Suppression of *MDR1* gene expression by chemically modified siRNAs

E. B. Logashenko, A. V. Vladimirova, A. A. Volkov, M. N. Repkova, A. G. Ven´yaminova, M. A. Zenkova, E. L. Chernolovskaya, and V. V. Vlassov*

Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy or Sciences, 8 prosp. Akad. Lavrent´eva, 630090 Novosibirsk, Russian Federation. Fax: +7 (383) 333 3677. E-mail: evg_log@niboch.nsc.ru

The ability of chemically modified siRNAs targeted to *MDR1* mRNA to inhibit P-glycoprotein expression and to restore sensitivity of cancer cells to antibiotic vinblastine was investigated. The effects of chemical modifications on RNA stability in cell culture medium and inhibition of *MDR1* gene expression were tested. We found that siRNAs containing 2´-*O*-methyl ribonucleotides within either sense or/and antisense strands display high stability in serum but exhibit a significant reduction in the biological activity. The protection of 3´-ends of siRNA by introduction of 3´—3´-inverted phosphodiester bonds and two 2´-*O*-methyl ribonucleotides in protruding 3´-ends considerably increase their biological activity, which allows a 30-fold decrease in the cytostatic agent concentration required for cancer cell death. The data obtained demonstrate that the chemically modified siRNAs can be considered as potential therapeutics, which enhances the efficiency of cancer chemotherapy.

Key words: RNA interference, siRNA, dsRNA, P-glycoprotein, *MDR1*, multidrug resistance.

The *MDR1* gene encodes P-glycoprotein, an ATP-dependent transmembrane protein, which acts as a membrane pump and efficiently decreases the intracellular concentrations of different compounds. This gene is an important therapeutic target, as its overexpression is associated with most cases of multiple-drug resistance (MDR) in cells, which is a a possible cause of chemotherapy-based treatment failure.[1,2] Therefore, studies aimed at the development of agents for selective repression of either P-glycoprotein activity or *MDR1* gene expression have been actively carried out in recent years. A direct approach to inhibition of *MDR1* expression may be based on a decrease in the amount of its mRNA in a cell induced by small interfering RNAs, which act by an RNA-interference mechanism. Small interfering RNAs (siRNAs) are short (containing 19—21 bp) double-stranded RNA fragments[3] with two overhanging nucleotides at the 3´-ends of the strands. In the cells, they are involved in a multiprotein complex called RISC (RNA-induced silencing complex), which is responsible for directed degradation of the complementary mRNA target.[4]

An insufficient duration of the interference effect caused by the siRNA degradation under the action of cell and serum ribonucleases is an important obstacle holding up the use of siRNA for inhibition of expression of therapeutically significant genes. As a rule, after a single transfection of siRNA into a cell, a decrease in the concentration of the mRNA target in the cell persists for 2—4 days, after which the mRNA level is gradually restored.[5—7] The introduction of chemical modifications can increase the nuclease resistance of siRNA and the duration of its biological effect. A comparison of the efficiency of chemically modified siRNAs revealed the factors that ensure their activity: (1) to retain the biological activity, the modified oligoribonucleotide should maintain the A-form of the helix and the major groove structure,[8] (2) the 2´-OH group is not necessary for the biological activity,[9] and (3) the 5´-end of the antisense strand of the duplex should not be modified.[9]

Previously,[10] we have studied restoration of sensitivity of the drug-resistant human cancer KB-8-5 cell line to vinblastine upon treatment with siRNAs directed to different regions and identified a number of anticancer siRNAs. The efficiency of action of siRNA was shown to depend to a lesser extent on the position of the target sequence in the mRNA than that of antisense oligonucleotides.

In this work we studied the inhibition of *MDR1* gene expression and restoration of sensitivity of cancer cells to the citostatic by means of chemically modifiied siRNAs. It was shown that protection of the 3´-ends of siRNA by the 3´—3´-(inverted) phosphodiester bond and introduc-

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 7, pp. 1227—1235, July, 2006.

1066-5285/06/5507-1275

tion of two 2′-*O*-methyl units into the 3′-overhanging ends essentially increase the biological activity of siRNA (as compared with the nonmodified analog) and duration of its action, which allows a 30-fold decrease in the cytostatic concentration needed to kill cancer cells. The introduction of modifications into the central region of siRNA and/or complete modification of one of the strands resulted in a pronounced repression of their biological activities.

Experimental

The following chemicals were used: acrylamide, *N*,*N*′-methylenebisacrylamide, TEMED, tris(hydroxymethyl)aminomethane (Tris), EDTA, sodium acetate, IMDM medium (Iscove's Modified Dulbecco's Medium), fetal calf serum, penicillin, streptomycin, amphotericin, trypsin, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide, mouse antibodies to human P-glycoprotein and β-actin, *p*-coumaric acid, luminol (Sigma, USA), Oligofectamine (Invitrogen, USA), *N*-protected 5′-*O*-dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilylribonucleoside-3′-*N*,*N*-diisopropyl(2-cyanoethyl) phosphoramidite, 5′-*O*-dimethoxytrityl-2′-*O*-methylribonucleoside-3′-*N*,*N*-diisopropyl(2-cyanoethyl) phosphoramidites, nucleoside-bound polymer carriers (Glen Research, USA), and reagents for oligonucleotide synthesis and deprotection (Merck, Germany and Sigma, USA). All solutions were prepared using MilliQ water (Millipore, USA).

