Reversion of the multiple-drug resistance phenotype mediated by short interfering RNAs

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Repression of MDR1 gene expression and restoration of cancer cell sensitivity to cytostatic agents were studied with the use of synthetic siRNAs directed to different MDR1 mRNA regions. Short interfering RNAs that enhance sensitivity of drug-resistant cells to vinblastine were revealed. The ability of various siRNAs to repress gene expression does not correlate with the efficiency of antisense oligonucleotides complementary to the same mRNA regions. An siRNA sample remained intact in a medium during a period of time sufficient to exhibit biological activity. The results of our study suggest that siRNAs can be considered as a basis for therapeutic drugs for enhancement of the efficiency of antitumor chemotherapy.

Key words: RNA interference, siRNA, double-stranded RNA, P-glycoprotein, MDR1 gene.

The multiple-drug resistance (MDR) syndrome is a major impediment to successful chemotherapy.[1] Most cases of MDR are associated with hyperexpression of the MDR1 gene coding the transmembrane protein P-glycoprotein (P-gp),[2,3] which provides ATP-dependent transport of cytotoxic agents of various structures from cells.

Inhibition of P-gp activity by antagonist agents permitted to use in medical practice is of limited utility because of their toxicity and insufficient efficacy. A possible way of overcoming MDR and increasing the efficiency of the treatment of neoplasia can be based on selective repression of MDR1 gene expression using gene-directed biologically active substances combined with conventional chemotherapy.

In a number of studies, it was demonstrated that the MDR phenotype of tumor cells can, in principle, be reversed with the use of antisense oligonucleotides complementary to particular mRNA regions of the MDR1 gene. However, the levels of inhibition of expression thus attained were insufficient for achieving the therapeutic effect.[4—6]

Recent investigations of the mechanisms of regulation of gene activity have revealed a new mechanism of repression of gene expression (RNA interference), which resides in the ability of double-stranded RNAs (dsRNAs) to cause specific degradation of mRNA targets, whose sequences are complementary to one of dsRNA strands.[7] Long dsRNAs, when entered the cells, are subject to endonuclease-catalyzed cleavage to give short (19—21 bp) double-stranded fragments[8] with two overhanging nucleotides at the 3´-ends of the strands. These short duplexes, which are called siRNAs (short interfering RNAs), involved in complexes with proteins form catalytic structures responsible for directed degradation of the complementary mRNA target.[9] Nowadays, RNA interference is extensively used to control gene expression and as a method for studying the functional genomic of eukaryotic organisms.

The mechanism of RNA interference in mammalian cells can be triggered with the use of short synthetic RNA duplexes, *viz.*, siRNAs.[10] It was demonstrated that siRNAs exhibit activity even at very low concentrations and can efficiently repress expression of particular genes.

Earlier,[11] we have demonstrated that synthetic siRNAs efficiently repress MDR1 gene expression in a human carcinoma cell culture, which opens up prospects for the design of agents enhancing the efficiency of antitumor chemotherapy.

In the present study, we examined repression of MDR1 gene expression and restoration of tumor cell sensitivity with respect to cytostatic agents using synthetic siRNAs directed to different MDR1 mRNA regions. We demonstrated that the efficiency of siRNA is virtually independent of the complementary region and its position in the mRNA structure of the MDR1 gene.

Experimental

Materials. In the present study, T4 polynucleotide kinase (Fermentas, Lithuania), acrylamide, *N*,*N*´-methylenebisacrylamide, *N*,*N*,*N*´,*N*´-tetramethylethylenediamine, tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid

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(edta) and its disodium salt, Rhodamine-123, sodium acetate, Iscove's Modified Dulbecco's Medium (IMDM), fetal calf serum, penicillin, streptomycin, amphotericin, trypsin, 3-(4,5-dimethythiazolyl)-2,5-diphenyltetrazolium bromide (MTT), fluorescent dye Hoechst-33258, anti-P-glycoprotein and anti-β-actin antibodies, *p*-coumaric acid, luminol (Sigma, USA), oligofectamine (Invitrogen, USA), and γ-[^{32}P]-ATP with a specific activity > 1 PBq mol^{-1} (Biosan, Russia) were used.

All solutions were prepared with the use of MilliQ water (Millipore, USA).

