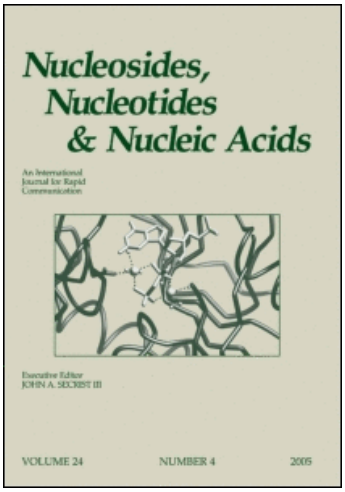

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Perfluoroarylazide Derivatives of 2'-O-Modified Oligoribonucleotides: Efficient Reagents for RNA Photomodification

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PERFLUOROARYLAZIDE DERIVATIVES OF 2′-O-MODIFIED OLIGORIBONUCLEOTIDES: EFFICIENT REAGENTS FOR RNA PHOTOMODIFICATION

D. S. Novopashina, R. N. Kulikov, M. A. Kuznetsova, A. G. Venyaminova, M. A. Zenkova, and V. V. Vlassov ▫ *Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia*

▫ *Site-specific photomodification of the 5′-terminal fragment of MDR1 mRNA by perfluoroarylazide derivatives of 2′-O-modified (2′-O-methyl or 2′-O-tetrahydropyranyl) oligoribo- and oligodeoxyribonucleotides was investigated. The conjugates built of 2′-O-modified oligoribonucleotides demonstrate beneficial features compared with their deoxyribo analogs: the extent of RNA modification by 2′-O-modified oligoribonucleotides and oligodeoxyribonucleotide conjugates was 40—50% and 20%, respectively.*

Keywords 2′-O-Modified oligoribonucleotides, Perfluoroarylazide, Site-specific RNA modification, *MDR1* mRNA, Oligonucleotide conjugates

INTRODUCTION

Significant progress in the design of various reactive oligonucleotide derivatives and their application in a number of important areas of molecular biology has been achieved.[1–3] Conjugates of oligonucleotides with molecules capable of irreversibly modifying nucleic acids or proteins provide opportunities of selective modification of these biopolymers. Among these molecules photoinducible reagents are of substantial interest as useful tools for investigation of structure and functions of biopolymers. It has been shown that oligonucleotides conjugated to perfluoroarylazides are capable of modifying DNA, RNA, and proteins.[1,2,4–6] These compounds can be easily synthesized and have a number of advantages over other reactive oligonucleotide derivatives: they are inert in the dark and can be "switched on" at will by UV-irradiation, which results in fast reaction with

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biopolymer. 2′-O-Modified oligoribonucleotides are beneficial due to their improved hybridisation properties, an increased selectivity, and resistance to intracellular nucleases.[7–9] Recently, photomodificaton of short RNA and DNA targets by perfluoroarylazide derivatives of 2′-O-modified oligoribonucleotides was described.[10]

Here we describe the study of photomodification of the 190 nucleotides long 5′-terminal fragment of *MDR1* mRNA by conjugates of 2′-O-modified oligoribo- and deoxyribonucleotides bearing *p*-azidotetrafluorobenzamide group at the 3′-phosphate. We have found that efficient site-specific photomodification of the RNA is achieved by using 2′-O-modified oligoribonucleotide conjugates.

RESULTS AND DISCUSSION

2′-O-Modified (2′-O-methyl or 2′-O-tetrahydropyranyl) dodecaribonucleotides and dodecadeoxyribonucleotide complementary to the region 155–166 of *MDR1* mRNA were synthesized by the solid phase H-phosphonate method according to protocols developed earlier[11–12] with an overall yield of 10–18% after two successive HPLC isolations. To synthesize oligonucleotides with phosphate group at the 3′-end, the polymer bearing 2-[2-(4, 4′-dimethoxytrityloxy)ethylsulfonyl]ethyl group was used. The nucleoside composition of 2′-O-modified oligonucleotides was confirmed by the complete enzymatic digestion of oligonucleotides by snake venom phosphodiesterase and alkaline phosphatase (see Experimental section) followed by the quantitative RP-HPLC analysis of the digest products.

Conjugation of perfluoroarylazide to the terminal 3′-phosphate of 2′-O-modified ribo- and deoxyribooligonucleotides via aliphatic diamine linkers was performed according to published procedure.[13] Figure 1 displays the structures of the

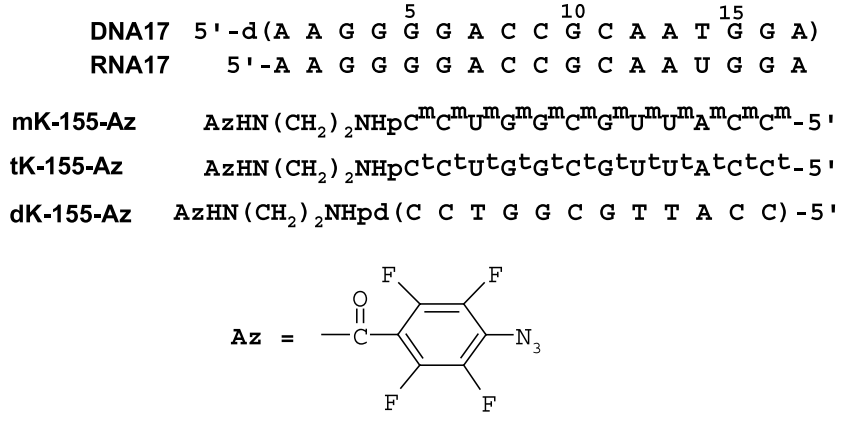

FIGURE 1 RNA and DNA oligonucleotides and perfluoroarylazide oligonucleotide conjugates used in the study. Abbreviations: N^m-2′-O-methylribonucleotide; N^t-2′-O-tetrahydropyranyl-ribonucleotide; **dN**-deoxyribonucleotide; **Az**-4-azido-2,3,5,6-tetrafluorobenzoyl group; **K-155**, synthetic oligonucleotides, complementary to *MDR1* mRNA sequence 155–166.

