10-23 DNA enzymes for targeting *MDR1* mRNA

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The properties of the 31-mer 10-23 DNA enzyme and its analog with the terminal 3´-3´ internucleotide linkage, which are complementary to an mRNA region of the multi-drug resistance gene *MDR1*, were investigated. DNA enzymes can selectively cleave RNA with high efficiency in a catalytic mode as exemplified by a synthetic 18-mer fragment of *MDR1* mRNA.

Key words: 10-23 DNA enzyme, RNA cleavage, multi-drug resistance gene *MDR1*.

Considerable progress has been achieved in the chemistry of oligonucleotides and their derivatives and the *in vitro* and *in vivo* biological effects of these compounds were extensively studied. As a result, synthetic oligonucleotides that are capable of inhibiting gene expression at the mRNA level are presently considered as a new generation of promising therapeutic drugs, which can selectively suppress virus infections and tumors. The use of catalytic nucleic acids, *viz.*, oligonucleotides, which have characteristic secondary structures and can cleave mRNA in a catalytic mode, is a rapidly progressing field of research into the strictly selective action on particular mRNAs.[1,2]

Recently, sequences of DNA enzymes, *viz.*, oligodeoxyribonucleotides capable of catalyzing RNA cleavage, were found by the *in vitro* selection technique.[3] The 10-23 DNA enzyme proved to be the most efficient. This DNAenzyme contains a conserved catalytic domain flanked by two variable sequences, which are responsible for the complementary recognition of target RNA. In the presence of magnesium ions, the 10-23 DNA enzyme can cleave the phosphodiester bond between the purine and pyrimidine nucleotides of target RNA, which are located between the recognition regions.

The aim of the present study was to design the modified 10-23 DNA enzyme for the cleavage of mRNA of the multi-drug resistance gene *MDR1*. Its product, *viz.*, P-glycoprotein, removes drugs from cells thus hindering the treatment of tumors with chemotherapeutic agents.[4,5] We chose a region of *MDR1* mRNA in the vicinity of the translation initiation site (nucleotides 122—137), which is accessible for binding to oligonucleotides,[6] as the cleavage target.

Experimental

The reagents were purchased from ICN, Sigma, Aldrich (USA), Fluka (Switzerland), Merck (Germany), and Reakhim (Russia). [γ-^{32}P]Adenosine 5´-triphosphate (3000 Ci mol^{-1}) was from the Physical Energy Institute (Russia). The 18-mer fragment of *MDR1* mRNA, *viz.*, 5´-UCCAAGGAGCGCGUGGUC, was synthesized by the standard solid-phase phosphoramidite method on an automated ASM-102U synthesizer (Biosset, Russia).

The enzymatic reactions were carried out with the use of T4 polynucleotide kinase (EC 2.7.1.78) (Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences) and *E.coli* RNase H (EC 3.1.4.34) (Hybaid, UK).

Water was sterilized by adding diethyl pyrocarbonate (to 0.02%) and keeping at 37 °C for 12 h or at ~20 °C for 2 days.

The radioactive label was introduced into the RNA target as described previously.[7]

The cleavage reaction was carried out in a buffer containing 50 *mM* Tris*-HCl (pH 7.5) and 10 *mM* $MgCl_2$ at 37 °C. The concentration of RNA in the reaction mixture was varied from $0.5 \cdot 10^{-7}$ to $5.0 \cdot 10^{-7}$ mol L^{-1}. The concentration of the DNA enzymes was varied from $1.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-9}$ mol L^{-1}. To estimate the kinetic parameters, the cleavage reactions were conducted in the presence of an excess of the RNA target. The initial rate of cleavage was determined from the kinetic curves. Based on the results obtained, a straight line was plotted on the Lineweaver—Burk coordinates and the constants K_m and k_{cat} were determined from intercepts on the coordinate axes. To study the dependence of the extent of cleavage on the concentration of magnesium ions, RNA was incubated with the DNA enzymes in a buffer containing 50 *mM* Tris-HCl (pH 7.5) and $MgCl_2$ at a required concentration.