The following buffers were used: PBS buffer (1.7 m*M* KH_2PO_4, 5.2 m*M* Na_2HPO_4, pH 7.4, 150 m*M* NaCl); buffer A (30 m*M* HEPES—KOH, pH 7.4, 100 m*M* KOAc, 2 m*M* $Mg(OAc)_2$); buffer B (6 *M* urea, 0.01% Bromophenol Blue); TBE buffer (0.1 *M* Tris—boric acid, pH 8.3, 2 m*M* Na_2edta); buffer C (50 m*M* Tris, pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 100 m*M* 2-mercaptoethanol, 0.01% Bromophenol Blue); buffer D (47.9 m*M* Tris, 38.6 m*M* glycerol, pH 8.3, 10% methanol).

Synthesis of siRNA. The synthesis of 21-mer oligoribonucleotides (Table 1) was carried out by the solid-phase phosphoramidite method[12] on an automated ASM-102U synthesizer (Biosset, Russia). The oligoribonucleotides were purified by gel electrophoresis under denaturing conditions. The oligonucleotides were desalted on a Sep-Pak C18 cartridge (Millipore, USA) followed by precipitation with 96% ethanol in the presence of 0.3 *M* sodium acetate. The oligonucleotide sequences were confirmed by sequencing by Donis-Keller's method.[13]

The duplexes were prepared as follows. Two complementary oligoribonucleotides (each at a concentration of 10 μmol L^{-1}) were incubated in the buffer A at 90 °C for 3 min and then slowly cooled to 37 °C for 1 h. The formation of duplexes was monitored by electrophoresis in 20% native polyacrylamide gel (PAAG) containing the TBE buffer.

[^{32}P]-Labeling of the 5′-terminal position of siRNA. The [^{32}P]-label was introduced at position 5′ of the antisense and sense strands of the siE duplex using [γ^{32}P]-ATP and T4 polynucleotide kinase.[14] The siRNA samples had the specific activity of (1—5)·10^5 cpm pmol^{-1}.

Determination of intactness of siRNA. A reaction mixture ((10 μL) containing a [^{32}P]-labeled (50000 cpm) antisense or sense strand in siE and IMDM or IMDM containing 10% fetal calf serum (200 μL) was incubated at 37 °C for 24 h. Samples were withdrawn after 1, 4, and 24 h. The reaction was terminated by adding 10-fold volume of a 2% lithium perchlorate solution in acetone. Then RNA was isolated by centrifugation, washed with acetone (100 μL), and dissolved in buffer B. Hydrolysis products were analyzed by electrophoresis in 18% PAAG containing 8 *M* urea and the TBE buffer. The degree of cleavage was determined as the ratio of radioactivity in the band of the starting RNA to the total radioactivity of the sample applied onto the lane. The assignment of the cleavage sites was made by comparing the random cleavage products of 5′-[^{32}P]-labeled strands of the siE duplex with RNase T1 and 2 *M* imidazole.[15]

Cell lines. Experiments were carried out using KB-3-1 human epidermoid carcinoma cells (Institute of Cytology of the Russian Academy of Sciences, St.-Petersburg) and KB-8-5 cells having the multiple-drug resistance phenotype,[16,17] which were kindly provided by Professor M. Gottesman (USA). The KB-3-1 cells were cultured in IMDM containing 10% fetal calf serum, antibiotics (100 units of penicillin and 0.1 mg per milliliter of streptomycin), and the antimicotic agent amphotericin (0.25 μg mL^{-1}) in the atmosphere of 5% CO_2 at 37 °C. The KB-8-5 cells were cultured under the same conditions in the presence of 300 n*M* vinblastine. On the day before transfection, cells were placed in a 96-well plate (3000 cells in 100 μL of IMDM per well) or onto glass coverslips in a 24-well plate (10000 cells in 500 μL of IMDM per well).

Transfection of siRNA. Transfection was carried out with oligofectamine. The cultural medium was replaced with fresh serum- and antibiotic-free IMDM (80 μL per well in a 96-well plate and 300 μL per well in a 24-well plate). Oligofectamine was mixed with serum-free IMDM according to the protocol of the manufacturer of oligofectamine (0.8 μL of oligofectamine and 2.2 μL of IMDM per well in a 96-well plate, 2 μL of oligofectamine and 5.5 μL of IMDM per well in a 24-well plate) and incubated at room temperature for 5—10 min. Synthetic siRNAs were mixed with serum-free IMDM so that the concentration of siRNA ranged from 1 to 150 nmol L^{-1} after the addition to the cells (17 μL for a 96-well plate and 42.5 μL for a 24-well plate). Solutions of oligofectamine and siRNA were combined, incubated at room temperature for 20 min to form a complex, and added to the cells. The cells were incubated with siRNA in the serum- and antibiotic-free medium in the atmo-