conjugates. The conjugates are denoted as follows: **dK-155-Az**, **mK-155-Az**, and **tK-155-Az**, where **K-155** are synthetic oligonucleotides, complementary to sequence 155–166 in *MDR1* mRNA; **Az** is a 4-azido-2,3,5,6-tetrafluorobenzoyl group; **mK-155** is 2′-O-methylribo-; **tK-155** is 2′-O-tetrahydropyranyl-ribo-; and **dK-155** is deoxyribooligonucleotide.

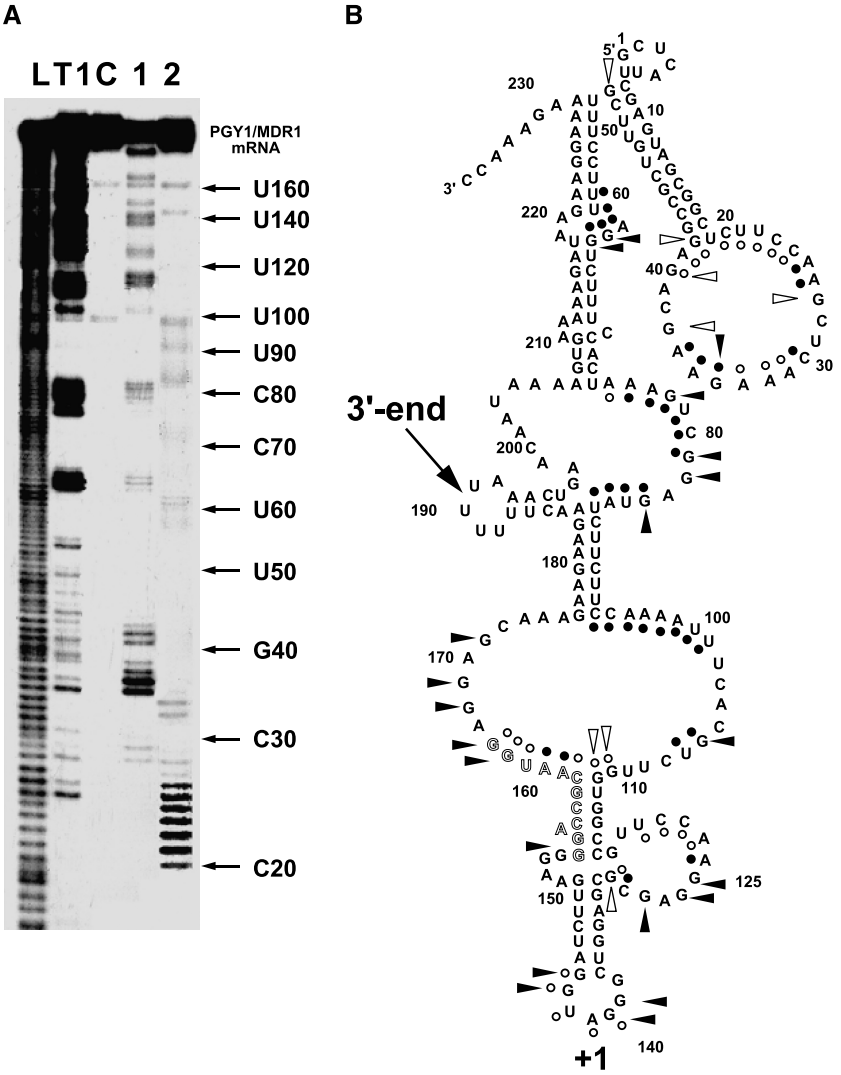

FIGURE 2 Probing of secondary structure of in vitro transcript of *MDR1* mRNA. A. Autoradiogram showing enzymatic cleavage sites on *MDR1* mRNA. Lanes L and T1, imidazole and RNase T1 ladder, respectively; Lane C, incubation control; Lanes 1 and 2, RNA cleaved with 10 U/ml RNase T1 and 0.1 U/ml RNase ONE, respectively. B. Secondary structure model of the 190-nucleotide-long 5′-terminal fragment of *PGY1/MDR1* mRNA. Filled and open figures indicate strong and weak sites reactive to RNase T1 (triangles) and to RNase ONE (circles). The sequence complementary to the oligonucleotide **K-155** is denoted by gray.

