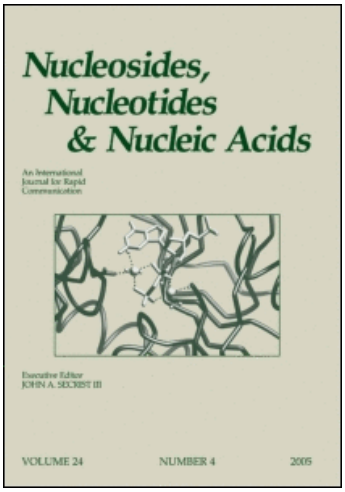

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Silencing of MDR 1 Gene in Cancer Cells by siRNA

E. B. Logashenko[a]; A. V. Vladimirova[a]; M. N. Repkova[a]; A. G. Venyaminova[a]; E. L. Chernolovskaya[a]; V. V. Vlassov[a]

[a] Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia

Online publication date: 10 July 2004

To cite this Article Logashenko, E. B. , Vladimirova, A. V. , Repkova, M. N. , Venyaminova, A. G. , Chernolovskaya, E. L. and Vlassov, V. V.(2004) 'Silencing of MDR 1 Gene in Cancer Cells by siRNA', Nucleosides, Nucleotides and Nucleic Acids, 23: 6, 861 — 866

To link to this Article: DOI: 10.1081/NCN-200026032

URL: http://dx.doi.org/10.1081/NCN-200026032

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 6 & 7, pp. 861–866, 2004

Silencing of MDR 1 Gene in Cancer Cells by siRNA

E. B. Logashenko,* A. V. Vladimirova, M. N. Repkova, A. G. Venyaminova, E. L. Chernolovskaya, and V. V. Vlassov

Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia

ABSTRACT

Inhibition of p-glycoprotein (PGP) expression and reverse of multidrug resistance (MDR) phenotype in KB-8-5 cells by synthetic 21-bp double-stranded oligoribonucleotides were investigated. siRNA constructs for the efficient down regulation of MDR1 that are active in nanomolar concentrations and cause reversal of MDR phenotype in cells were developed.

Key Words: RNA-interference; siRNA; dsRNA; P-glycoprotein; MDR1.

INTRODUCTION

The phenomenon of multiple drug resistance restricts considerably the possibilities of anticancer chemotherapy. The MDR phenotype associated with overexpression of the PGP/MDR1 gene is clinically the most significant form of multidrug resistance. The MDR1 gene encodes PGP, a transmembrane protein, which acts as an unspecific efflux-pump involved in the control of the intracellular drugs accumulation.[1]

Chemical agents capable of circumventing the MDR are known, however, the clinical use of such compounds is limited by their toxicity and poor specificity. Attempts have been made to control expression of the MDR1 gene using antisense

*Correspondence: E. B. Logashenko, Institute of Chemical Biology and Fundamental Medicine, 8 Lavrentiev Ave., Novosibirsk, 630090, Russia; E-mail: evg_log@niboch.nsc.ru.

DOI: 10.1081/NCN-200026032 1525-7770 (Print); 1532-2335 (Online)

approach. However, oligonucleotides developed failed to show the needed inhibiting activity.[2,3]

Recent studies on the regulation of gene expression led to the discovery of a potent mechanism that suppresses gene expression when double-stranded RNAs with nucleotide sequences identical to those of the corresponding genes are introduced into cells.[4] It was shown that treatment of mammalian cells by 21–22 nt RNA duplexes with two nucleotides protruding at the 3′ ends (siRNA) leads to degradation of cognate mRNAs.[5]

We investigated effect of different 21-bp double-stranded oligoribonucleotides on expression of the MDR1 gene in KB-8-5 cells in order to identify the constructs capable of efficient silencing the gene.

EXPERIMENTAL

RNA Preparation

21-Nucleotide oligodeoxyribonucleotides were synthesized using 5′-O-dimethoxytrityl-2′-O-tert-butyl-dimethylsilyl phosphoramidite chemistry and automatic synthesizer ''ASM-102U'' (Biosset, Russia). Deprotected oligoribonucleotides were purified by non-denaturing PAGE. Ribooligonucleotides (10 μM) were annealed in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7,4, 2 mM magnesium acetate) for 3 min at 90°C followed by 1 h incubation at 37°C. siRNA species were designed to target different sites within human MDR1 mRNA (accession no. M14758).