Synthesis of siRNAs. Oligoribonucleotides were synthesized by the phosphoramidite method on an automated ASM-102U synthesizer (Biosset, Russia) on a 0.2 μmol scale by a procedure[11] optimized for the given synthesizer. The attachment of 3′-*O*-dimethoxytritylthymidine[12] to the polymer carrier CPG-500 (Sigma, USA) containing carboxy groups was carried out as described previously.[13] A mixture of a 0.1 *M* solution of *N*-methylimidazole and a 0.45 *M* solution of tetrazole in acetonitrile was used as the activating reagent for oligonucleotide synthesis.[14] The condensation time was 10—20 min. After completion of the synthesis, cleavage of the oligonucleotides from the polymer and deprotection of the heterocyclic bases were carried out with a mixture of concentrated NH_4OH and ethanol (3 : 1) at 56 °C for 16 h. The 2′-*O*-silyl groups were removed by treatment with a triethylamine trihydrofluoride—*N*-methyl-2-pyrrolidinone—triethylamine mixture (4 : 6 : 3) at 65 °C for 1 h. The deprotected oligonucleotides were isolated by electrophoresis in 20% native polyacrylamide gel under denaturing conditions. After elution from the gel, oligonucleotides were desalted on a Sep-Pac C18 cartridge (Millipore, USA) and precipitated as Li^+ salts. The structures of the isolated oligonucleotides were confirmed by MALDI-TOF mass spectra (REFLEX III, Bruker Daltonics, Germany).

The duplexes were prepared as follows. Two complementary oligoribonucleotides at a concentration of 10^{-5} mol L^{-1} were incubated separately in buffer A (30 *mM* HEPES-KOH, pH 7.4, 100 *mM* potassium acetate, 2 *mM* magnesium acetate) for 3 min at 90 °C and then slowly (over a period of 1 h) cooled to 37 °C. The formation of duplexes was monitored by electrophoresis in 20% native PAAG containing a TBE buffer (0.1 *M* Tris—boric acid, pH 8.3, 2 *mM* Na_2EDTA).

^{32}P-Labeling of oligoribonucleotides. The ^{32}P-labeling of oligoribonucleotides was carried out by phosphorylation of the 5′-OH group with [γ^{32}P]-ATP and T4 polynucleotide kinase.[15] A mixture (10 μL) containing [γ^{32}P]-ATP (0.1 mQi), T4 buffer (50 *mM* Tris-HCl, pH 7.6, 10 *mM* $MgCl_2$, 1 *mM* DTT, 0.1 *mM* spermidine), 0.01 optical units of the A_{260} oligoribonucleotide, and 10 activity units of T4 polynucleotide kinase was incubated for 1 h at 37 °C. Using 8% denaturing PAAG electrophoresis, [5′-^{32}P]-RNA was isolated. After completion of the electrophoresis, the band of the labeled oligoribonucleotide was sliced and eluted from the gel (by passive elution) with 0.3 *M* sodium acetate, pH 5.5, and precipitated with ethanol. The RNA precipitate was dissolved in water and stored at −20 °C. The specific activity of [5′-^{32}P]-RNA was 2 mCi $nmol^{-1}$.

Random cleavage of oligoribonucleotide with 2 *M* imidazole.[16] A reaction mixture (20 μL) containing [5′-^{32}P]-oligoribonucleotide (6 nmol L^{-1}) and buffer L (2 *M* imidazole, pH 7.0, 1 *mM* EDTA, 250 μg mL^{-1} of the total *E.coli* tRNA) was incubated at 90 °C for 50 min. After completion of the reaction, RNA was precipitated with 2% lithium perchlorate (200 μL) in acetone. The RNA precipitate was separated by centrifugation (13000 rpm, 10 min, 4 °C), washed, dried, and dissolved in buffer B (8 *M* urea, 0.025% Bromophenol Blue, 0.025% Xylene Cyanol).

Oligoribonucleotide cleavage with ribonuclease T1 under denaturing conditions.[17] A reaction mixture (27 μL) containing [5′-^{32}P]-oligoribonucleotide (3 nmol L^{-1}) and buffer T1 (6 *M* urea, 25 *mM* sodium citrate, pH 4.5, 1 *mM* EDTA, 250 μg mL^{-1} of total *E.coli* tRNA) was incubated at 50 °C for 10 min. The initial mixture was separated into three portions, and RNase T1 was added to each portion (2, 4, and 6 activity units, respectively). The mixtures were incubated again at 50 °C for 10 min. The reaction was stopped by adding TBE buffer (1 μL) in a tenfold concentration. The samples were frozen and stored before analysis at −20 °C.

Determination of siRNA nuclease resistance in IMDM containing 5% fetal bovine serum (FBS). A reaction mixture (200 μL) containing IMDM, 5% FBS, and [5′-^{32}P]-siRNA (3 nmol L^{-1}) was incubated at 37 °C; aliquot portions of the mixture being taken at regular intervals. The aliquots were mixed with an equal volume of buffer B and quickly frozen. The samples were stored at −20 °C. Prior to application on 18% denaturing PAAG, the samples were incubated at 65 °C for 5 min. The degree of cleavage was determined as the ratio of the radioactivity in the band of the starting siRNA to the total radioactivity of the sample applied on a gel lane. The cleavage sites were assigned by comparing the cleavage products in the FBS medium with the random cleavage with RNAse T1 and in a 2 *M* imidazole buffer.

Cell lines. Cells of the KB-8-5 line possessing the multiple-drug resistance phenotype[18,19] kindly provided by Professor M. Gottesman (NIH, USA) were used. The KB-8-5 cells were cultured in IMDM containing 10% FBS, antibiotics (penicillin, 100 units mL^{-1}, and streptomycin, 0.1 mg mL^{-1}), and the antimicotic amphotericin (0.25 μg mL^{-1}), in the atmosphere of 5% CO_2 at 37 °C in the presence of vinblastine (300 nmol L^{-1}). On the day before transfection, the cells were planted in a 96-well plate (3000 cells in 100 μL of the medium per well).

Transfection of siRNA. Transfection of siRNA into the KB-8-5 cells was carried out with Oligofectamine. Prior to transfection, the culture medium was replaced by a fresh serum- and antibiotic-free IMDM (80 μL per a 96-well plate). Oligo-

fectamine was mixed with IMDM (0.8 μL of Oligofectamine to 2.2 μL of IMDM per well) and incubated for 10 min at room temperature. Synthetic siRNA was mixed with IMDM (17 μL of the solution per well) so that the concentration of siRNA upon the addition to the cells ranged from 5 to 100 nmol L^{-1}. Solutions of Oligofectamine and siRNA in the medium were combined, incubated at room temperature for 20 min to form a complex, and added to the cells (20 μL of the solution per well of a 96-well plate). Transfection was carried out at 37 °C for 4 h in the atmosphere of 5% CO_2; then 50 μL of IMDM containing 30% FBS was added in each well and these were incubated at 37 °C for 72—120 h.