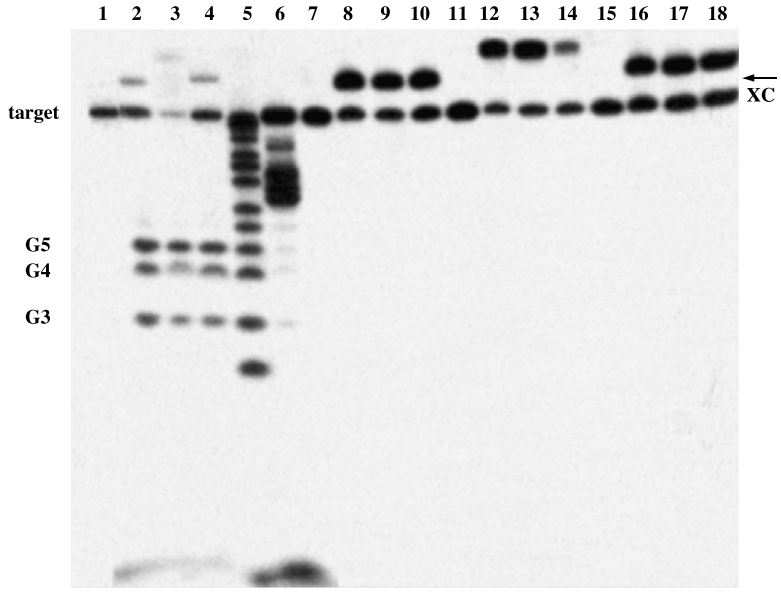

FIGURE 3 Photomodification of the [5′-^{32}P]-DNA17 by conjugates **dK-155-Az** (Lanes 2, 7–10), **tK-155-Az** (Lanes 3, 11–14), and **mK-155-Az** (Lanes 4, 15–18). Autoradiogram of 20% polyacrylamide/8 M urea gel. Lane 1, piperidine treatment of nonmodified DNA17; Lane 5, A + G ladder; Lane 6, C + T ladder; lanes 2–4, photomodification products treated with piperidine; Lanes 7, 11, and 15, reaction mixtures before irradiation; Lanes 8, 12, and 16, irradiation at 5°C; Lanes 9, 13, and 17, irradiation at 20°C; Lanes 10, 14, and 18, irradiation at 40°C. Conditions of the photomodification and piperidine treatment are described in the Experimental section.

The ability of the photoreactive conjugates to modify nucleic acids was investigated in experiments with synthetic 17-mer ribo- (RNA17) and deoxyribo- (DNA17) oligonucleotides and 190 nucleotide long fragment of *MDR1* mRNA (here and after RNA190) as targets. The sequence of RNA17 and DNA17 corresponds to the *MDR1* mRNA region 151–167 (Figure 1). Oligonucleotide conjugates bound to the complementary sequences in the targets should deliver the arylazide group attached to their 3′ ends, to the cluster of four guanine residues G_3-G_6, in the RNA17 and DNA17; or G_{153}-G_{156} in the *MDR1* mRNA (See Figures 1 and 2). It is known that guanines exhibit the highest sensitivity to photomodification by arylazides.[4]

TABLE 1 Photomodification of RNA and DNA Targets by Perfluoroarylazide Conjugates of Oligonucleotides

	Photomodification yield, %[a,b]						
	DNA17			RNA17			RNA190
Conjugate	5°C	20°C	40°C	5°C	20°C	40°C	37°C
tK-155-Az	69	68	42	68	68	36	51
mK-155-Az	59	58	60	59	61	21	40–50
dK-155-Az	65	64	58	59	50	32	20

[a]Reaction conditions are described in the Experimental section.
[b]Experimental error was ≤10%.

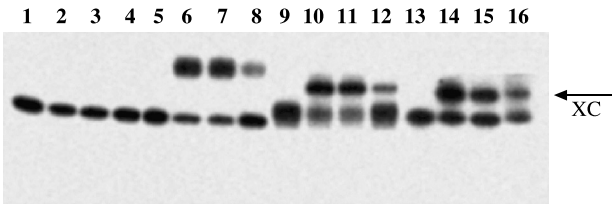

FIGURE 4 Photomodification of [5′-^{32}P]-RNA17 by conjugates **tK-155-Az** (Lanes 5–8), **mK-155-Az** (Lanes 9–12), and **dK-155-Az** (Lanes 13–16). Autoradiogram of 20% polyacrylamide / 8M urea gel. Lane 1, RNA17 before irradiation; Lanes 2–4, RNA17 irradiated at 5, 20, and 40°C, respectively in the absence of conjugates; Lanes 5, 9, and 13, reaction mixtures before irradiation; Lanes 6, 10, and 14, irradiation at 5°C; Lanes 7, 11, and 15, irradiation at 20°C; Lanes 8, 12, and 16, irradiation at 40°C. Conditions of the photomodification reaction are described in the Experimental section.

The photomodification was induced by irradiation of reaction mixture with UV-light ($303 < \lambda < 365$ nm) at 5, 20, and 40°C and at 37°C in the case of RNA190. The conjugates **dK-155-Az**, **mK-155-Az**, and **tK-155-Az** modify DNA17 with similar efficiencies at 5 and 20°C, (Figure 3, Table 1). At 40°C a considerable decrease of modification extent was observed in the case of **tK-155-Az**, apparently due to decreased stability of heteroduplex formed by DNA17 and this conjugate.[10,12] To determine sites of DNA modification, samples of DNA17 irradiated in the presence of the conjugates, were treated with 10% piperidine and analyzed by electrophoresis in 20% polyacrylamide/8 M urea gel (Figure 3). DNA17 was modified predominantly at G3, G4, and G5 residues by all the conjugates (Figure 3).

In the case of RNA17, high extents of the photomodification at 5 and 20°C were observed (Figure 4, Table 1). After the modification of RNA17 by the conjugate **mK-155-Az**, smeared bands with electrophoretic mobility low than that of RNA17 were observed. We suggested that these are stable complexes of the RNA17 with 2′-O-methylribo oligonucleotide conjugate that were not destroyed

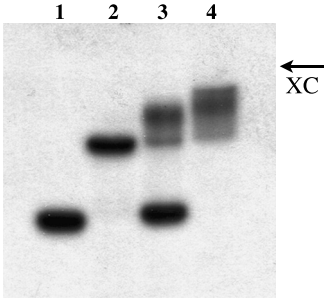

FIGURE 5 Stability of complexes formed by 2′-O-methyl oligonucleotide and RNA17 under the denaturing 20% PAGE conditions. Lane 1, [^{32}P]-**mK-155**, Lane 2, [^{32}P]-RNA17 target; Lane 3, [^{32}P]-**mK-155**+ [^{32}P]-RNA17; Lane 4, **mK-155-Az**+ [^{32}P]-RNA17. Concentrations of RNA17 and the conjugate were $1 \cdot 10^{-7}$ M and $1 \cdot 10^{-5}$ M, respectively.

under the denaturing PAGE conditions used. This suggestion was confirmed in the experiment with [^{32}P]-labeled **mK-155** (Figure 5).