Cell Culture and Transfection

Human KB-3-1 cells were grown at 37°C in IMDM supplemented with 10% FBS and antibiotic antimycotic solution (100 units/ml penicillin, 0,1 mg/ml streptomycin and 0,25 μg/ml amphotericin) (''Sigma''). For KB-8-5 cells, 0,01 mkg/ml PMA and 300 nM vinblastine were added to the culture medium for the maintenance of the MDR phenotype. Cells were regularly passaged to maintain exponential growth. The day before transfection, cells were trypsinized, fresh medium without antibiotics was added and the cells were transferred to 24 or 96 well plates. Transfection of siRNA was carried out using Oligofectamine™ (''Invitrogen'') according to the manufacturer's protocol. MDR1 gene silencing and restoration of the KB-8-5 cells sensitivity to vinblastine were examined 48–96 h after transfection.

MTT Assay

Cells were transferred in 96-well plate, treated by siRNAs and incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air for 4 days in the presence of 300 nM vinblastine. Cell number was assessed at the end of the incubation period with a colorimetric assay based on reduction of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by living cells.[6] Absorbance of samples was measured at 570 nm using Multiscan RC (Labsystems). Results are expressed as a percentage of living cells.

Rhodamine Efflux

To investigate the accumulation of rhodamine-123 by the cells from the culture medium, the day before treatment KB-3-1 cells activated by adding 1 nM vinblastine, non-activated KB-3-1 cells and KB-8-5 cells were transferred on glass coverslips. The treatment with siRNAs was carried out as described above. 48 h after the siRNA treatment the cells were washed with PBS and incubated in the presence of 0,6 μg/ml rhodamine-123 for 15 min at 37°C. Then the cells were washed with PBS, incubated in PBS without rhodamine-123 (5% CO2, 37°C, 10 min) and fixed by 4% formaldehyde solution for 10 min. The cells were stained with 200 ng/ml Hoechst 33258 in PBS for nucleus visualization for 20 min. Then the cells were washed twice with PBS and were mounted in PBS:glycerin (1:1) buffer and examined under fluorescence microscope using Zeiss Axiophot camera with a 40 × lens and MetaMorph Imaging Software.

Efflux of fluorescent dye was measured by quantitative computerized fluorescent imaging in individual cells. Efficacy of the cells phenotype conversion was counted using the following equation:

$$R/B = \frac{Rcell/Scell - Rbgr/Sbgr}{Bcell/Scell - Bbgr/Sbgr}$$

R, red (rhodamine-123 signal); B, blue (Hoechst 33258 signal)

Rcell, Bcell, red and blue signal intensities in the cell; Scell, cell area; Sbgr, background area; Rbgr, Bbgr, background of red and blue signals intensities.

RESULTS AND DISCUSSION

We tested six siRNAs targeted to different sequences of MDR1 mRNA (Table 1).

The constructs siE, siB and siD were chosen taking into account the data of experiments with antisense oligonucleotides.[7] In these experiments oligonucleotide E demonstrated high inhibitory effect on the mRNA MDR, oligonucleotide B was less active and oligonucleotide D showed no effect (these oligonucleotides are shown in Table 1 boldface). The results were in agreement with the hybridization properties of the oligonucleotides. The constructs siU, siM and siI were chosen according to the reported set of rules for the design of siRNA.[8]

Restoration of KB-8-5 cells sensitivity to vinblastine after treatment with siRNAs was examined by MTT assay (Fig. 1). In these experiment we used concentrations of our constructs up to 150 nM. We found that the constructs siE, siM, siB and siD delivered with Oligofectamine™ induced concentration dependent enhancement of the cells sensitivity to vinblastin resulting in cell death. Constructs siI, siU and Oligofectamine™ itself showed negligible effect on cells viability. Interestingly, that oligonucleotide D did not show any antisense activity,[7] but siD was almost as effective as siE.