* Tris is tris(hydroxymethyl)aminomethane.

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Hydrolysis of RNA with *E.coli* RNase H was performed in a buffer containing 20 *mM* HEPES*-KOH (pH 8.0), 4 *mM* $MgCl_2$, 5 *mM* KCl, and 0.05% bovine serum albumin. The concentration of RNA in the reaction mixture was $1.0 \cdot 10^{-7}$ mol L^{-1} and the concentration of the oligodeoxyribonucleotides was $1.0 \cdot 10^{-6}$ mol L^{-1}. The reaction mixtures (10 μL) were incubated at 20 °C for 15 min. Then 0.01 IU RNase H was added and the mixture was incubated at 37 °C for 15 min.

The nucleotide material was precipitated from the reaction mixtures with a 2% $LiClO_4$ solution in acetone. The precipitate was dissolved in an 8 *M* urea solution containing 0.025% of bromophenol blue and xylene cyanole FF and then applied onto 20% denaturating polyacrylamide gel for electrophoretic analysis.

To determine the degree of RNA cleavage, the autoradiograph of the gel on a Renex X-ray film (Germany) was digitized with the use of the Gel-Pro Analyzer program package (Media Cybernetics, Inc., USA). The extent of cleavage was calculated as a ratio of the peak areas of the products to the sum of the peak areas of the products and the peak area of the starting oligonucleotide. The relative error in the determination of the cleavage extent did not exceed 20%.

Results and Discussion

The DNA enzyme under study is a 31-mer oligodeoxyribonucleotide containing a 15-mer catalytic domain flanked by two octadeoxyribonucleotides (Fig. 1). Since the ultimate aim of our investigation is to design catalytic structures for the *in vivo* use, we attempted to enhance the stability of the designed DNA enzyme with the use of a minimal chemical modification. It is known that the major oligonucleotide degradation pathway in cells involves the 3′-exonucleolytic cleavage.[8] To protect the 3′-terminus of the DNA enzyme, we used an approach proposed previously.[9,10] The 3′-terminal 3′—5′ phosphodiester bond was replaced by the inverted 3′—3′ bond. The ability of the DNA enzyme (**1**) and its 3′-modified analog (**2**) to cleave mRNA was examined with the use of the synthetic 18-mer fragment of *MDR1* gene mRNA.

The presence of the Mg^{2+} ions in the reaction mixture is the necessary condition for the RNA cleavage by the 10-23 DNA enzyme. We studied the effect of the concentration of $MgCl_2$ on the degree of RNA cleavage by DNA enzymes **1** and **2**. It appeared that the efficiency of the DNA enzyme in the reaction performed in a catalytic mode (in the presence of a 100-fold excess of the RNA target with respect to the DNA enzyme) was enhanced as the concentration of the magnesium ions was increased from 1 to 200 mmol L^{-1} (Fig. 2, *a*). By contrast, when the DNA enzyme and the RNA target were taken in an equimolar ratio, the extent of RNA cleavage reached 80% as the concentration of Mg^{2+} was increased

