol/l). The sensitivity of the HL60 cell line to MTX was determined by a standard growth inhibition assay (Itoh *et al.*, 2002). Briefly, cells were cultured in two different conditions: in the drug-free medium, and the medium containing drug. Viable cells were counted using trypan blue. The 50% growth inhibitory concentration (IC_{50}) level was defined as the concentration that inhibited growth by 50% relative to untreated controls. Relative resistance represents the ratio of the IC_{50} of the resistant cell lines to that of the parental cell lines. The results are expressed as mean values of three experiments \pm SD.

RESULTS

siRNA reduced *MRP1* mRNA level: At first, *MRP1* expression in the HL60 cell line was compared to the expression of *MRP1* in lymphocytes of 10 healthy individuals (Fig. 1). The final results were expressed as the ratio of *MRP1* to β 2M transcript level in each sample. The mean of *MRP1* expression mean was 0.235 in resistant HL60 but was 0.0219 in healthy samples.

Real time RT-PCR analysis demonstrated that within 36 h of siRNA transfection the expression level of the *MRP1* started to reduce and continued to do so until 48 h to the point at which *MRP1* mRNA signal decreased to 18.63 % of the initial signal (Fig. 2). After the above time period, the *MRP1* mRNA expression

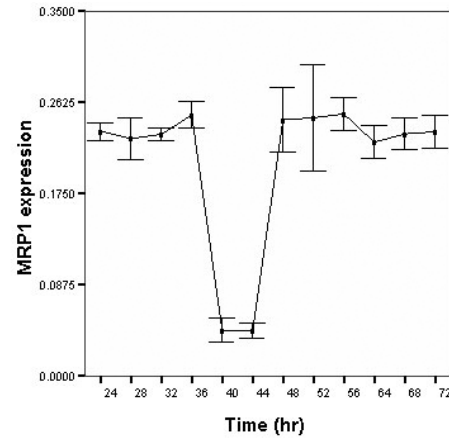


Figure 2. Analysis of siRNA mediated silencing of *MRP1* mRNA expression determined by quantitative real-time RT-PCR. Relative *MRP1* expression level normalized against β 2M expression level in siRNA transfected cells. The minimum *MRP1* mRNA was detected between 36 to 48 hours. The level of mRNA represented as mean \pm SD.

started to increase and reached the original mRNA expression level. The best result was achieved by the 1:3 ratio (6 mg) of siRNA: Metafectene complex (Fig. 3). Therefore, treatment of cells by siRNA decreased the level of *MRP1* mRNA expression in the human promyelocytic cell line in a time dependent manner.

Increase in drug sensitivity of the siRNA- treated cells: The siRNA-mediated reversal of the MDR phenotype was assessed by comparison of the IC_{50} values determined by the cell viability assay of in siRNA-treated tumor cells versus untreated controls. Cytotoxicity experiments were performed 48 h after treatment with siRNA molecules. As shown in Figure 4, the sensitivity of the MDR cells to the drug

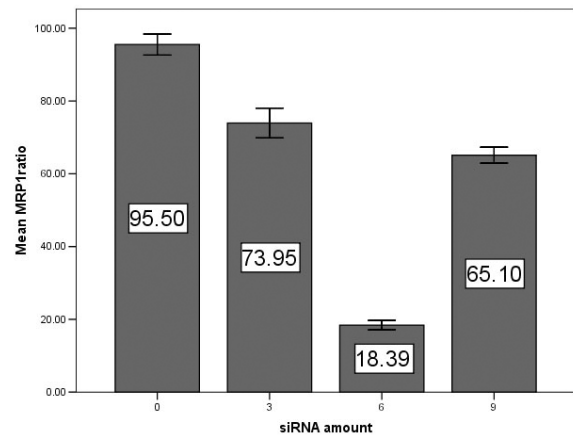


Figure 3. siRNA knockdown of *MRP1* mRNA. Human promyelocytic cells were transfected with 0 to 9 mg *MRP1* siRNA. The best results achieved by 1:3 ratios (6 mg) of siRNA: Metafectene complex which decreased the *MRP1* transcript level to 18.63% of initial amount.

increased significantly by the introduction of *MRP1*-targeted siRNA. siRNA treatment caused a 100 fold reversal of cellular resistance (P -value: 0.008).