Table 1. Types of siRNA used without repression of MDR1 gene expression (GenBank No. M14758)

Notation	Sequence	Complementary region	Functional region in MDR1 mRNA
siB	5′-UUCCAAGGAGCGCGAGGUC**GG**-3′ 3′-**GC**AAGGUUCCUCGCGCUCCAG-5′	403—423	AUG codon
siE	5′-AUCAUCCAUGGGGCUGGAC**UU**-3′ 3′-**GG**UAGUAGGUACCCCGACCUG-5′	598—618	Coding region
siD	5′-GGCUUGACAAGUUGUAUAU**GG**-3′ 3′-**AA**CCGAACUGUUCAACAUAUA-5′	557—577	Coding region
siE	5′-CUUCCGAACCGUUGUUUCU**UU**-3′ 3′-**UU**GAAGGCUUGGCAACAAAGA-5′	3133—3143	Coding region
siU	5′-UGCAGACUUAAUAGUGGUG**UU**-3′ 3′-**UU**ACGUCUGAAUUAUCACCAC-5′	4141—4161	3′ UTR Region
siI	5′-GUGUCAGGCUUUCAGAUUU**CC**-3′ 3′-**UU**CACAGUCCGAAAGUCUAAA-5′		Intron region

sphere of 5% CO_2 at 37 °C for 4 h, after which fetal calf serum was added to the concentration of 10% and the mixture was incubated at 37 °C for 72—120 h.

Determination of cell sensitivity to vinblastine (MTT test). The sensitivity of KB-8-5 line cells to vinblastine after incubation with siRNA was determined by the MTT test.[18] The cells were seeded in 96-well plates (3000 cells per well), transfected with various siRNA at concentrations varying from 1 to 150 nmol L^{-1}, as described above, and incubated in the presence of 300 n*M* vinblastine for 96 h. An MTT solution was added to the cells up to the final concentration of 0.5 mg mL^{-1} and incubated for 3 h. After removal of the medium, the formazan crystals that formed were dissolved in dimethyl sulfoxide (100 μL), and the absorption difference at 570 and 630 nm was measured on a Multiscan RC multichannel photometer (Labsystems). The results were represented as the percentage of surviving cells. The amount of cells in the control sample incubated in the presence of oligofectamine without the addition of siRNA was taken as 100%.

Measurements of P-gp functional activity. Cells of the KB-8-5 line were placed in a 24-well plate onto glass coverslips and transfected with various siRNAs, as described above. In the experiments, KB-3-1 cells, KB-3-1 cells activated with 3 n*M* vinblastine, and KB-8-5 cells that have not been treated with siRNA, were used as the control. Within 72 h after the transfections, the cells were washed with the PBS buffer and incubated in the presence of Rhodamine-123 (0.6 μg mL^{-1}) for 15 min. Then the cells were incubated in PBS containing no Rhodamine for 10 min and fixed with a 4% formaldehyde solution in PBS at 37 °C. To visualize the nuclei, the cells were stained with a solution of the fluorescent dye Hoechst-33258 in PBS (200 ng mL^{-1}) for 20 min. The cells were twice washed with PBS, the glasses from the plate were transferred to glass coverslips into a drop of a 1 : 1 PBS—glycerol solution. The cells were visualized with an Axioskop 2 Plus microscope (Zeiss, Germany) equipped with a CCD camera (Zeiss, Germany) with a magnification 400x. The data were processed using the MetaMorph Imaging Software. The relative Rhodamine content was calculated by the formula

$$R/B = \frac{R_c/S_c - R_b/S_b}{B_c/S_c - B_b/S_b},$$

where R is the intensity of the red signal of Rhodamine-123, B is the intensity of the blue signal of Hoechst-33258, R_c and B_c are the intensities of the red and blue signals, respectively, in cells, S_c is the surface area of the cell, R_b and B_b are the intensities of the red and blue signals, respectively, of the background, and S_b is the surface area of the background.