RNA photomodification by the conjugates was considerably affected by reaction temperature. A smooth decrease in the RNA modification yield was observed for **dK-155-Az** with temperature increasing from 5 to 40°C. In the case of 2′-O-modified oligoribonucleotides a sharp decrease of photomodification extent was observed when temperature rose from 20 to 40°C, despite the fact that 2′-O-methyl oligonucleotides and their perfluoroarylazide conjugates form very stable duplexes with complementary RNA sequences.[10,12] These results indicate that efficiency of RNA photomodification depends not only on the duplex stability but also on some other temperature-sensitive factors; e.g., increase of conformational freedom of arylazide residue.

To demonstrate the ability of the synthesized reagents to modify RNA molecule with pronounced secondary structure, photomodification of the *MDR1* mRNA fragment was performed. Recently, secondary structure of the 5′-terminal fragment of *MDR1* mRNA containing 670-nucleotides was established.[14] Since the shortened RNA fragment (190 nucleotides long) might have a different secondary structure, we compared the structures of the fragments of *MDR1* mRNA containing 190 and 670 nucleotides by probing with ribonuclease T1 and RNase ONE.[15] Comparison of the probing data revealed identity of the RNA cleavage patterns produced by ribonucleases T1 and ONE in experiments with both fragments

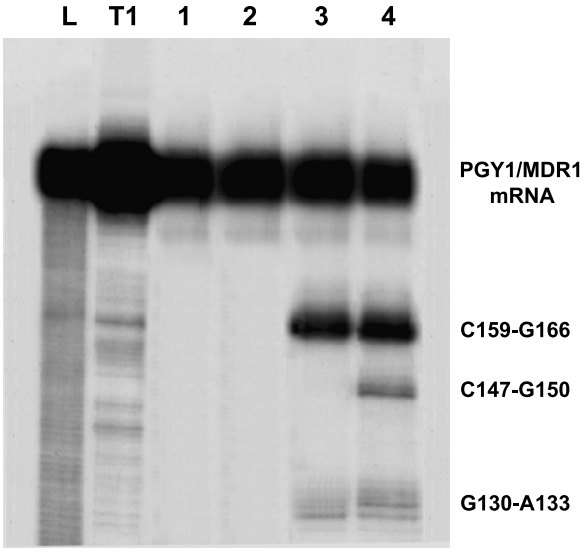

FIGURE 6 Ribonuclease H footprinting of *MDR1* mRNA bound to the oligonucleotide **dK-155** and to the conjugate **dK-155-Az**. Autoradiograph of 8% polyacrylamide / 8 M urea gel. Lanes L and T1, imidazole and RNase T1 ladders; Lane 1, RNA incubated with RNase H; Lane 2, RNA incubated with oligodeoxynucleotide **dK-155**; Lanes 3 and 4, RNA incubated with RNase H (1 U/mL) for 30 min at 37°C in the presence of $1 \cdot 10^{-5}$ M oligonucleotide **dK-155** and $1 \cdot 10^{-5}$ M conjugate **dK-155-Az**, respectively. Reaction conditions: 25 mM HEPES, pH 7.5, 100 mM KCl, 0.05 mM EDTA, 10 mM $MgCl_2$, 100 μg/mL tRNA carrier.

(probing data for 670 nucleotide long fragment are not shown), which indicates that secondary structures of the 5′-terminal 190-nucleotides long sequence in these RNAs have similar foldings (Figure 2).

The ability of the oligonucleotides and the conjugates under consideration to form duplexes with complementary sequences of RNA190 was tested by footprinting with RNase H. [5′-^{32}P]-Labeled RNA190 was cleaved by RNase H in the presence of the oligodeoxyribonucleotide **dK-155** or the conjugate **dK-155-Az**. Analysis of the reaction products (Figure 6) revealed cleavage sites within the RNA190 sequence C_{159}-G_{166}, which corresponds to the complementary sequence of **dK-155**. Some faint cuts were observed in the region G_{130}-A_{133} both in the case of **dK-155** and **dK-155-Az**, and in the region C_{147}-G_{150} in the case of the conjugate. These cuts can be explained by some mismatched complexes formation.

Photomodification of the RNA190 was carried out at 37°C under the same conditions used for the short RNA target. Autoradiograph of the gel obtained after separation of the products of RNA photomodification is shown in Figure 7A.