* HEPES is *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

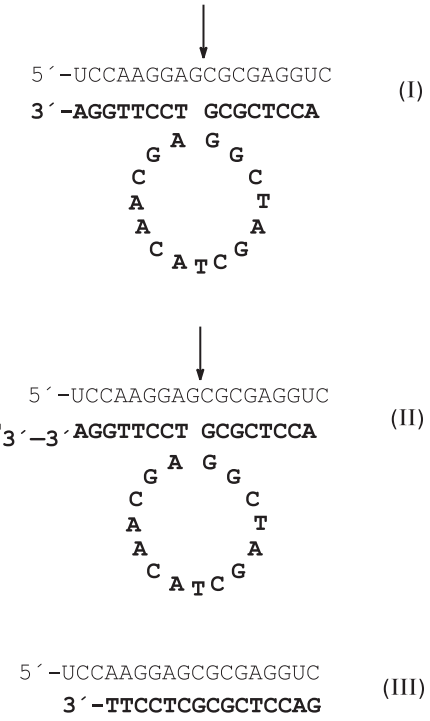

Fig. 1. Model system for the examination of RNA cleavage by the 10-23 DNA enzyme (duplex I), its 3′-modified analog (duplex II), and RNase H in the duplex III with the complementary pentadecadeoxyribonucleotide.

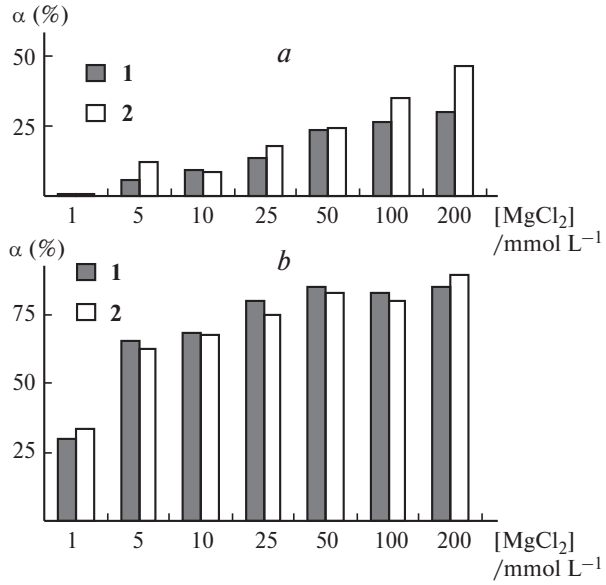

Fig. 2. Dependence of the extent of RNA cleavage (α) on the concentration of $MgCl_2$ ([$MgCl_2$]). The reactions were carried out in a buffer containing 50 *mM* Tris-HCl (pH 7.5) and $MgCl_2$ in required concentrations at 37 °C. The concentration of RNA in the reaction mixture was $1.0 \cdot 10^{-7}$ mol L^{-1}. The concentrations of the DNA enzyme (**1**) and its 3′-modified analog (**2**) were $1.0 \cdot 10^{-9}$ (*a*) and $1.0 \cdot 10^{-7}$ mol L^{-1} (*b*). The reaction time was 4 h (*a*) and 2 h (*b*).

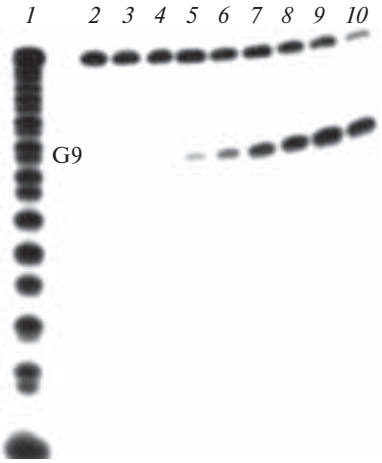

Fig. 3. Cleavage of RNA by the 3′-modified DNA enzyme (**2**). The reactions were carried out in a buffer containing 50 *mM* Tris-HCl (pH 7.5) and 10 *mM* $MgCl_2$ at 37 °C. The concentration of RNA and the DNA enzyme in the reaction mixture was $1.0 \cdot 10^{-7}$ mol L^{-1}; *1*, limited hydrolysis of target RNA by 0.05 *M* $NaHCO_3$ (pH 9.5, 90 °C, 10 min); *2*, RNA 4 h after incubation in the absence of the DNA enzyme; *3*, RNA 4 h after incubation with the DNA enzyme in the absence of $MgCl_2$; *4—10*, RNA after incubation with the DNA enzyme for 0, 5, 15, 30, 60, 120, and 240 min respectively.

to 50 mmol L^{-1} and then the extent of cleavage remained virtually unchanged as the $MgCl_2$ concentration was further increased (Fig. 2, *b*).