Western blot assay. To determine the amount of P-glycoprotein, we washed the cells growing in a 96-well plate (after incubation for 72 h) with PBS, subjected to lysis with buffer C, and heated at 96 °C for 5 min. The proteins were separated in 10% PAAG containing sodium dodecyl sulfate.[19] After electrophoresis, the proteins were transferred to an Immobilon-P PVDF membrane (Millipore) in buffer D using semidry electrotransfer on a Semi-Phor instrument (Hoefer, USA). To decrease nonspecific binding, the membrane was incubated (after the transfer) for ~14 h in 5% fat-free dry milk dissolved in the PBS buffer. The P-gp- and β-actin-containing bands of the membrane were sliced and incubated with the primary monoclonal antibodies anti-P-glycoprotein or anti-β-actin (in dilutions of 1 : 5000 and 1 : 10000, respectively) in 5% fat-free dry milk in PBS at room temperature for 1 h. The membrane was washed three times (for 10 min in each step) with 5% fat-free dry milk in PBS containing 0.1% Tween-20 and incubated with a conjugate of rabbit antibodies against mouse immunoglobulins coupled with peroxidase (the conjugate was kindly supplied by P. P. Laktionov, Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk) for 1 h. The membrane was washed and the protein—antibody complex was visualized by chemoluminescence. For this purpose, the membrane was incubated in a mixture of solution *1* (140 μL; 68 m*M* *p*-coumaric acid in DMSO), solution *2* (14 mL; 1.25 m*M* luminol in 0.1 *M* Tris-HCl, pH 8.5), and 30% H_2O_2 (5 μL) for 1 min and exposed together with an X-ray film for 0.5—1 min. Then the X-ray film was developed and scanned. The band intensities were evaluated using the GelPro32 program.

Results and Discussion

To study the influence of the binding region of siRNA to mRNA on the efficiency of repression of human MDR1 gene expression, we synthesized five siRNAs (see Table 1) addressed to different mRNA regions and then tested them in experiments with epidermoid carcinoma KB-8-5 cells. We used siRNA homologous to the intron region of the MDR1 gene (siI) as the control; siE, siB, and siD served as homologous MDR1 mRNA regions. Interactions of the latter with antisense oligonucleotides have been examined in earlier studies,[6,20] where the antisense oligonucleotides E and B (complementary to the coding region and the region of the AUG codon of MDR1 gene mRNA) were demonstrated to decrease the level of expression of MDR1 mRNA in KB-8-5 cells by 90 and 40%, respectively, whereas the oligonucleotide D complementary to the coding region did not cause any change in the level of this mRNA. The nontranslated region of MDR1 gene mRNA served as a target for siU, because studies of some genes[21] demonstrated that it is this region that is most sensitive to siRNA. We chose siM directed to the coding region of mRNA using the method developed[22] for the design of siRNA.

The efficiency of repression of MDR1 gene expression with siRNA was estimated from restoration of sensitivity of KB-8-5 cells treated with siRNA to vinblastine. The cell sensitivity to vinblastine was determined by the MTT test, which is based on the ability of living cells to convert a solution of the tetrazolium salt (MTT) into intensely colored insoluble formazan and allows one to estimate the amount of living cells in samples by spectrophotometry.[18]

Incubation of cells in the presence of vinblastine for 72 h after treatment with siRNA does not noticeably change cell viability, which is associated apparently with the fact that the lifetime of P-glycoprotein is 42—72 h

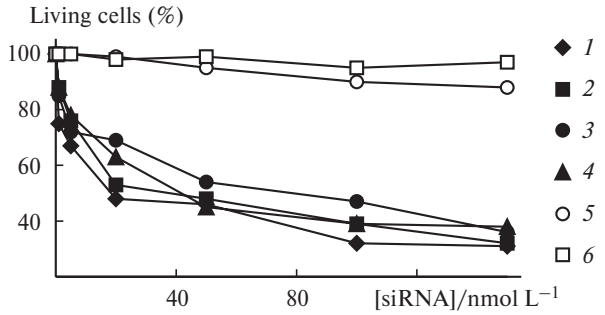

Fig. 1. Changes in the sensitivity of KB-8-5 cells to vinblastine under the action of siRNAs: siE (*1*), siM (*2*), siB (*3*), siD (*4*), siU (*5*), and siI (*6*). The KB-8-5 cells were treated with siRNAs and incubated in the presence of 300 n*M* vinblastine for 96 h. The number of living cells in the control experiment was taken as 100%; the cells were incubated with oligofectamine in the absence of siRNA. The experimental error was no higher than 10%. The average values from three independent experiments are given.