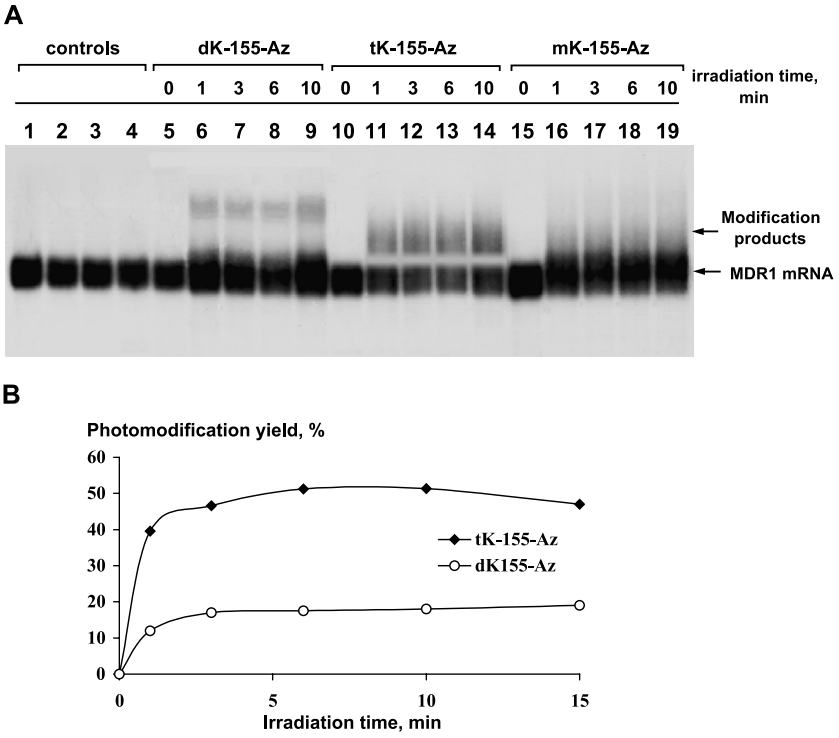

FIGURE 7 Photomodification of the 5′-[^{32}P] labeled *MDR1* mRNA by the conjugates **dK155-Az**, **tK-155-Az**, and **mK-155-Az**. A. Autoradiograph of 8% polyacrylamide/8 M urea gel. Lane 1, irradiated RNA; Lanes 2–4, RNA incubated with conjugates **dK-155-Az**, **tK-155-Az**, and **mK-155-Az**; Lanes 5–9, 10–14, and 15–19, RNA irradiated in the presence of $1 \cdot 10^{-5}$ M conjugates **dK155-Az**, **tK-155-Az**, and **mK-155-Az**, respectively. The conditions of the photomodification reaction are described in the Experimental section. Irradiation time is indicated on the top. B. Kinetics of the RNA photomodification by conjugates **tK-155-Az** and **dK-155-Az**. The reaction conditions are described in the Experimental section.

Photomodification of RNA190 occurred with the highest efficiency (up to 50%) in the reaction with conjugate **tK-155-Az**. The products of RNA modification by conjugate **mK-155-Az** have the electrophoretic mobility similar to that of RNA190, probably due to stable complex formed by 2′-O-methyl oligonucleotide and RNA target that survives under standard electrophoretic denaturing conditions (see above). Therefore, the extent of RNA190 modification by this conjugate could not be determined accurately by using gel electrophoresis (Figure 7A, lanes 16–19). RNA modification by conjugate **dK-155-Az** occurs less efficiently (20%), which can be attributed to spatial organization of the RNA/DNA duplex. As can be seen from Figure 7B, the reaction proceeded rapidly and reached a plateau in a few minutes.

Identification of the sites of *MDR1* mRNA photomodification with the conjugates was carried out by primer-directed reverse transcription (Figure 8). It was found that the reaction occurred at G_{156} and, to much smaller extent, at G_{155}. These guanine residues are located near the 5′ end of oligonucleotide **K155** bound to RNA190. In the case of **mK-155-Az**, additional modification is observed at C_{161} within the sequence involved in the complex formation. Conjugates of 2′-O-modified oligoribonucleotides demonstrated two folds higher efficiency in reaction with *MDR1* mRNA than similar oligodeoxyribonucleotide conjugate. Apparently, this is due to differences in organization of duplexes and, consequently, the arrangement of the reactive groups with respect to the major and minor grooves in the RNA/RNA and RNA/DNA duplexes.

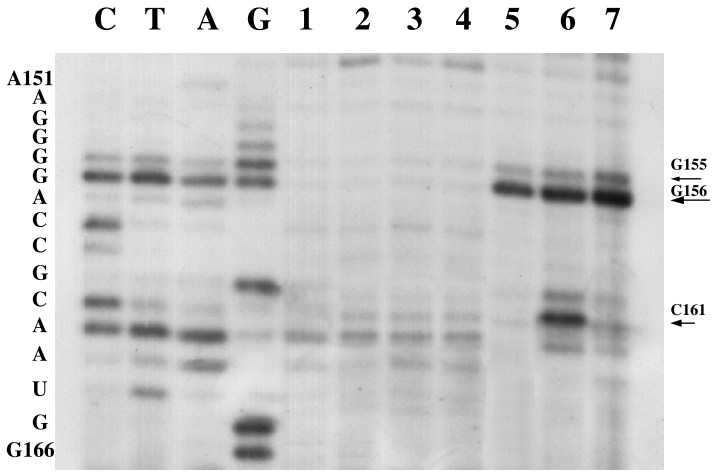

FIGURE 8 Identification of the sites of *MDR1* mRNA photomodification with the conjugates **tK-155-Az**, **mK-155-Az**, and **dK-155-Az** by primer-directed reverse transcription. Autoradiograph of 8% polyacrylamide/8 M urea gel. Lanes C, T, A, and G represent corresponding dideoxy sequencing reactions. Lanes 1–4, controls, intact *MDR1* mRNA and *MDR1* mRNA incubated at 37°C in the dark with the conjugates **tK-155-Az**, **mK-155-Az**, and **dK-155-Az**, respectively; Lanes 5–7, *MDR1* mRNA, irradiated in the presence of conjugates **tK-155-Az**, **mK-155-Az**, and **dK-155-Az** at 37°C. Sites of RNA photomodification are indicated on the right; *MDR1* mRNA sequence is shown on the left panel.