Determination of cell sensitivity to vinblastine (MTT test). The restoration of sensitivity of KB-8-5 cells to vinblastine after incubation with siRNA was determined by the MTT test.[20] The cells were seeded into 96-well plates (3000 cells per well) and transfected with various siRNAs at concentrations from 5 to 100 nmol L^{-1}, as described above. One day after transfection, vinblastine was added to the cells up to a concentration of 10—300 nmol L^{-1} and the mixture was incubated at 37 °C for 1—5 days in the atmosphere of 5% CO_2. After incubation, a solution of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was added up to the final concentration of 0.5 mg mL^{-1} and the mixture was incubated for additional 3 h under the same conditions. After removal of the medium, the formazan crystals formed in the cells were dissolved in dimethyl sulfoxide (100 μL) and the optical density was determined on a Multiscan RC multichannel photometer (Labsystems, Finnland) as the absorption difference at 570 and 630 nm. The data were represented as the percentage of surviving cells with respect to the control. The amount of cells in the control sample incubated in the presence of Oligofectamine without the addition of siRNAse was taken as 100%.

Western blot assay. To determine the amount of P-glycoprotein, the cells growing in the 96-well plate incubated for 1—8 days in the presence of siRNA (20 nmol L^{-1}) in a cytostatic-free medium were washed with PBS (1.7 *mM* KH_2PO_4, 5.2 *mM* Na_2HPO_4, pH 7.4, 150 *mM* NaCl) lysated with buffer C (50 *mM* Tris, pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 100 *mM* β-mercaptoethanol, 0.01% Bromophenol Blue), and heated at 96 °C for 5 min. The proteins were separated in 10% PAAG containing sodium dodecylsulfate.[21] After electrophoresis, the proteins were transferred to a Immobilon-P PVDF membrane (Millipore, USA) in buffer D (47.9 *mM* Tris, 38.6 *mM* glycine, pH 8.3, 10% methanol) using semidry electrotransfer on a Semi-Phor instrument (Hoefer, USA). To reduce nonspecific binding, the membrane was incubated after the transfer for ~16 h in 5% fat-free dry milk dissolved in PBS buffer. The membrane bands containing P-glycoprotein and β-actin were sliced and incubated with the primary monoclonal mouse antibodies anti-human 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% solution of fat-free dry milk in PBS containing 0.1% Tween-20 and incubated for 1 h with a conjugate of rabbit antibodies against mouse immunoglobulins coupled with peroxidase (the conjugate was kindly provided by P. P. Laktionov, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the RAS). The membrane was washed, and the protein—antibody complex was visualized by chemiluminescence. To this end, the membrane was incubated for 1 min in a mixture consisting of solution 1 (68 *mM* *p*-coumaric acid in DMSO) (140 μL), solution 2 (1.25 *mM* luminol in 0.1 *M* Tris-HCl, pH 8.5) (14 mL), and 30% H_2O_2 (5 μL) and exposed together with an X-ray film for 0.5—1 min. The film was developed and scanned and band intensities were evaluated using the GelPro32 program.

Results and Discussion

To study the effect of chemical modifications of siRNA on the efficiency and duration of repression of human *MDR1* gene expression, a series of modified siRNAs homologous to the 598—618 nucleotide sequence of mRNA of the *MDR1* gene were synthesized. The siRNAs contained different numbers of 2′-*O*-methylribonucleotides (B^m) and/or 3′—3′-inverted phosphodiester bonds (below referred to as 3′—3′-inversion) on the ends for the protection of oligoribonucleotides from endonucleases, for example, 3′—3′-inverted thymidine (Tinv) (Fig. 1).

In order to study the degradation of natural and modified siRNAs in a culture medium containing 5% FBS, siRNAs Em, Em/E, E, Ei/E, and Ei [5′-^{32}P]-labeled at the antisense strand (siRNA consisted of the nonlabeled sense and labeled antisense strands) were incubated in the presence of a serum at 37 °C. Aliquots were withdrawn at certain time intervals. The siRNA cleavage products were analyzed by electrophoresis in 18% PAAG under denaturing conditions. In the serum-containing medium, siRNA undergoes fast degradation; typical cleavage products appear even in the first 3 min of incubation (Fig. 2, *a*). Cleavage of siRNA in the serum-containing medium is not random. For each of the siRNA studied, a certain set of cleavage products is formed (Fig. 2). For siRNA Ei, the major sites of cleavage by nucleases are the following: C^3pC^4, C^4pA^5, $C^{10}pA^{11}$, and $U^{12}pG^{13}$ (see Fig. 2, *a*); no cleavage at other sites was observed even on long-term incubation (24 h).

The highest stability was exhibited by siRNA Em: even upon incubation for 24 h, at least 95% of the starting material remsin intact (see Fig. 2, *b*, curve *1*). siRNA Em/E consisting of fully 2′-*O*-methylated sense and nonmodified antisense strands was less stable (see Fig. 2, *b*, curve *2*): after incubation for 5 min, about 64% of this siRNA remained intact, while by the end of incubation, the amount of siRNA Em/E decreased to 24%.

Higher susceptibilities to the action of serum nucleases were found for siRNAs E, Ei/E, and Ei (see Fig. 2, *b*, curves *3*—*5*). Even after 15 min of incubation, about 85% of these siRNA were split, while after 4 h full degradation occurred. It is noteworthy that the introduction of 3′—3′-phosphodiester (inverted) bond into siRNAs did not increase their stability against nucleases.