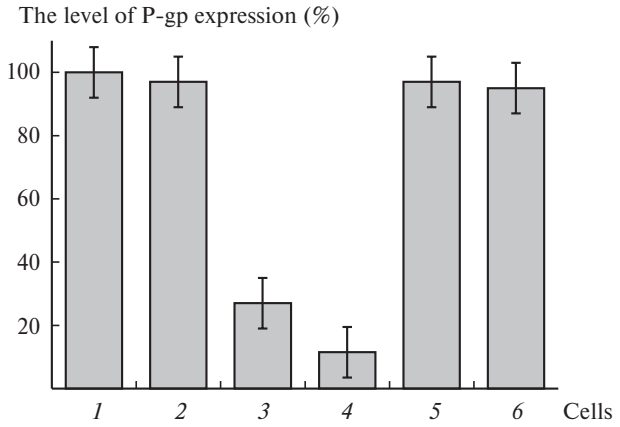

Fig. 2. Analysis of the P-glycoprotein content of cells transfected with various siRNAs by Western blot hybridization: *1*, untreated cells; *2* cells incubated in the presence of oligofectamine; 5 (*3*) and 20 nM siE (*4*); 5 (*5*), and 20 nM siU (*6*).

(depending on the cell line).[23] However, when cells were incubated for 96—120 h in a medium with vinblastine, the degree of cell death was observed to depend on the concentration of siE, siM, siB, or siD as a result of an increase in the cell sensitivity to vinblastine (Fig. 1). For siE and siM, the amount of living cells decreases substantially already at the duplex concentration of 20 nmol L^{-1} (50% of living cells after 96 h). The amount of living cells decreases to 30% of the control value as the concentration of these siRNAs increases to 150 nmol L^{-1}, whereas an increase in the time of cell incubation in the presence of vinblastine to 120 h after treatment with siE, siB, siM, or siD at a concentration higher than 5 nmol L^{-1} leads to death of all cells (preliminary data are not reported). The siI construction (control siRNA homologous to the intron of the MDR1 gene) and the siU construction (homologous to the 3′-notranslated region of MDR1 gene mRNA) have no effect on cell viability in the presence of vinblastine even upon prolonged incubation. Control experiments demonstrated that all the siRNAs under study are nontoxic for cells and do not decrease their viability in the absence of vinblastine.

Therefore, four of six siRNAs under study (siE, siD, siB, and siM) restore sensitivity of drug-resistant human tumor cells to vinblastine, restoration of sensitivity occurring with similar efficiency for all four siRNAs. Consequently, the ability of different siRNAs to repress gene expression does not correlate with the efficiency of action of antisense oligonucleotides complementary to the same mRNA regions.

The specificity of repression of MDR1 gene expression with siRNAs was confirmed by determining the amount of the product of this gene, *viz.*, P-glycoprotein, in cells treated with oligonucleotide constructions. The amount of P-gp was determined by the Western blot assay using the protein β-actin as the internal control. As can be seen from Fig. 2, the amount of P-glycoprotein decreases to 28 and 17% already in 72 h after treatment of cells with the siE construction at a concentration of 5 and 20 nmol L^{-1}, respectively. At the same time, treatment of cells with oligofectamine or with siU in the presence of oligofectamine has no effect on the amount of P-glycoprotein in cells. In the samples under study, the amount of β-actin remained constant, which is indicative of the specificity of repression of P-glycoprotein expression with siRNAs.

The efficiency of expression of target gene expression and the duration of the effect depend substantially on the intactness of siRNA in biological media. We studied the kinetics and character of degradation of siRNA in a culture medium containing 10% serum and in the absence of the serum. For this purpose, the [^{32}P] label was introduced at position 5′ of the antisense and sense strands of siE. Duplexes consisting of one labeled and one unlabeled strands were incubated in a medium in the presence or in the absence of the serum. Aliquots were withdrawn at definite intervals of time, and total RNA was isolated and analyzed in 18% PAAG under denaturing conditions. In a serum-free medium, siRNAs remained intact over a rather long period of time. After incubation for 24 h, at least 95% of the starting duplex remained intact (Fig. 3, *a*). In the presence of 10% serum, siRNA is less stable. However, at least 77% of the starting duplex remained intact even under these conditions after incubation for 4 h. Investigation of degradation products of the antisense and sense strands clearly demonstrated that degradation is caused primarily by endonucleases rather than exonucleases (which is in contradiction with earlier data[24]), *i.e.*, no predominant cleavage of the overhanging ends of the ribo-duplex occurs. To the contrary, particular sites in each strand are specifically cleaved. Therefore, stability of