To investigate the effect of siRNA on the accumulation of foreign compounds in the cells, we used KB-8-5 cell line. Rhodamine-123 accumulation in cytoplasm of the cells was investigated using fluorescence microscope. Since rhodamine-123 is a substrate for PGP,[9] accumulation of this dye in the cell correlates with the drugs

Table 1. Oligoribonucleotides for silencing MDR1 gene.

siB (corresponding to 403-423 nt of the exon 1)
5′-**UUCCAAGGAGCGCGAGGUCGG**-3′
5′-GACCUCGCGCUCCUUGGAACG-3′
siE (corresponding to 598-618 nt of the exon 4)
5′-**AUCAUCCAUGGGGCUGGACUU**-3′
5′-GUCCAGCCCCAUGGAUGAUGG-3′
siD (corresponding to 557-577 nt of the exon 4)
5′-**GGCUUGACAAGUUGUAUAUGG**-3′
5′-AUAUACAACUUGUCAAGCCAA-3′
siM (corresponding to 3133-3143 nt of the exon 22)
5′-**CUUCCGAACCGUUGUUUCUUU**-3′
5′-AGAAACAACGGUUCGGAAGUU-3′
siU (corresponding to 4141-4161 nt of the 3′ UTR region)
5′-**UGCAGACUUAAUAGUGGUGUU**-3′
5′-CACCACUAUUAAGUCUGCAUU-3′
siI (corresponding to the intron 1)
5′-**GUGUCAGGCUUUCAGAUUUCC**-3′
5′-AAAUCUGAAAGCCUGACACUU-3′

accumulation and indicates the absence of p-glycoprotein activity. The results are shown in Fig. 2.

It was found that treatment of the cells with 20 nM siE, siB, siD and siM suppresses the mechanism removing the dye from the cytoplasm and results in appearance of the stained ''red'' cells. Treatment with siU and siI did not cause efflux reduction and the cells remained ''blue.''

Results of our experiments evidence that siRNA efficacy is less dependent on the target position than the activity of antisense oligonucleotides. The tested siRNA

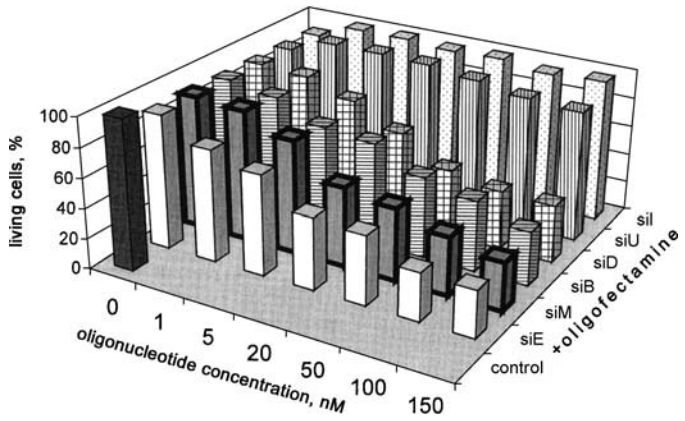

Figure 1. Restoration of KB-8-5 cells sensitivity to vinblastine. The restoration of KB-8-5 cells sensitivity to vinblastine after 96 h of incubation after treatment with different siRNAs in the presence of Oligofectamine^{TM}. Analyzing using MTT-assay.