Perfluoroarylazide conjugates of oligonucleotides have some features advantageous for structure-functional studies; e.g., for investigation of RNA-protein complexes. These conjugates have been successfully used for modification of human ribosomes within mRNA binding centers.[5] In the present study we compared photomodification of DNA and RNA targets different in their structure complexity and type of helix formed upon hybridization with photoreactive oligonucleotide conjugates. The conjugates modify short DNA and RNA targets with similar efficiencies: at 5 and 20°C the maximal achieved modification extent was 68% for both DNA17 and RNA17. It should be mentioned that at 20°C conjugate **dK-155-Az** was less efficient in the reaction with RNA17 than the 2′-O-modified conjugates (modification extent 68, 61, and 50% for conjugates **tK-155-Az**, **mK-155-Az**, and **dK-155-Az**, respectively). Our data clearly show that the conjugates are capable of efficient site-specific photomodification of natural RNA. The most efficient modification of *MDR1* mRNA fragment (up to 50%) was observed in reaction with 2′-O modified oligoribonucleotide conjugates. Thus, for the first time oligonucleotides conjugated with perfluoroarylazide were successfully applied for site-selective modification of long RNA fragment.

EXPERIMENTAL SECTION

General

Chemicals were obtained from Fluka (Switzerland), Merck (Germany), and Sigma (USA). Organic solvents were purchased from Reachim (Russia) and prepared for chemical synthesis of oligonucleotides using standard methods. 3′-H-Phosphonates of N-acyl, 5′-O-dimethoxytrityl protected 2′-O-methyl-, and 2′-O-tetrahydropyranyl- or deoxyribonucleosides were prepared as described in the literature.[11,16,17] *p*-Azidotetrafluorobenzoic acid N-hydroxysuccinimide ester was provided by Dr. T.M. Ivanova (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia). [γ-^{32}P]ATP with specific activity > 0.1 PBq · mol^{-1} was from Biosan (Russia). Heptadecaribonucleotide AAGGGGACCG-CAAUGGA was synthesized by Dr. M.N. Repkova (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia).

Attachment of the first nucleoside or 2-[2-(4,4′-dimetoxytrityloxy)ethylsulfonyl]-ethanol to the CPG-500 carrier (Sigma, USA) was carried out as described in Efimov et al.[18] Average loading was 35–45 μmol per 1 g of the carrier.

Oligonucleotides and their analogs were purified using successive anion exchange and reverse-phase HPLC on Waters chromatograph (USA). Column 4.6 × 250 mm (Polysil SA; Teor. Praktika, Russia) and linear gradient 0–0.3 M of KH_2PO_4, pH 6.5 in 30% acetonitrile was used for ion exchange chromatography. RP-HPLC isolation was performed on a Lichrosorb RP-18 (Merck, Germany) column (4.6 × 250 mm) in a linear gradient of acetonitrile (0–20%) in 0.05 M $LiClO_4$ in the case of oligo(2′-O-methylribonucleotides) and

oligodeoxyribonucleotides or 0–50% of acetonitrile in 0.05 M TEAB, pH 8.5 in the case of oligo(2′-O-tetrahydropyranylribonucleotides). Oligonucleotides were precipitated as lithium salts from aqueous solutions with 2% $LiClO_4$ in acetone.

Snake venom phosphodiesterase (EC 3.1.4.1) was purchased from Sigma (USA); T4 polynucleotide kinase (EC 2.7.1.78) and restriction enzyme Dra I were from Sibenzyme (Russia); T7 RNA polymerase (EC 2.7.7.6) was from Laboratory of Enzyme Bioorganic Chemistry (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia); alkaline phosphatase (EC 3.1.3.1) from calf intestine and RNase H (EC 3.1.4.34) were from Fermentas (Lithuania); RNase T1 (EC 3.1.4.8), RNase ONE (EC 3.1.4.22), and RNAsin were from Promega (USA); and AMV reverse transcriptase was from Life Science (USA). All solutions were prepared using MilliQ water (MilliPore, USA). The buffers were sterilized by incubation with diethylpyrocarbonate (0.02%) at 37°C for 12 h or/and by autoclaving.

Photomodification was carried out using a DRK-120 high-pressure mercury lamp of an OI-18A illuminator (LOMO, Russia) equipped with an UFS-2 light filter to select emission in the range of 303 to 365 nm; the total power density of the flux $W = 0.5 \cdot 10^{-4}$ W · cm^{-2}. Constant temperature was maintained using Multitemp II Thermostatic Circulator (LKB, Sweden).

Synthesis of Oligonucleotides

2′-O-Tetrahydropyranylribo 2′-O-methylribo and -deoxyribo oligomers were synthesized by manual solid-phase H-phosphonate method[11,12] in 1–2 μmol scale. A column with porous filter supplied with 50 mg of the carrier beads was used for each synthesis. The carrier with attached 2-[2-(4,4′-dimethoxytrityloxy)-ethylsulfonyl]ethanol was used for the synthesis of oligomers with 3′-phosphate. After removal of the carrier and N-protecting groups (30% NH_4OH, 56°C, 16 h) oligonucleotides were purified and precipitated as described above. Nucleoside composition of oligonucleotides was analyzed by digestion with a mixture of snake venom phosphodiesterase and alkaline phosphatase followed by RP-HPLC analysis on a Nucleosil C-18 column (2 × 62 mm, 5 μm, Macherey-Nagel, Germany) in a linear gradient of acetonitrile (0–30%) in 50 mM $LiClO_4$; 0.3–0.5 A_{260} units of respective oligonucleotide were converted to nucleosides by incubation with a mixture of 0.01 u snake venom phosphodiesterase and 0.34 u alkaline phosphotase in 30 μL of 20 mM Tris-HCl buffer, pH 7.8, containing 1 mM $MgCl_2$ at 37°C for 5 h. In the case of dodeca(2′-O-tetrahydropyranylribonucleotide), incubation at acidic pH (0.01 M HCl, 50°C, 2 h) preceded nuclease digestion.