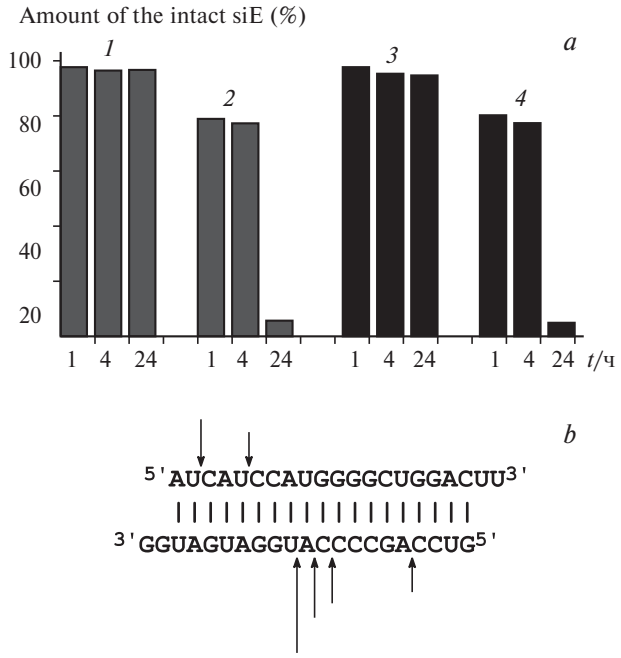

Fig. 3. *a*. Intactness of siE in IMDM: *1* and *2*, the amounts of the intact [^{32}P]-labeled sense strand in siE; *3* and *4*, the amount of the intact [^{32}P]-labeled antisense strand in siE; *1* and *3*, after incubation in IMDM; *2* and *4*, after incubation in IMDM containing 10% fetal calf serum. *b*. Main regions of cleavage of the siE construction upon incubation in IMDM containing 10% fetal calf serum at 37 °C. The arrow length is proportional to the degree of RNA cleavage at this phosphodiester bond.

duplexes in biological media is determined by the oligoribonucleotide sequence and can substantially influence the induced biological effects.

The MDR1 gene, *viz.*, the mediated MDR phenotype, is characterized by efficient P-glycoprotein-mediated transport of cytotoxic agents from cells. Reversion of the MDR phenotype was tested from the accumulation of Rhodamine-123 in cells, because this dye is a P-glycoprotein substrate.[25] Cells containing active P-glycoprotein translocate Rhodamine-123 from the cytoplasm into the intercellular space. If MDR1 gene expression is repressed and P-glycoprotein is absent, Rhodamine-123 is accumulated in the cytoplasm. Cells of the following lines that were not treated with siRNA were used as the control: KB-8-5 cells characterized by a high level of MDR1 expression, drug-sensitive KB-3-1 cells, and KB-3-1 cells, in which the MDR1 gene was activated by incubation in the presence of vinblastine (3 nmol L^{-1}). Cells were treated with siRNA and incubated with Rhodamine-123 over a period of time sufficient for accumulation of the dye in the cells. Then the cells were washed, fixed, and stained. The cell nuclei were visualized with the fluorescent dye Hoechst-33258, which stains nucleic acids blue. The photographs of the samples taken with the use of a luminescence microscope are shown in Fig. 4. The results of our study demonstrated that treatment of KB-8-5 cells with siE and siD at a concentration of 20 nmol L^{-1} represses translocation of Rhodamine-123 from the cytoplasm, and the cells remain red, *i.e.*, acquire the drug-sensitive phenotype (see Fig. 4, *d*, *e*). Analogous data were obtained for KB-8-5 cells treated with siB and siM. Treatment of cells with 20 n*M* siU and siI duplexes does not influence dye transport and the cells remain blue, *i.e.*, retain the drug-resistant phenotype (see Fig. 4, *f*).

The data obtained using a luminescence microscope were processed with the use of the MetaMorph Imaging