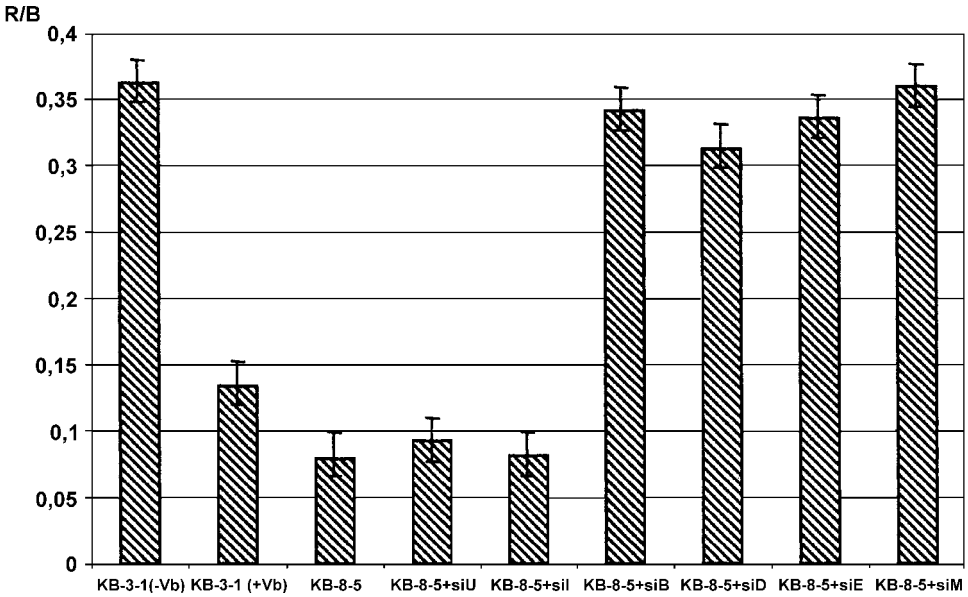

Figure 2. MDR1 phenotype conversation. The following cells were used as controls: KB-3-1 (-Vb)–with non activated MDR1 gene, KB-3-1 (+Vb)–with MDR1 gene activated by vinblastine, KB-8-5–drug resistant cell line characterized by high level of MDR 1 expression The cells phenotype conversion was analyzed using the method of rhodamine efflux. R/B—ratio of red signal of rhodamine-123 to blue signal of hoechst 33258.

constructs are active in nanomolar concentrations and cause reversal of MDR phenotype in the drug resistant cancer cells. These oligonucleotides can be considered as prototype therapeutics increasing the efficacy of anticancer drugs.

ACKNOWLEDGMENTS

This work was supported by NO-008-X1 of the U.S. Civilian Research and Development Foundation for Independent States of the Former Soviet Union (CRDF), by RFBR (grant 03-04-48550, 02-04-48550), SS-1384.2003.4, State Program Gene-Targeted Biologically Active Compounds as Antiviral and Anticancer Drugs, by Grant of Russian Ministry of education on fundamental research in the natural science No E02-6.0-51, SB RAS (Interdisciplinary grant) and RAS (program Basic Sciences to Medicine).

REFERENCES

1. Muller, C.; Goubin, F.; Ferrandis, E.; Cornil-Scharwtz, I.; Bailly, J.D.; Bordier, C.; Benard, J.; Sikic, B.I.; Laurent, G. Evidence for transcriptional control of human

mdr1 gene expression by verapamil in multidrug-resistant leukemic cells. Mol. Pharmacol. **1995**, *47*, 51–56.
2. Motomura, S.; Motoji, T.; Takanashi, M.; Wang, Y.H.; Shiozaki, H.; Sugawara, I.; Aikawa, E.; Tomida, A.; Tsuruo, T.; Kanda, N.; Mizoguchi, H. Inhibition of P-glycoprotein and recovery of drug sensitivity of human acute leukemic blast cells by multidrug resistance gene (*MDR1*) antisense oligonucleotides. Blood **1998**, *91*, 3163–3171.
3. Wang, L.; Chen, L.; Walker, V.; Jacob, T.J.C. Antisense to *MDR1* mRNA reduces P-glycoprotein expression, swelling-activated C1-current and volume regulation in bovine ciliary epithelial cells. J. Physiol. **1998**, *511*, 33–44.
4. Sharp, P.A. RNA interference–2001. Genes Dev. **2001**, *15*, 485–490.
5. Tuschl, T.; Zamore, P.D.; Lehmann, R.; Bartel, D.P.; Sharp, P.A. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. **1999**, *13*, 3191–3197.
6. Carmichael, J.; DeGraff, W.G.; Gazdar, A.F.; Minna, J.D.; Mitchell, J.B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res. **1987**, *47*, 936–942.
7. Kostenko, E.; Dobrikov, M.; Pyshnyi, D.; Petyuk, V.; Komarova, N.; Vlassov, V.; Zenkova, M. 5′-bis-Pyrenylated oligonucleotides displaying excimer fluorescence provide sensitive probes of RNA sequence and structure. Nucleic Acids Res. **2001**, *29*, 3611–3620.
8. Elbashir, S.M.; Harborth, J.; Weber, K.; Tuschl, T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods **2002**, *26*, 199–213.
9. Christians, U.; Sewing, K.-F. Cyclosporin metabolism in transplant patients. Pharmacol. Ther. **1993**, *57*, 291–345.

Received December 2, 2003
Accepted April 23, 2004