Synthesis of Oligonucleotide Conjugates Bearing 4-Azido-2,3,5,6-Tetrafluorobenzamide Groups at the 3′-Phosphates (dK-155-Az, tK-155-Az, and mK-155-Az)

Synthesis of oligonucleotide conjugates was performed as described in the literature.[10,19] Briefly, 6.8 mg (25 μmol) of triphenylphosphine, 5.3 mg (25 μmol) of dipyridyldisulfide, and 5 mg (40 μmol) of N,N-dimethylaminopyridine were

added to oligonucleotide solution (3–5 OD_{260}) (cetyltrimethylammonium salt) in dry 60 μL of DMFA-DMSO mixture (1:1) and the reaction mixture was vortexed for 20 min at room temperature. Then, 1 μL (15 μmol) of ethylenediamine was added and the reaction mixture was incubated for 1 h at constant vortexing and washed with ether. Oligonucleotide was precipitated by 2% $LiClO_4$ in acetone, dissolved in 100 μl of 3 M $LiClO_4$, and precipitated with acetone. The resulting oligonucleotide derivative, bearing aliphatic amino group at the 3′-phosphate, was dissolved in 15 μL 2:1 mixture of 0.1 M $NaHCO_3$ (pH 8.3) and DMFA, and a solution of N-hydroxysuccinimide ester of *p*-azidotetrafluorobenzoic acid (1.8 mg, 6 μmol) in 20 μL of DMFA was added stepwise (10, 5, and 5 μL every 30 min) to the oligonucleotide; a coupling reaction was performed for 1.5 h at room temperature. Then, oligonucleotide conjugate was precipitated by 2% $LiClO_4$ in acetone. The synthesized conjugates were isolated by RP-HPLC as described above. The conjugates were homogeneous as assayed by 20% PAGE under denaturing conditions followed by "Stains-All" staining. Molar absorbances (ε_{260}) of oligonucleotide conjugates containing *p*-azidotetrafluorobenzamide group were calculated as a sum of ε_{260} of corresponding unmodified oligonucleotide and ε_{260} of the $N_3C_6F_4CO$ group (23,200 $M^{-1} \cdot cm^{-1}$).[20]

[5′-^{32}P]-Labeling of RNA and DNA Targets

[^{32}P]-Labeling of oligonucleotides r(AAGGGGACCGCAAUGGA) and d(AAGGGGACCGCAAUGGA) (RNA17 and DNA17, respectively) was performed as follows: 15 μL of the 30 μM oligonucleotide solution in 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mCi [γ-^{32}P]ATP, and 5 U of T4 polynucleotide kinase was incubated for 30 min at 37°C; then 1 μl of 10 mM ATP and 5 u of T4 polynucleotide kinase were added and the reaction was incubated for 30 min at 37°C. Labeled oligonucleotide was precipitated by 2% $LiClO_4$ in acetone and purified by electrophoresis in 20% polyacrylamide/8 M urea gel. [^{32}P]-RNA17 was eluted from the gel with 0.3 M NaAc, pH 5.0, containing 10 mM EDTA, 0.1% Na dodecylsulfate and precipitated by ethanol; [^{32}P]-DNA17 was eluted with 0.3 M $LiClO_4$ containing 0.5% Triton X-100 and precipitated by 2% $LiClO_4$ in acetone.

Preparation of In Vitro Transcripts of *MDR1* mRNA

The 190 nucleotides long *MDR1* mRNA fragment was prepared by in vitro transcription using T7 RNA polymerase and plasmid pMDR-670, linearized with restriction enzyme DraI, according to published protocol.[14] Standard reaction mixture (V = 300 μL) containing 50 mg/ml DNA; 40 mM Tris-HCl, pH 8.1; 16 mM $MgCl_2$; 2 mM spermidine; 10 mM dithiothreitol; 1 mM of each nucleoside triphosphate; and 200 U/mL T7 RNA polymerase was incubated at 37°C for 2 h. After phenol/chloroform extraction, the RNA transcript was purified by size-exclusion chromatography on Speen-column (Sigma), ethanol-precipitated, and dissolved in MilliQ water. Concentration of RNA was determined spectrophotometrically.

5′-End Labeling of *MDR1* mRNA

In vitro transcript of *MDR1* mRNA was dephosphorylated as described in Maniatis et al.[21] Reaction mixture (V = 50 μl) containing 400 pmol of in vitro transcript *MDR1* mRNA; 50 mM Tris-HCl, pH 8.5; 1 mM Na_2EDTA; 2.5 mM dithioerythreitol; 2% formamide; 200 U/mL RNAsin; and 10 U/mL alkaline phosphatase was incubated at 37°C for 30 min, then a new portion of alkaline phosphatase was added up to the concentration 20 U/mL, and the reaction was again incubated for 30 min. After phenol/chloroform extraction, dephosphorylated RNA was ethanol-precipitated, pelleted by centrifugation, washed with ethanol, dried, and dissolved in MilliQ water. [5′-^{32}P]-Labeling of RNA was performed according to the standard protocol.[22] Labeled RNA was purified in 8% polyacrylamide/8 M urea gel, eluted from the gel by extraction with 300 μL 0.3 M NaAc, pH 5.0 overnight, and precipitated as described above.