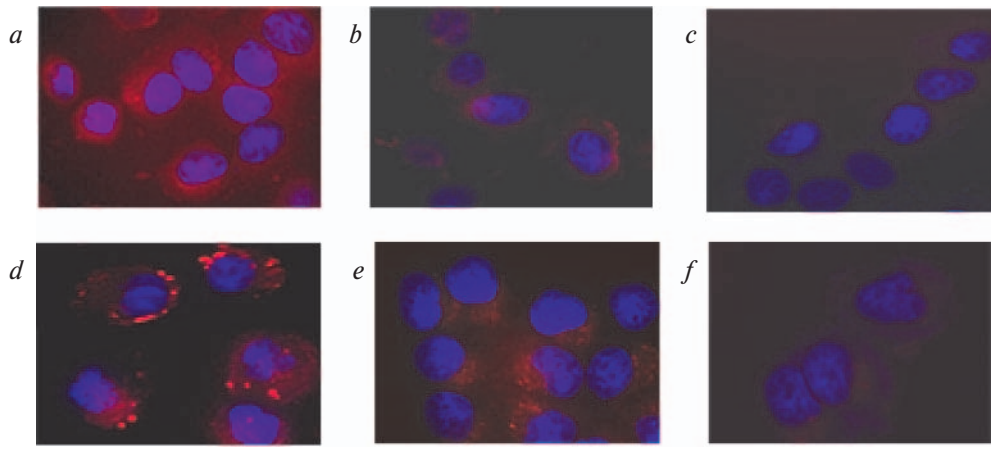

Fig. 4.* Accumulation of Rhodamine-123 in cells; photographs of cells under a fluorescence microscope field, 400x magnification; nuclei stained with the dye Hoechst-33258 are shown in blue, Rhodamine-123 accumulated in the cytoplasm is shown in red; *a*—*c*, photographs of control samples: KB-3-1 cells cultured in a medium in the absence of vinblastine (KB-3-1 (–Vb), *a*) and activated with 3 n*M* vinblastine (KB-3-1 (+Vb), *b*) KB-8-5 cells (*c*); *d*, *e*, and *f*, photographs of samples of KB-8-5 cells treated with 20 n*M* siE, siD, and siU, respectively, after incubation for 72 h.

* Figure 4 is available in full color in the on-line version of the journal (http://www.springeronline.com) and on the web site of the journal (http://russchembull.ru).

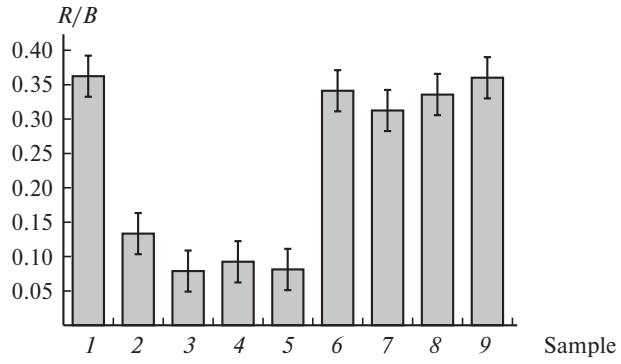

Fig. 5. Quantitative data obtained by analyzing the image (see Fig. 4) with the use of the MetaMorph Imaging Software (see the Experimental): KB-3-1 (–Vb) (*1*), KB-3-1 (+Vb) (*2*), KB-8-5 (*3*), KB-8-5+siU (*4*), KB-8-5+sil (*5*), KB-8-5+siB (*6*), KB-8-5+siD (*7*), KB-8-5+siE (*8*), and KB-8-5+siM (*9*).

Software (see Experimental). The ratio of the red signal (Rhodamine-123) to the blue signal (Hoechst 33258) normalized to the surface area of cells was used as the quantitative characteristic of the efficiency of dye accumulation in cells. For each sample, no less than ten cells were calculated. As can be seen from Fig. 5, drug-sensitive KB-3-1 cells accumulate 4.5-fold larger amount of the dye than drug-resistant KB-8-5 cells. The action of 3 n*M* vinblastine on KB-3-1 cells leads to activation of dye transport from cells and the amount of the dye in the cells in only one-and-a-half times larger than that in KB-8-5 cells. Treatment of KB-8-5 cells with siRNAs (20 n*M* siE, siB, siD, or siM) efficiently blocks the dye transport from cells and reverses the MDR phenotype, as evidenced by Rhodamine-123 accumulation in these cells. A comparison of the quantitative data demonstrated that drug-resistant KB-8-5 cells treated with siRNA constructions accumulate the dye with the same efficiency as KB-3-1 cells that were not treated with cytostatic agents. Therefore, the dye and drug transport from cells is efficiently repressed with siRNA.

Nowadays, extensive experimental data on repression of various gene expression in mammalian cells with siRNA are available. Analysis of the published data shows that the levels of repression of gene expression and the required siRNA concentrations vary substantially for different RNA targets and cells. Efficient siRNAs are, as a rule, identified by an experimental consideration of a series of sequences. The effect of the position of the target sequence in mRNA on the efficiency of action of siRNA remains an open question. There are contradictory data on the influence of the efficiency of RNA interference on the structure of the RNA target and/or the ability of an interfering ds-oligoribonucleotide to form a complementary complex with the target and the dependence of interference on other factors, for examples, on intactness of oligoribonucleotides in media and serums or the efficiency of the formation of a RISC complex with different-sequence siRNAs.