The influence of siRNA stability in the culture medium on their ability to suppress the expression of target genes was studied. The repression of *MDR1* expression

Designation of siRNA	Sequence
E	5′-AUCAUCCAUGGGGCUGGACUU-3′ 3′-GGUAGUAGGUACCCCGACCUG-5′
Ei	5′-AUCAUCCAUGGGGCUGGACU^{m}U^{m}Tinv-3′ 3′-TinvG^{m}G^{m}UAGUAGGUACCCCGACCUG-5′
Em	5′-A^{m}U^{m}C^{m}A^{m}U^{m}C^{m}C^{m}A^{m}U^{m}G^{m}G^{m}G^{m}G^{m}C^{m}U^{m}G^{m}G^{m}A^{m}C^{m}U^{m}U^{m}-3′ 3′-G^{m}G^{m}U^{m}A^{m}G^{m}U^{m}A^{m}G^{m}G^{m}U^{m}A^{m}C^{m}C^{m}C^{m}C^{m}G^{m}A^{m}C^{m}C^{m}U^{m}G^{m}-5′
Emi	5′-A^{m}U^{m}C^{m}A^{m}U^{m}C^{m}C^{m}A^{m}U^{m}G^{m}G^{m}G^{m}G^{m}C^{m}U^{m}G^{m}G^{m}A^{m}C^{m}U^{m}U^{m} Tinv-3′ 3′-TinvG^{m}G^{m}U^{m}A^{m}G^{m}U^{m}A^{m}G^{m}G^{m}U^{m}A^{m}C^{m}C^{m}C^{m}C^{m}G^{m}A^{m}C^{m}C^{m}U^{m}G^{m}-5′
E/Em	5′-A U C A U C C A U G G G G C U G G A C U U-3′ 3′-G^{m}G^{m}U^{m}A^{m}G^{m}U^{m}A^{m}G^{m}G^{m}U^{m}A^{m}C^{m}C^{m}C^{m}C^{m}G^{m}A^{m}C^{m}C^{m}U^{m}G^{m}-5′
Em/E	5′-A^{m}U^{m}C^{m}A^{m}U^{m}C^{m}C^{m}A^{m}U^{m}G^{m}G^{m}G^{m}G^{m}C^{m}U^{m}G^{m}G^{m}A^{m}C^{m}U^{m}U^{m}-3′ 3′-G G U A G U A G G U A C C C C G A C C U G-5′
E/Ei	5′-AUCAUCCAUGGGGCUGGACUU-3′ 3′-TinvG^{m}G^{m}UAGUAGGUACCCCGACCUG-5′
Ei/E	5′-AUCAUCCAUGGGGCUGGACU^{m}U^{m}Tinv-3′ 3′-GGUAGUAGGUACCCCGACCUG-5′
Emi/E	5′-A^{m}U^{m}C^{m}A^{m}U^{m}C^{m}C^{m}A^{m}U^{m}G^{m}G^{m}G^{m}G^{m}C^{m}U^{m}G^{m}G^{m}A^{m}C^{m}U^{m}U^{m}Tinv-3′ 3′-G G U A G U A G G U A C C C C G A C C U G-5′
E/Emi	5′-A U C A U C C A U G G G G C U G G A C U U-3′ 3′-TinvG^{m}G^{m}U^{m}A^{m}G^{m}U^{m}A^{m}G^{m}G^{m}U^{m}A^{m}C^{m}C^{m}C^{m}C^{m}G^{m}A^{m}C^{m}C^{m}U^{m}G^{m}-5′

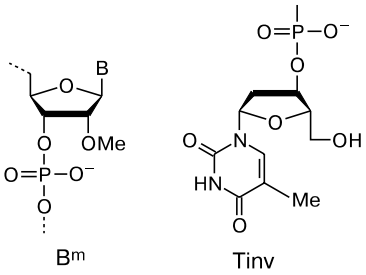

Fig. 1. Sequences of siRNA used for inhibition of *MDR1* gene expression and their chemical modifications.

with siRNA decreases the amount of P-glycoprotein in the cytoplasmic membrane; since the cells are cultured in a medium containing the cytostatic vinblastine, the vinblastine concentration in the cytoplasm gradually increases, which results in cell death. The cell sensitivity to vinblastin was determined by the MTT test, which is based on the ability of living cells to convert tetrazole-based compounds (MTT) into intensely colored formazan crystals, thus allowing an estimation of the amount of surviving cells in the samples by spectrophotometry.[20]

The experiment was carried out in the following way. The KB-8-5 cells growing in a 96-well plate were transfected with different amounts of siRNA in combination with Oligofectamine and 4 h after the transfection, vinblastine was added up to a concentration of 300 nmol L^{-1}. After 96-h incubation of the cells under these conditions, the amount of living cells was determined by the MTT test. The results show (Fig. 3) that both the native siRNA E and completely modified analogs, siRNA Em, Emi, and Ei, induce a concentration-dependent restoration the sensitivity of cells to vinblastine and cell death (see Fig. 3, column diagrams *1*—*4*). However, complete replacement of natural ribonucleotides by 2′-*O*-methylated analogs in siRNA results in a pronounced decrease in efficiency (see Fig. 3, column diagrams *2*, *3*). The incubation of cells for 96 h in the presence of vinblastine and siRNA E (20 nmol L^{-1}) decreased the amount of living cells to 35% relative to the control sample, whereas the incubation in the presence of fully 2′-*O*-methylated siRNA Em (20 nmol L^{-1}) did not affect the cell viability. Only when the concentration of siRNA Em was increased to 100 nmol L^{-1}, was a toxic action of vinblastine detected. Thus, 2′-*O*-methylated siRNA Em exhibits a reduced biological activity. Similar results were obtained in the case of 2′-*O*-methylated siRNA Emi containing a 3′—3′-inversion at the 3′-ends of both strands (see Fig. 3, column diagram *3*). The protection of the 3′-ends of siRNA by 3′—3′-inversion and the introduction of two 2′-*O*-methyl units into the 3′-overhanging ends of siRNA (siRNA Ei) increased the degree of restoration of cell sensitivity to vinblastine: in the presence of this siRNA (20 nmol L^{-1}), only 10% of cells survived (see Fig. 3, column diagram 4).

In order to prove that chemically modified siRNAs cause cell death in the presence of vinblastine through repression of the target gene expression rather than through their own toxicity, the cells of the parent drug-sensitive KB-3-1 line were incubated in the presence of siRNAs under study in the absence of a cytostatic. This showed that the siRNAs themselves are non-toxic for cells (preliminary data are not given).