Probing of RNA Structure with Ribonucleases ONE and T1

Secondary structure of *MDR1* mRNA fragment was probed with RNase T1 and RNase ONE as described in Ehresmann et al.[15] Reaction mixtures (V = 10 μl) containing 50 mM HEPES, pH 7.0; 200 mM KCl; 5 mM $MgCl_2$; $1 \cdot 10^{-7}$ M [5′-^{32}P]-RNA fragment; and 1 mg/mL total tRNA from *E. coli* as a carrier were incubated 2 min at 37°C, 1 min at 0°C, and 10 min at 25°C, then RNase ONE or RNase T1 as added up to concentrations ranging from 0.1 to 0.5 U/mL and from 10 to 30 U/mL, respectively. The reaction mixtures were incubated at 25°C for 15 min. The RNA cleavage products were ethanol-precipitated and analyzed in 8% polyacrylamide/8 M urea gel. Imidazole and RNase T1 ladders were run in parallel. Imidazole ladder was prepared by incubation of [5′-^{32}P]-RNA in 2 M imidazole, pH 7.0; 1 mM EDTA; and 250 μg/mL tRNA-carrier, at 90°C for 10 min. T1 ladder was prepared under standard conditions.[23]

RNase H Footprinting of *MDR1* mRNA · Oligodeoxyribonucleotide Heteroduplexes

RNase H footprinting of *MDR1* mRNA oligonucleotide complexes was performed as follows.[24] Reaction mixture (V = 10 μL) containing $1 \cdot 10^{-7}$ M [5′-^{32}P]-*MDR1* mRNA and $1 \cdot 10^{-5}$ M oligonucleotide **dK-155** or conjugate **dK-155-Az**, 25 mM HEPES, pH 7.5, 100 mM KCl, 0.05 mM EDTA, 10 mM $MgCl_2$, 100 μg/mL tRNA-carrier, was incubated for 30 min at 37°C. Then RNase H was added up to concentration 1 U/mL, and the mixture was incubated at 37°C for 30 min. Reactions were quenched by ethanol precipitation. The RNA cleavage products were analyzed in 8% polyacrylamide/8 M urea gel.

Photomodification of Synthetic RNA and DNA Targets

Samples (V = 10 μL) containing $1 \cdot 10^{-7}$ M 5′-[^{32}P]-labeled target and $1 \cdot 10^{-5}$ M conjugate (**tK-155-Az**, **mK-155-Az**, or **dK-155-Az**) in 10 mM Tris-HCl

buffer, pH 7.4, containing 100 mM NaCl, 0.1 mM EDTA were placed into the wells of an immunological plate and irradiated for 10 min at 5, 20, or 40°C as described above. The nucleic acids were precipitated by 2% $LiClO_4$ in acetone and analyzed in 20% polyacrylamide/8 M urea gel. Gel was dried and autoradiographed for 16 h. To obtain quantitative data, the autoradiograph was digitized by Gel-Pro Analyzer (Media Cybernetics, Inc., USA). Relative experimental error was $\leq$ 10%. To determine DNA17 modification sites, the reaction mixture after irradiation at 5°C was treated with 10% aqueous piperidine (30 min at 100°C) followed by precipitation with 2% $LiClO_4$ in acetone and analysis by electrophoresis as described above.

Photomodification of *MDR1* mRNA

Reaction mixture (V = 10 μl) containing $1 \cdot 10^{-7}$ M [5′-^{32}P]-RNA; $1 \cdot 10^{-5}$ M conjugate (**tK-155-Az**, **mK-155-Az**, or **dK-155-Az**); 50 mM Tris-HCl, pH 7.2; 0.2 M KCl; and 100 μg/mL tRNA carrier was incubated for 10 min at 37°C in the dark, then UV-irradiated for 1, 3, 6, and 10 min. After irradiation, RNA was ethanol-precipitated. The products of RNA photomodification were analyzed in 8% polyacrylamide/8 M urea gel. To get quantitative data, the gels were dried and autoradiographed for 1 day, the bands corresponding to *MDR1* RNA and its modification products were cut out of the gel, and their radioactivity was measured by Cherenkov counting. Relative experimental errors did not exceed 10%.

Identification of Photomodification Sites

MDR1 mRNA, photomodified as described above, was purified by electrophoresis in an 8% polyacrylamide/8 M urea gel, eluted overnight from the gel by 300 μL 0.3 M NaAc, pH 5.0, and ethanol-precipitated, then RNA was pelleted by centrifugation, washed with ethanol, and dried. Modified RNA (0.05 μg) was dissolved in 5 μL of solution containing 60 nmol of a specific [5′-^{32}P]-RT primer d($^{5'}$CCCCCGTTCTTCTTC$^{3'}$), incubated 1 min at 90°C, then 1 min at 0°C, and 10 min at 25°C. Then 1 μL of RT-buffer (250 mM Tris-HCl, pH 8.3; 30 mM $MgCl_2$; 120 mM KCl) was added, and the reaction was incubated again for 10 min at 25°C. Then 2 μL of RT-buffer, dNTP mix (2 mM), AMV reverse transcriptase (100 U/mL) and H_2O up to 15 μL were added to the reaction mixture. Reactions were performed at 37°C for 1 h, the products were ethanol-precipitated and analyzed by electrophoresis in 8% polyacrylamide/8 M urea gel. To localize modification sites, extension reactions of unmodified RNA190 performed in the presence of corresponding dideoxynucleoside triphosphate were run in parallel.[14,15]

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