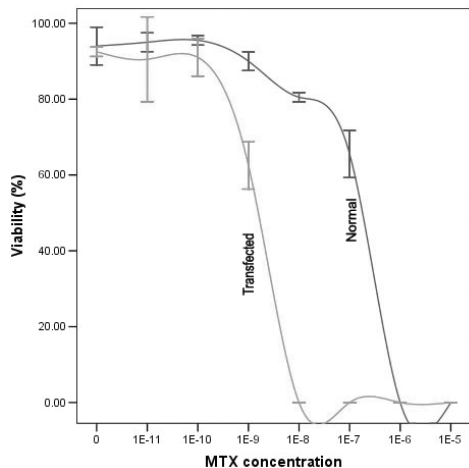


Figure 4. Effect of siRNA on drug sensitivity of human promyelocytic cells and dose-response curves of HL60 to MTX. Normal cells (blue line) are more resistance when compared to siRNA transfected cells (green line). The results are expressed as mean values of three experiments \pm SD.

DISCUSSION

The improvement of treatments using chemosensitizers to reverse the MDR phenotype has been modest due to tertiary resistance, toxicity problems and pharmacokinetic interactions (Fojo and Bates, 2003; Seiden *et al.*, 2002). Whereas the search for effective, less toxic chemosensitizers continues, molecular strategies to block the expression of *MRP1* via the use of antisense oligodeoxynucleotides (ODNs) and hammerhead ribozymes have also been proposed (Kuss *et al.*, 2002; Matsumoto *et al.*, 2004; Niewiarowski *et al.*, 2000; Osada *et al.*, 2003; Rebowski *et al.*, 2001). Although, experimental results indicated that these strategies are effective *in vitro*, their clinical use is limited due to difficulties in delivery, stability, and potency. In contrast, RNA interference is part of naturally occurring cellular machinery, and it has been widely assumed that siRNAs are more effective than conventional antisense oligonucleotides (Xu *et al.*, 2004). With our increasing understanding of the mechanism of RNAi mediated gene silencing, using siRNAs to combat drug resistance may have an impact as a therapeutic tool (Liscovitch and Lavie, 2002; Ryther *et al.*, 2005).

Recent studies have shown that transient transfection of cells with chemically synthesized siRNA directed against *MDR1* can modulate the MDR pheno-

type (Nieth *et al.*, 2003; Wu *et al.*, 2003). Although selection for drug resistance in cell lines usually results in the dominance of a special kind of ABC transporter, such a situation is not observed in clinical samples. In some cases of acute leukemia, the combined overexpression of *MDR1* and *MRP1* has been implicated in the MDR phenotype. It may be possible to use a combination of siRNAs against the repertoire of ABC transporters to combat MDR phenotype in cancer patients.

Recently, the RNAi strategy has been used to down-regulate *MRP1* expression in human aortic endothelial cells to evaluate the *MRP1* role in modulation of oxidative stress and glutathione reductase activity (Mueller *et al.*, 2005). In the present study, the modulation of the *MRP1* mediated MDR phenotype was demonstrated by siRNA-triggered RNA. Treatment of MDR cells with *MRP1* siRNA effectively down-regulated *MRP1* expression and enhanced the sensitivity to chemotherapy. This approach could be applicable in developing future strategies to reverse the MDR phenotype and increase the efficiency of chemotherapeutic drugs.

Acknowledgements

Authors wish to thank Dr. Dyzagee for donating HL60 cells. This work was supported by the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

References

- Buchner T (1997). Treatment of adult acute leukemia. *Curr Opin Oncol.* 9: 18-25.
- Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC (1992). Overexpression of a transporter gene in a multidrug resistant human lung cancer cell line. *Science.* 258: 1650-1654.
- Cole SP, Deeley RG (1998). Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. *Bioessays.* 20: 931-935.
- Lockhart AC, Tirona RG, Kim RB (2003). Pharmacogenetics of ATP-binding Cassette Transporters in Cancer and Chemotherapy. *Mol Cancer Ther.* 2: 685-698.
- Ding Y, Chan CY, Lawrence CE (2004). Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res.* 32: W135-W141.
- Fojo T, Bates S (2003). Strategies for reversing drug resistance. *Oncogene* 22: 7512-23.
- Gottesman MM, Fojo T, Bates SE (2002). Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer.* 2: 48-58.
- Henschel A, Buchholz F, Habermann B (2004). DEQOR: a web-based tool for the design and quality control of siRNAs. *Nucleic Acids Res.* 32: W113-W120.
- Itoh Y, Tamai M, Yokogawa K, Nomura M, Moritani S, Suzuki H, Sugiyama Y, Miyamoto K (2002). involvement of multidrug

