ISSN 0026-8933, Molecular Biology, 2013, Vol. 47, No. 1, pp. 75–84. © Pleiades Publishing, Inc., 2013.
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GENOMICS.
TRANSCRIPTOMICS

UDC 577.113.6:578.832.1:578.282

Design of Deoxyribozymes for Inhibition of Influenza A Virus Reproduction

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Received February 29, 2012; in final form April 22, 2012

Abstract—Influenza A viruses play a significant role in human and animal pathologies that cause epidemics and epizootics. Therefore, the development of new anti-flu drugs has become increasingly urgent. Deoxyribozymes can be considered as promising antiviral agents due to their ability to efficiently cleave RNA molecules with high specificity. In this study, a number of genomic sequences of the most relevant influenza A virus subtypes, i.e., H5N1, H3N2, and H1N1, were analyzed. Conserved regions were revealed in the five least variable segments of the fragmented viral RNA genome, and potential sites of their cleavage with 10-23 deoxyribozymes were determined. We designed and synthesized 46 virus-specific 33-mer deoxyribozymes with the general structure of 5'N_8AGGCTAGCTACAACGAN_9. Screening of the antiviral activity of these agents in combination with lipofectin on the Madin-Darby Canine Kidney cells infected with highly pathogenic avian influenza virus A/chicken/Kurgan/05/2005(H5N1) revealed 17 deoxyribozymes that suppressed the titer of virus cytopathicity by more than 2.5 log$TCID_{50}$/mL (i.e. the neutralization index of the virus was more than 300), five of which suppressed the virus titer by a factor of 1000 or more. The most active deoxyribozymes appeared to be specific to segment 5 of the influenza A virus genome, which encoded NP nucleoprotein.

DOI: 10.1134/S0026893312060040

Keywords: deoxyribozymes, influenza A virus, cell culture, inhibition of viral replication

INTRODUCTION

Influenza A viruses belong to the *Orthomyxoviridae* family. Their genome consists of eight single-stranded RNA segments with negative polarity, each of which encodes one to two protein products, i.e., hemagglutinin (HA), neuraminidase (NA), three subunits of RNA-dependent RNA polymerase (PB1, OB2, and PA), nucleoprotein (NP), matrix protein (M1), membrane protein (M2), and unstructured proteins (NS1 and NEP/NS2) [1, 2]. A spherically shaped virion with a diameter of 80–120 nm is a nucleocapsid surrounded with a lipid bilayer coated with two kinds of glycoprotein spikes, i.e., hemagglutinin and neuraminidase. These surface antigens determine the subtype of the virus, i.e., H5N1, H3N2, H1N1, etc. To date, 16 hemagglutinin subtypes (H1–H16) and 9 neuraminidase subtypes (N1–N9) of the influenza A virus have been isolated from birds.

Influenza A viruses occupy a significant place in the infectious diseases of humans and animals that cause periodic epidemics and epizootics. Since the beginning of the 20th century, influenza viruses have caused three global epidemics that killed more than 50 million people. Over the last decade, the World Health Organization was concerned about the alternate appearance of new strains of highly pathogenic avian influenza virus H5N1 and swine influenza virus H1N1, which can be transmitted to humans. Part of the newly emerging strains is resistant not only to the previously used drugs of the adamantane series, amantadine and rimantadine (inhibitors of membrane protein M2), but also to modern flu drugs, oseltamivir and zanamivir (inhibitors of viral neuraminidases) [3–5]. It should also be noted that neuraminidase inhibitors are ineffective at the beginning of therapy after two or three days after infection; moreover, there have been recent reports of cases of acute mental disorders in patients treated with oseltamivir [6]. Thus, the drug problem in relation to influenza A viruses is far from being solved.

Abbreviations: dz is DNAzyme; ribozyme is RNAzyme; MDCK is Madin-Darby canine kidney cell; H5N1 is a highly pathogenic subtype of avian influenza A virus.

Currently, the development of new therapeutic preparations, including antiviral drugs based on the use of native and chemically modified nucleic acids, is underway. These drugs include antisense oligonucleotides, nucleozymes (ribo- and deoxyribozymes), aptamers, and short interfering RNAs, which are able to inhibit the gene expression and function of certain proteins or have some other specific functions [7–9]. Today, about 100 similar drugs are in various stages of clinical trials [10]. Two of them have already entered the pharmaceutical market, i.e., thiophosphorylated oligonucleotide Vitravene, for the treatment of rhinitis caused by cytomegalovirus, and aptamer Macugen, for the treatment of the wet form of senile macular degeneration. It is known that the principle of the action of antisense oligonucleotides is the formation of DNA–RNA hybrid duplexes with complementary regions of target mRNA, which leads to its cleavage with cellular RNase H and/or, depending on chemical modifications in antisense oligonucleotides, inhibits mRNA translation. The advantage of nucleozymes is their catalytic activity, i.e., the ability to cleave complementary RNA sequences. Unlike native ribozymes (RNAzymes), native deoxyribozymes (DNAzymes) are still not found. At the same time, artificial nucleozymes may be prepared by in vitro selection [11, 12]. Currently, a number of nucleozymes with RNase activity are known; they have a high catalytic rate and a high turnover number and can cleave a wide range of RNA substrates [13–15]. Due to the relatively high resistance to serum and cellular nucleases in vivo, DNAzymes are superior to RNAzymes.

The goal of this work was to design DNAzymes addressed to conserved RNA regions of currently topical H5N1, H3N2, and H1N1 subtypes of influenza A virus and a comparative study of the ability of these DNAzymes to inhibit the virus reproduction based on the example of highly pathogenic avian influenza virus H5N1.