In order to determine the strand (sense or antisense one) whose modification has a more pronounced effect on the siRNA activity, we compared the abilities of siRNAs containing modifications in one or both strands to restore the sensitivity of the KB-8-5 cells to

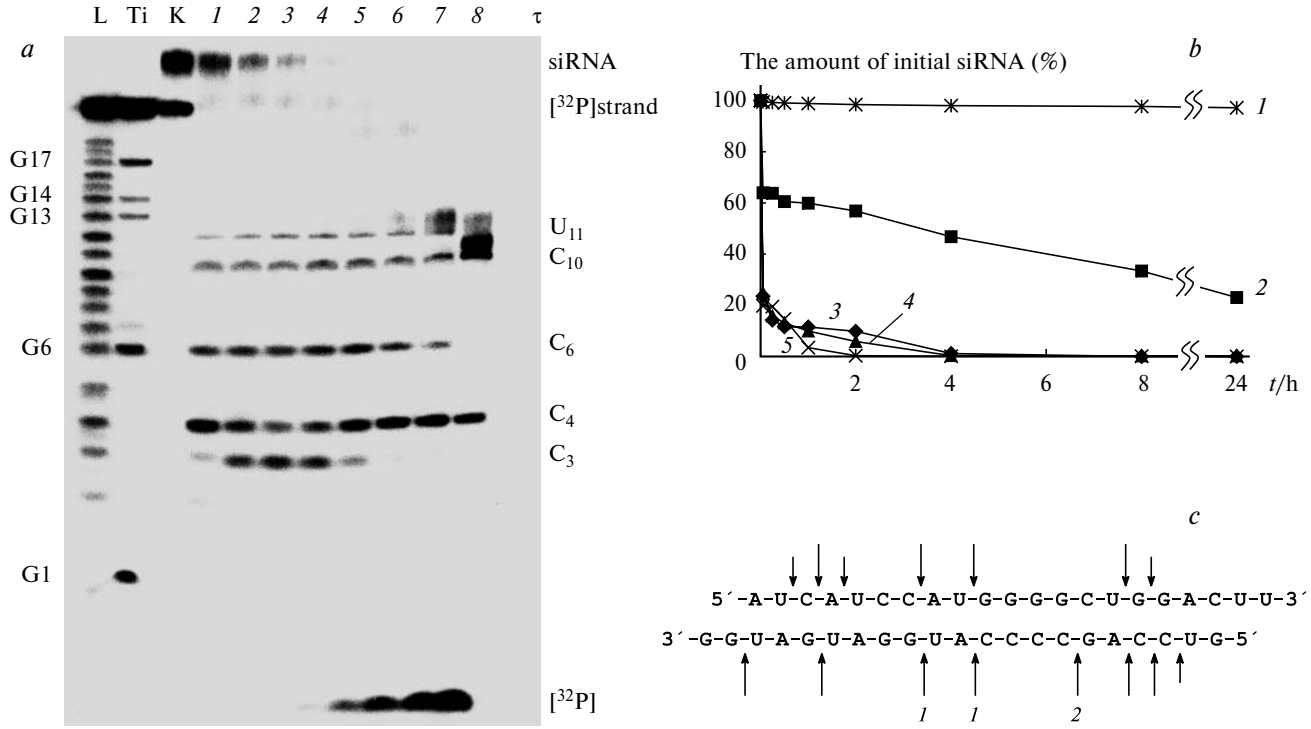

Fig. 2. Stability of [5´-^{32}P]-siRNA E and its modified analogs in IMDM containing 5% FBS. *a*. Radioautograph of 18% PAAG containing 8 *M* urea after separation of siRNA Ei cleavage products. Incubation time (τ): 3 (*1*), 15 (*2*), 30 min (*3*), 1 (*4*), 2 (*5*), 4 (*6*), 8 (*7*), 24 h (*8*). K is the initial siRNA Ei. On the left of the gel are the cleavage sites of the [5´-^{32}P]-labeled strand of siRNA Ei by RNase T1; on the right, cleavage sites by serum nucleaseas are given. *b*. Kinetics of siRNA cleavage in IMDM containing 5% FBS: siRNA Em (*1*), Em/E (*2*), E (*3*), Ei/E (*4*), Ei (*5*). *c*. The overall distribution pattern of the cleavage sites of siRNA E and its modified analogs by nucleases: the arrows show the cleaved bonds; the size of the arrows reflects the cleavage intensity for the particular bond; (*1*) sites not subjected to cleavage within siRNA Em/E; (*2*) site subjected to cleavage only within siRNA Ei.