Based on the results obtained, the subsequent studies of RNA cleavage were carried out under the nearly physiological conditions (50 *mM* Tris-HCl (pH 7.5), 10 *mM* $MgCl_2$, 37 °C). In all cases, the residue G9 of target RNA was the only cleavage site. A typical autoradiograph of electrophoretic separation of the reaction products is shown in Fig. 3.

The dependence of the extent of RNA cleavage on the reaction time was studied at different DNA enzyme : target RNA ratios (Fig. 4). The kinetic parameters of RNA cleavage by the designed 10-23 DNA enzymes are given in Table 1. The efficiency of RNA cleavage by DNA enzymes may vary within rather wide ranges depending on the length and sequence of the substrate-binding regions of the DNA enzyme.[2] The kinetic parameters determined in this study are in agreement with the published data on the constants K_m and k_{cat} for the cleavage of short RNA substrates by the 10-23 DNA enzymes.[3,11,12] Interestingly, the introduction of the additional thymidine residue, which is not involved in the formation of a duplex with the RNA substrate and is bound through the 3′—3′ phosphodiester bond, at the 3′-terminus of the DNA enzyme leads to the enhancement of the catalytic efficiency k_{cat}/K_m (Table 1). The influence of the change in polarity of the 3′-terminal phosphodiester bond in the 10-23 DNA enzyme on the efficiency of cleavage of the 25-mer fragment of *c-myc*

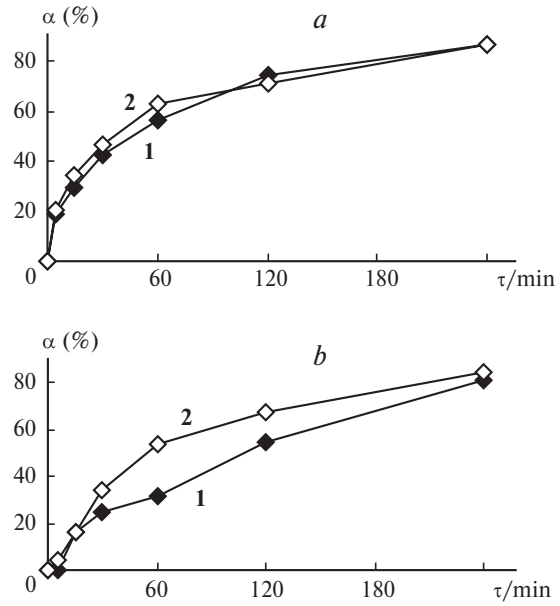

Fig. 4. Kinetic curves for cleavage of target RNA by the DNA enzyme (**1**) and its 3′-modified analog (**2**). The reactions were carried out in a buffer containing 50 *mM* Tris-HCl (pH 7.5) and 10 *mM* $MgCl_2$ at 37 °C. The concentration of RNA in the reaction mixture was $1.0 \cdot 10^{-7}$ mol L^{-1}. The concentrations of the DNA enzymes were $1.0 \cdot 10^{-6}$ (*a*) and $1.0 \cdot 10^{-8}$ mol L^{-1} (*b*).

mRNA was examined in the study.[12] In this case, the 3′-terminal nucleotide occurred in a duplex with RNA and the inversion of the 3′-terminal bond led to either an increase or decrease in K_m and k_{cat} depending on the lengths of the substrate-binding regions of the DNA enzyme. The authors of the cited study believed that the inversion of the 3′-terminal internucleotide linkage leads to a decrease in stability of the duplex of the DNA enzyme with the RNA substrate, which, in turn, causes a decrease in the rate of formation of the enzyme-substrate complex but simultaneously accelerates dissociation of the reaction products from the complex. It can be assumed that in our case, the dangling 3′-terminal thymidine residue bound through the 3′—3′ internucleotide linkage also causes a change in stability of the duplex with RNA. As a result, at this lengths of the flanking