- resistance-associated protein 2 in *in vitro* cisplatin resistance of rat hepatoma AH66 cells. *Anticancer Res.* 22: 1649-1654.
- Kim VN (2005). Small RNAs: Classification, Biogenesis, and Function. *Mol cells.* 19: 1-15.
- Kuss BJ, Corbo M, Lau WM, Fennell DA, Dean NM, Cotter FE (2002). *In vitro* and *in vivo* downregulation of MRP1 by antisense oligonucleotides: a potential role in neuroblastoma therapy. *Int J Cancer.* 98: 128-133.
- Liscovitch M, Lavie Y (2002). Cancer multidrug resistance: A review of recent drug discovery research. *IDrugs* 5: 349-355.
- Lockhart AC, Tirona RG, Kim RB (2003). Pharmacogenetics of ATP-binding Cassette Transporters in Cancer and Chemotherapy. *Mol Cancer Ther.* 2: 685-698.
- Loe DW, Deeley RG, Cole SP (1996). Biology of the multidrug resistance-associated protein, MRP. *Euro J Cancer.* 32: 945-957.
- Marie JP, Zhou DC, Gurbuxani S, Legrand O, Zittoun R (1996). MDR1/P-glycoprotein in haematological neoplasms. *Eur J Cancer.* 32: 1034-1038.
- Matsumoto Y, Miyake K, Kunishio K, Tamiya T, Seigo N (2004). Reduction of expression of the multidrug resistance protein (MRP)1 in glioma cells by antisense phosphorothioate oligonucleotides. *J Med Invest.* 51: 194-201.
- Mueller CF, Widder JD, McNally JS, McCann L, Jones DP, Harrison DG (2005). The role of the Multidrug Resistance Protein-1 in Modulation of Endothelial Cell Oxidative Stress. *Circ Res.* 97: 637-644.
- Nieth C, Priebisch A, Stege A, Lage H (2003). Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). *FEBS Lett.* 545: 144-150.
- Niewiarowski W, Gendaszewska E, Rebowek G, Wojcik M, Mikolajczyk B, Goss W, Soszynski M and Bartosz G (2000). Multidrug resistance-associated protein-reduction of expression in human leukaemia cells by antisense phosphorothioates oligonucleotides. *Acta Biochim Pol.* 47: 1183-1188.
- Osada H, Tokunaga T, Abe Y, Asai S, Miyachi H, Hatanaka H, Tsugu A, Kijima H, Yamazaki H, Shima K, Ueyama Y, Osamura Y, Nakamura M (2003). Reversal of drug resistance mediated by hammerhead ribozyme against multidrug resistance-associated protein 1 in a human glioma cell line. *Int J oncol.* 22: 823-827.
- Rebowek G, Wojcik M, Boczkowska M, Gendaszewska E, Soszynski M, Bartosz G, Niewiarowski W (2001). Antisense hairpin loop oligonucleotides as inhibitors of expression of multidrug resistance-associated protein 1: Their stability in fetal calf serum and human plasma. *Acta Biochim Pol.* 48: 1061-1067.
- Rots MG, Pieters R, Kaspers GJ, van Zantwijk CH, Noordhuis P, Mauritz R, Veerman AJ, Jansen G (1999). Differential methotrexate resistance in childhood T- versus common/preB-acute lymphoblastic leukemia can be measured by an *in situ* thymidylate synthase inhibition assay, but not by the MTT assay. *Blood* 93: 1067-1074.
- Ryther RC, Flynt AS, Phillips JA, Patton JG (2005). siRNA therapeutics: big potential from small RNAs. *Gene Ther.* 12: 5-11.
- Seiden MV, Swenerton KD, Matulonis U, Campos S, Rose P, Batist G, Ete E, Garg V, Fuller A, Harding MW, Charpentier D (2002). A phase II study of the MDR inhibitor biricodar (INCEL, VX-710) and paclitaxel in women with advanced ovarian cancer refractory to paclitaxel therapy. *Gynecol Oncol.* 86: 302-10.
- Sonneveld P (2000). Multidrug resistance in haematological malignancies. *J Intern Med.* 247: 521-34.
- Silva JM, Sachidanandam R, Hannon G (2003). Free energy lights the path toward more effective RNAi. *Nat Genet.* 35: 303-305.
- Truss M, Swat M, Kielbasa SM, Schafer R, Herzel H and Hagemeyer C (2005). HuSiDa-the human siRNA database: access database for published functional siRNA sequences and technical details of efficient transfer into recipient cells. *Nucleic Acids Res.* 1: 108-11.
- Verma NK, Dey CS (2004). RNA-mediated gene silencing: mechanisms and its therapeutic applications. *J Clin Pharm Ther.* 29: 395-404.
- Wu H, Hait WN, Yang JM (2003). Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. *Cancer Res.* 63: 1515-1519.
- Xu D, Kang H, Fisher M, Juliano R (2004). Strategies for Inhibition of MDR1 Gene Expression. *Mol Pharmacol.* 66: 268-275.
- Yuan B, Latek R, Hossbach M, Tuschl T, Lewitter F (2004). siRNA selection server: an automated siRNA Oligonucleotide prediction server. *Nucleic Acids Res.* 32: W130-W134.