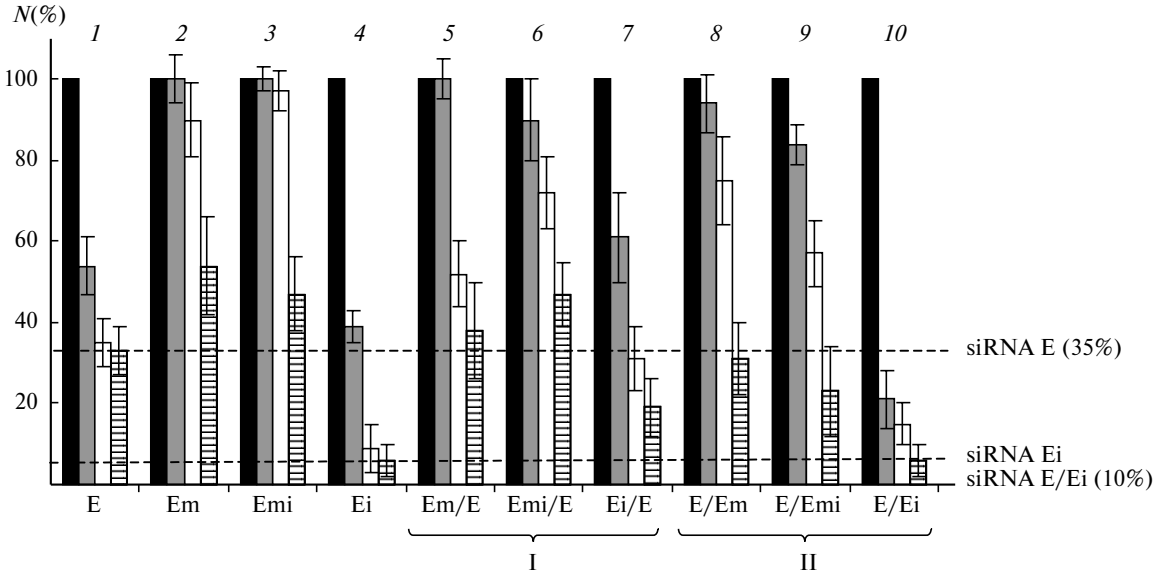

Fig. 3. Effect of modification of the sense (I) and antisense (II) siRNA strands on the efficiency of restoration of the sensitivity of the KB-8-5 cell line to vinblastine. The cells were incubated for 96 h in the presence of only Oligofectamine (black columns), in the presence of the siRNA/Oligofectamine complex in concentrations of 5 nmol L^{-1} (gray columns), 20 nmol L^{-1} (white columns), and 100 nmol L^{-1} (hatched columns) in a medium containing 300 nmol L^{-1} of vinblastine. The amount of living cells (*N*) was estimated by the MTT-test. The amount of living cells incubated with Oligofectamine in the absence of siRNA was taken as 100%.

vinblastine (see Fig. 3, column diagrams *5—10*). The siRNAs containing only one fully 2′-*O*-methylated strand (siRNA Em/E, E/Em) were found to be more active than fully modified siRNA Em. The modification of the sense strand suppresses the biological activity of the siRNA to a lesser extent than modification of the antisense strand (see Fig. 3, column diagrams *5* and *8*). The siRNAs bearing a double modification (2′-*O*-methylation and 3′—3′-inversion) in the sense and antisense strands demonstrated a somewhat different action. In this case, higher activity was found for the siRNA bearing modifications in the antisense strand (see Fig. 3, column diagrams *6* and *9*). The performance of the siRNA containing a 3′—3′-inversion and only two 2′-*O*-methyl units on the 3′-end of one or both strands was much higher than that of the native siRNA E (see Fig. 3, column diagrams *4*, *7*, *10*). As for fully methylated siRNA, the siRNA containing modifications in the antisense strand exhibited a *ca*. threefold higher activity than the siRNA modified at the sense strand (see Fig. 3, column diagrams *7* and *10*). It is noteworthy that siRNA E/Ei exhibited the same activity as siRNA Ei.

The kinetics of the decrease in the amount of living KB-8-5 cells during incubation in the presence of siRNA and vinblastine is shown in Fig. 4. It can be seen that 2′-*O*-methylated siRNAs (Em and Emi) cause a slight decrease in the amount of living cells three days after the transfection in the presence of vinblastine in a 300 nmol L^{-1} concentration. However, the survived cells retain the capacity for division, and five days after the transfection, their population is restored and does not differ from the control. For lower concentrations of vinblastine (100 and 10 nmol L^{-1}), these siRNAs did not induce cell death.

Small interfering RNA E efficiently restored cell sensitivity to vinblastine and caused their death with both high (100 and 300 nmol L^{-1}) and low (10 nmol $L^{-1)}$ vinblastine concentrations. In particular, 70 and 30% cells were killed at vinblastine concentrations of 300 and 10 nmol L^{-1}, respectively, whereas the amount of cells in the control sample doubled over the same period of time (see Fig. 4, *a* and *b*, column diagram E). On the fifth day of incubation of the cells with siRNA E, a slight increase in the number of living cells was observed. siRNA Ei was the most efficient in restoration of cell sensitivity to vinblastine: after 96 h of incubation, their amount decreased by 90, 70, and 60% relative to the initial value at vinblastine concentrations of 300, 100, and 10 nmol L^{-1}, respectively (see Fig. 4, column diagram Ei). On the fifth day after transfection, the amount of living cells continued to decrease even when the vinblastine concentration was 10 nmol L^{-1}. Thus, it is with siRNA Ei that a single treatment inhibits expression of *MDR1* gene and restores the sensitivity of human cancer cells to vinblastine most efficiently and durably.

The duration of the inhibiting action of siRNA was studied by determining the amount of the product of *MDR1* gene, *viz.*, P-glycoprotein, in the cells treated with siRNA E and siRNA Ei using the Western blot assay with the protein β-actin as the internal standard (Fig. 5). Since the amount of the cells decreased almost to zero under the action of these siRNAs and vinblastine over a period of 5 days, to determine the P-glycoprotein level, the cells were incubated with siRNA in a cytostatic-free medium. Under these conditions, the amount of P-glycoprotein decreased even in the cells untreated with siRNA (see Fig. 5), which is indicative of the restoration of the normal phenotype of the cells in the absence of a cytostatic. However, under the action of siRNA, on the fourth day (*i.e.*, when the cell death in the vinblastine-containing medium is observed), the amount of P-glycoprotein decreased to 45% in the case of siRNA E and to 35% in the case of siRNA Ei, whereas the P-glycoprotein level in the control cells was 65% of the original level.

The introduction of chemical modifications into siRNA is an important tool for improving their pharmaceutical properties. To attain sufficient stability of siRNAs in blood, it is necessary to enhance their stability against exo- and endonucleases. The modifications can improve the pharmacokinetic properties of siRNA *in vivo* by mediating the binding to blood components and increasing the time of siRNA circulation in blood. In addition, certain chemical modifications can facilitate penetration of siRNA into various cells.