Table 1. Kinetic parameters of RNA cleavage by DNA enzymes **1** and **2**

DNA enzyme	K_m /nmol L^{-1}	k_{cat} /min^{-1}	k_{cat}/K_m /L μmol^{-1} min^{-1}
1	98.7	0.021	0.21
2	22.8	0.025	1.1

Note. The concentration of the DNA enzymes was $1 \cdot 10^{-8}$ mol L^{-1}. The concentration of target RNA: $0.5 \cdot 10^{-7}$, $0.75 \cdot 10^{-7}$, $1.0 \cdot 10^{-7}$, $2.5 \cdot 10^{-7}$, and $5.0 \cdot 10^{-7}$ mol L^{-1}; 50 *mM* Tris-HCl (pH 7.5), 10 *mM* $MgCl_2$, 37 °C.

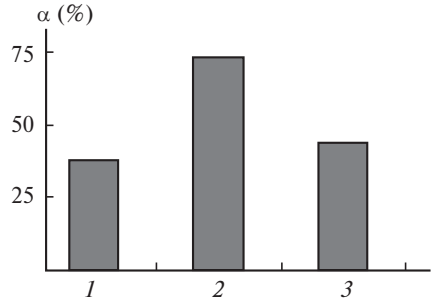

Fig. 5. Cleavage of RNA in the duplexes I (*1*, *2*) and III (*3*) in the absence (*1*) and presence of RNase H (*2*, *3*). The reactions were carried out in a buffer containing 20 *mM* HEPES-KOH (pH 8.0), 4 *mM* $MgCl_2$, 5 *mM* KCl, and 0.05% bovine serum albumin at 37 °C. The concentration of RNA in the reaction mixture was $1.0 \cdot 10^{-7}$ mol L^{-1}. The concentration of the DNA enzyme and pentadecadeoxyribonucleotide was $1.0 \cdot 10^{-6}$ mol L^{-1}.

regions, the catalytic efficiency of 3′-modified DNA enzyme **2** is enhanced. In the future, we plan to study the effect of the length of the flanking regions on the kinetic parameters of the RNA cleavage by this 3′-modified DNA enzyme.

Once inside the cell, the DNA enzyme can initiate hydrolysis of the mRNA target both due to its own catalytic activity and through hydrolysis of RNA in the DNAenzyme—RNA hybrid duplex by ribonuclease H. Actually, it appeared that in the presence of *E.coli* RNase H, the extent of RNA cleavage in the duplex with the DNA enzyme (Fig. 1, duplex I) is increased by the value close to the extent of RNA cleavage in the duplex III with the complementary pentadecadeoxyribonucleotide (Fig. 5). Hence it follows that under the conditions used, the presence of the 15-mer catalytic loop has no effect on the capability of the 10-23 DNA enzyme for activating RNase H.

To our knowledge, the 10-23 DNA enzymes have not been used previously for cleaving *MDR1* mRNA. The employment of hammerhead ribozymes for this purpose was described in the literature. The catalytic efficiency (k_{cat}/K_m) of RNA cleavage varied from 14.3 to 0.36 L μmol $^{-1}$ min^{-1} depending on the conditions of cleavage and the length of the RNA target.[13,14] Hammerhead ribozymes were successfully used for the cleavage of extended fragments of *MDR1* mRNA[13–15] and for inhibition of *MDR1* gene expression in the cell culture.[16,17] These results suggest that the 10-23 DNA enzymes, which are characterized by an analogous mechanism of activity and higher stability in biological media, show promise as efficient inhibitors of *MDR1* gene expression.

To summarize, the 10-23 DNA enzyme and its 3′-modified analog can selectively and highly efficiently cleave the synthetic fragment of *MDR1* gene mRNA in a catalytic mode.

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