In the present study, we demonstrated that the MDR phenotype can be reversed with short double-stranded RNAs (siRNAs). All the siRNAs under study, which are directed to the coding region of the AUG codon of the MDR1 mRNA, repress MDR1 gene expression and restore cell sensitivity to vinblastine, the activity being observed at a concentration higher than 20 nmol L^{-1}. We demonstrated that the technology of repression of gene expression with siRNA imposes less stringent requirements upon the choice of the target sequence in mRNA and hybridization properties of the ds-oligoribonucleotides used compared to the antisense approach. Efficient repression (30—60%)[26—28] of MDR1 gene expression with antisense oligonucleotides is observed with the use of oligonucleotides at high concentrations (10—50 μmol L^{-1}) and/or the introduction of chemical modifications of different nature (thiophosphate,[26,27] 2′-*O*-methyl units,[26,28] or bis-pyrenyl groups[6]) into an antisense oligonucleotide. Rather efficient inhibition with oligonucleotides has been achieved only in one study,[6] where the level of MDR1 mRNA was decreased by 90% with the use of an analog of an antisense oligonucleotide (at a concentration of 50 μmol L^{-1}) complementary to the same mRNA region as that of siE used in the present study.

Of all the known anti-MDR siRNAs, the siRNAs examined in the present study proved to be most efficient. Earlier,[29] the influence of siRNA (homologous to the region of the nucleotide sequence 79—99 of the MDR1 gene mRNA) on MDR1 gene expression in MCF-7/BC19, MCF-7/AdrR, and A2780Dx5 cells has been studied, and partial restoration of cell sensitivity to cytostatic agents, a slight decrease in the mRNA level, and a decrease in the P-glycoprotein level by 45% have been documented. However, this is a short-time effect. The maximum decrease in the RNA level was observed 24 h after the addition of siRNA. After 72 h, the initial RNA level is restored. In the study,[30] siRNAs (nucleotide sequences 503—523 and 3050—3070) were used to inhibit expression of the endogenous and exogenous MDR1 genes in EPG85-257RDB and EPP85-181RDB cells. Treatment of cells with siRNAs at a concentration of 100 nmol L^{-1} led to reversion of the MDR phenotype by 87—79 and 48—58% in the case of endogenous and exogenous MDR1, respectively. Therefore, attempts to achieve efficient repression of endogenous MDR1 gene expression failed. These differences in the level of repression of MDR can be associated with both the characteristic features of various cell lines and the properties of the siRNAs used.

It is known that RNA duplexes are labile and undergo gradual degradation in a medium, particular, in the presence of a serum. The results of the present study demonstrated that siRNAs remain intact in a medium for at least 4 h, which is sufficient to exhibit biological action (see Fig. 3). Unlike antisense oligonucleotides whose degradation occurs primarily with the involvement of 3′-exonucleases, siRNAs are subject to endonuclease cleavage, which occurs predominantly at the phosphodiester bonds in the CA, AU, CC, and UC sequences. Chemical single-site modifications aimed at enhancing resistance of the identified sites to ribonucleases can substantially increase stability of siRNA in cells and, consequently, the time of repression of gene expression.

The siRNAs under consideration decrease the endogeneous P-glycoprotein level in cells to 17% of the initial level 72 h after treatment (see Fig. 2). Our data suggest that this decrease in the P-glycoprotein level is sufficient to cause cell death in the presence of vinblastine at previously tolerable concentrations. Consequently, the observed decrease in the P-glycoprotein level in cancer cells can be considered as therapeutically significant.

In many tumors, the multiple-drug resistance phenotype appears through several mechanisms. The question arises as to whether it is sufficient to block one of the transport paths of cytotoxic agents from cells for achieving the therapeutic effect. In KB-8-5 cells, MDR1 gene hyperexpression is accompanied by a high level of MRP gene expression, whose product also serves as a transmembrane pump with similar substrate specificity and can cause multiple-drug resistance.[31] We demonstrated that repression of P-glycoprotein expression with siRNA in drug-resistant KB-8-5 cells leads to accumulation of Rhodamine-123 (which serves as a substrate for both P-glycoprotein and the MRP protein) at the same concentration as that observed in drug-sensitive KB-3-1 cells. Expression of one MRP protein is insufficient for efficient transport of the dye and cytostatic agents and maintenance of the MDR phenotype. Therefore, repression of P-glycoprotein-mediated dye and drug transport provides accumulation of drugs and dyes in cells and restoration of sensitivity of these cells to chemodrugs even if there are additional ways of providing multiple-drug resistance.

The results of our study demonstrated that siRNAs, which we have constructed, can be considered as a basis for therapeutic drugs for enhancement of efficiency of cancer chemotherapy.

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