The preservation of siRNA in the cell and culture medium is an important factor responsible for the efficiency of repression of the target gene expression with siRNA and ensuring the duration of the effect. The serum is known to contain ribonucleases that belong to the RNase A, 3′-exonuclease, and phosphatase families. Ribonucleases cleave RNA mainly at single-strand sites and loops.[22] The nuclease stability of ribozymes and antisense oligonucleotides is increased with a number of chemical modifications, including 2′-*O*-methylation and introduction of inverted phosphodiester bonds at the 3′-ends of the molecule.[23—25]

Chemical modifications can be introduced into various regions of the RNA structure. It was shown that the 5′-end phosphate group of the antisense strand of siRNA is required for revealing the biological activity of siRNA and directed cutting of the target mRNA.[26] The synthetic siRNAs bearing a 5′-hydroxy group induce RNA interference, as they are phosphorylated in the cell with endogenous kinase. When the 5′-hydroxy end of the antisense strand is protected by the 5′-OMe group, the target gene expression is not suppressed, whereas modification of the sense strand at the 5′-OH group does not affect the siRNA activity.[26] The data concerning the modifications of the 3′-ends of the siRNA antisense strand are contradictory.

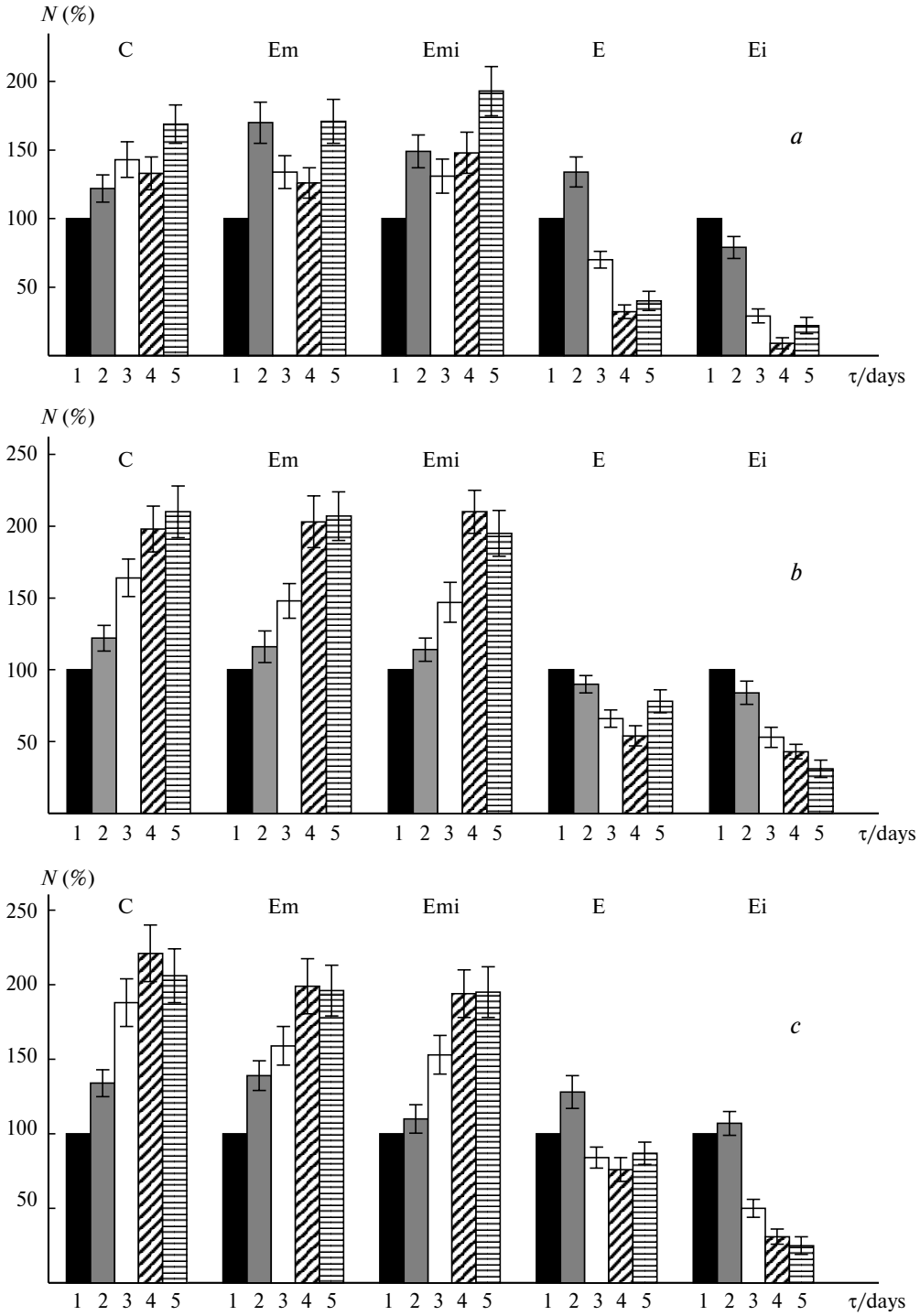

Fig. 4. Restoration of sensitivity of the KB-8-5 line cells to vinblastine after incubation in the presence of siRNA in a 20 nmol L^{-1} concentration containing chemical modifications: 300 (*a*), 100 (*b*), and 10 nmol L^{-1} (*c*) of vinblastine, respectively. Incubation time (τ) 1—5 days. The amount of living cells (*N*) was estimated by the MTT test. The cells treated only with Oligofectamine were taken as the control (C). The amount of living cells in the first 24 h after treatment with siRNA was taken as 100%.

Modification of the 3′-end of the antisense strand and both ends of the sense strand by introducing an inverted phosphodiester bond or an amino group attached through a six-methylene linker causes no decrease in the biological activity of siRNA,[27] whereas modification of the 3′-end of the antisense strand by 2-hydroxyethyl phosphate or 2′-*O*,4′-*C*-ethylenethymidine results in complete loss of activity.[28]

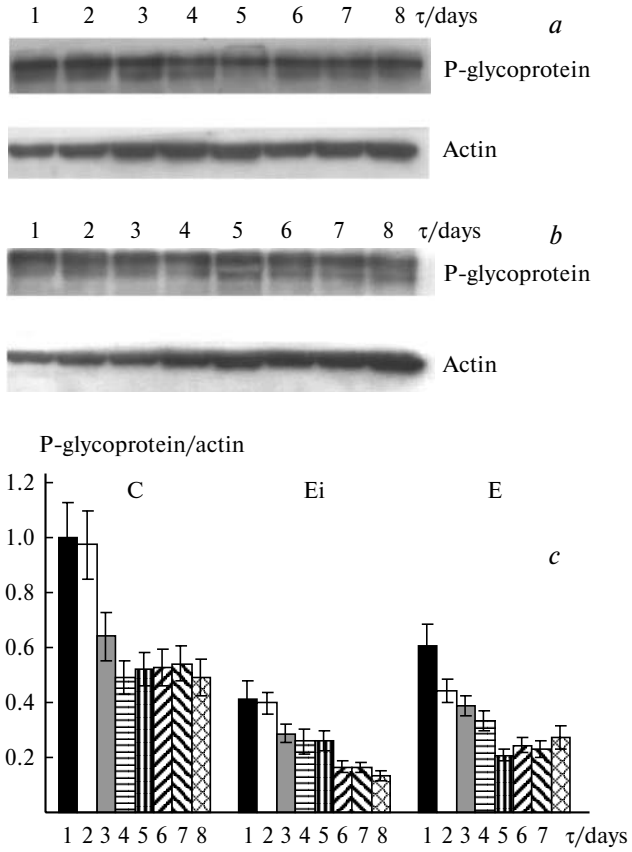

Fig. 5. Inhibition of *MDR1* gene expression in the KB-8-5 cell lines transfected by various siRNA (the results of Western blot hybridization). The cells treated only with Oligofectamine without adding siRNA were taken as the control (C). The KB-8-5 cells incubated in the presence of siRNA and Oligofectamine in a cytostatic-free medium for 1—8 days. The amount of β-actin in the cells was used as the reference. The P-glycoprotein : β-actin ratio in the cells within 24 h after the addition of the siRNA—Oligofectamine complex was taken as unity: (*a*) control cells, (*b*) cells incubated in the presence of the siRNA—Oligofectamine complex.

In the present work, ribonucleotides of siRNA E were replaced by their 2′-*O*-methylated analogs for protection from endoribonucleases, while 3′-ends of siRNA E were modified by introducing a 3′—3′-inverted bond for the protection from 3′-exonuclease (see Fig. 1). It was found that replacement of all siRNA ribonucleotides by their 2′-*O*-methylated analogs retards substantially the siRNA degradation (see Fig. 2, curve *1*), which is consistent with the results of earlier publications.[29,30] The introduction of a 3′—3′-inversion into siRNAs does not increase their nuclease resistance, which is also in line with the published results.[27] The pattern of siRNA cleavage in a serum-containing medium suggests that siRNA degradation is induced by endo- rather than exonucleases. Note that the UpG and CpA motives in which the phosphodiester bonds are cleaved most rapidly by ribonucleases of the RNase A family proved to be the primary nuclease-sensitive siRNA E sites (see Ref. 22). We found that the siRNA modification may change the cleavage pattern. Indeed, for the antisense strand of siRNA Em/E, the nuclease-sensitive C^7pA^8 and U^9pG^{10} sites disappear, which might be due to inability of endoribonuclease to bind efficiently to the duplex and to "fuse" the strand giving single-stranded sites; for the Ei duplex, an additional cleavage site, G^6pC^7, was identified.

A correlation of the lifetimes of the siRNAs in a culture medium with their biological activities showed that fully 2′-*O*-methylated siRNA Em (the most nuclease resistant siRNA) demonstrated the lowest biological activity. The highest biological activity was found for siRNA Ei whose half-life time was ~30 min. Partial modification of siRNA (3′—3′-inversion and double 2′-*O*-methylation of the 3′-overhanging ends) gives rise to a molecule with high performance efficiency. These results are consistent with publications,[5,29,31,32] demonstrating that modification of the overhanging 3′-ends does not decrease the biological activity of siRNA. Thus, the performance efficiency of the siRNA studied is not determined by only their lifetime in the culture medium.

The results obtained in the present study demonstrate that temporary *MDR1* gene suppression is sufficient to overcome the multiple-drug resistance syndrome and ensures the accumulation of chemotherapy drugs in the cells in concentrations needed for cell death (see Fig. 4). A study of the kinetics of the decrease in the amount of the KB-8-5 living cells during incubation in the presence of various siRNAs and vinblastine showed that even a single treatment of the cells with siRNA Ei resulted in cell death over a period of 5 days at the minimum vinblastine concentration used (10 nmol L^{-1}). Thus, siRNA Ei efficiently and durably inhibits the expression of *MDR1* gene and restores the sensitivity of human cancer cells to vinblastine.

The specificity of the inhibiting action of siRNA E and Ei was proved based on the decrease in P-glycoprotein level, the product of the *MDR1* gene in the cells, observed after the siRNA treatment. On the fourth day of incubation in a vinblastine-free medium, the amount of P-glycoprotein in the cells transfected with siRNA E and siRNA Ei was twice lower than that in the control cells. Comparison of these data with the data of MTT analysis shows that the cell death observed after treatment with siRNA in the presence of concentrations of vinblastine previously endured (or even lower) is correlated with the decrease in the P-glycoprotein level. Thus, the observed decrease in the P-glycoprotein level in cancer cells can be considered as therapeutically significant.

Our studies demonstrated that the efficient biological action of siRNA did not always directly correlate with its stability against nucleases. Despite the relatively low nuclease resistance, the chemically modified siRNA Ei we

synthesized, causes, after a single introduction, a durable (up to 5 days) decrease in the level of MDR 1 expression and an effective restoration of the multiple-drug resistance phenotype after a single introduction. Hence, this compound can be regarded as a basis for therapeutic drugs for combined chemotherapy of cancer.

This work was financially supported by the Russian Foundation for Basic Research (Project No. 03-04-48550-a), the RF Ministry of Education and Science (Program for the Support of Fundamental Research in Natural Sciences, Project No. E02-6.0-51), the Russian Academy of Sciences (Fundamental Research Program of the RAS "Fundamental Science for Medicine" and "Molecular Cell Biology", the Integration Projects of the Siberian Branch of the RAS No 20 and No. 5.10., The Federal Center for Science and Engineering Programs (Grant RI-012/001/254).

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Received January 24, 2006;
in revised form April 3, 2006