EXPERIMENTAL

We used a highly pathogenic strain of avian influenza virus A/chicken/Kurgan/05/2005 (H5N1) and Madin-Darby canine kidney (MDCK) cell culture from the collection of microorganisms and cell cultures, respectively, in State Research Center of Virology and Biotechnology VECTOR (Novosibirsk, Russia). Deoxyribozymes were synthesized by the standard phosphoramidite method on an automated synthesizer (Biossan, Russia) using commercial phosphoramidite synthons (Glen Research, United States). Concentrations of oligonucleotides were evaluated taking into account the structure of each sequence using the OligoAnalyzer 3.1 program (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer).

Common conserved sites in RNA of the H5N1, H3N2, and H1N1 subtypes of influenza A virus strains were searched using the special NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/flu) and the Influenza Sequence Database (http://flu.lanl.gov). For multiple sequence alignments, we used the standard ClustalW algorithm (http://www.ebi.ac.uk/clustalw) and the BioEdit 7.0 program [16].

The cleavage of model target RNA with DNAzyme in vitro was performed using 25-mer synthetic oligoribinucleotide target labeled at the 5'-end with [γ-^{32}P]ATP and T4 polynucleotide kinase. The reaction of [^{32}P]RNA (10 μM) with DNAzyme (10 μM) was carried out in a buffer containing 25 mM $MgCl_2$ and 50 mM Tris-HCl, pH 7.5, at 37°C. The reaction mixtures were analyzed by gel electrophoresis under denaturing conditions in 20% PAAG. Gels containing [^{32}P]RNA and cleavage products were radioautographed on CP-BU NIF films (AGFA, Belgium). The intensity of the spots on the radioautogram were evaluated using the GelPro program package (Media Cybernetics, United States). The extent of the cleavage was calculated by the $I : (I + I_0)$ ratio, where I and I_0 are the intensities of bands that correspond to the cleavage product and the initial RNA target, respectively.

Antiviral activity of DNAzymes was studied on the model of infected MDCK cells. MDCK cells were grown in 96-well plates in RPMI-1640 medium (Biolot, Russia) in the presence of 5% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) for 1–1.5 days until a monolayer was formed. Virus A/chicken/Kurgan/05/2005 was produced on 10-day-old developing chick embryos [17]. The concentration of the virus in virus-containing allantoic fluid was evaluated by titration on MDCK cells to be 8.5log $TCID_{50}$/mL (logarithms of 50% tissue cytopathic doses in 1 mL). The RPMI-1640 medium containing trypsin (2 μg/mL) was used for serial tenfold dilutions of the allantoic fluid. The diluted samples (100 μL/well) were added to the cell monolayer at a moi of 0.0001–1.0 $TCID_{50}$/cell). After 1 h of incubation at room temperature, virus-containing liquid was removed and cells were rinsed with medium. Then, RPMI-1640 medium containing the studied DNAzyme with a final concentration of 10 μM and lipofectin transfection agent (Invitrogen, United States) in twofold excess over the mass of DNAzyme was added to each well containing a cell monolayer. The control well contained 100 μL of RPMI-1640 medium with lipofectin and without DNAzyme. Cells were incubated for 5 h at 37°C in 5% CO_2. Medium with (experiment) and without (control) the preparation was removed and a new portion of medium containing trypsin (2 μg/mL) was added to each well. After 3 days of incubation, the cytopathic action of virus was registered using an inverted microscope. The

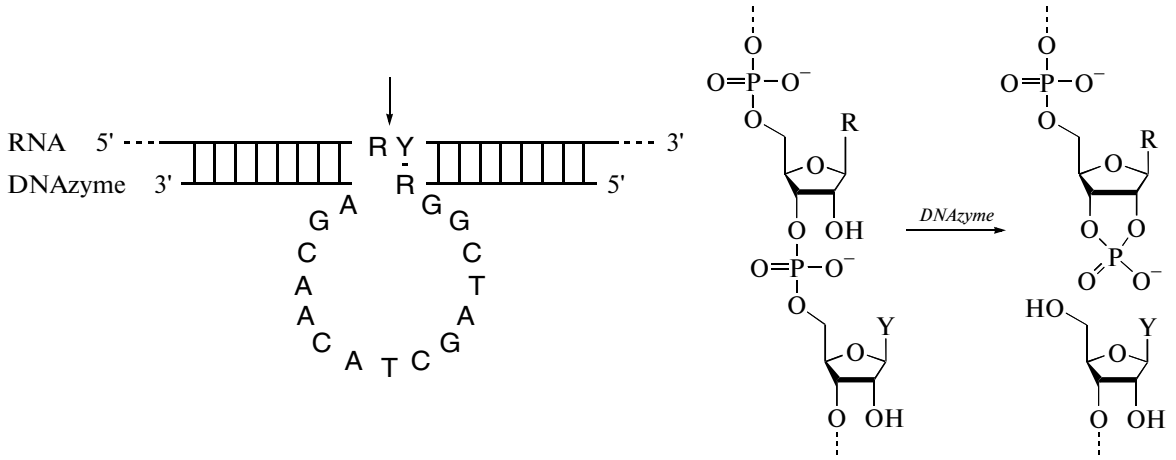

Fig. 1. Structure of 10-23 DNAzyme in complex with RNA substrate and scheme of the catalyzed cleavage of RNA with the formation of 2',3'-cyclophosphate at the 3'-end and the hydroxyl group at the 5'-end. "R" and "Y" designate purine and pyrimidine base/nucleotide, respectively.

presence of a virus in culture medium was confirmed by titration on MDCK cells. The measurements were performed after 3 days by the HA assay using 0.5% rooster erythrocytes [17].

RESULTS

Choice of General Structure of Virus-Specific DNAzymes

To date, one of the most promising reagents for the directed cleavage of RNA molecules of various origins are deoxyribozymes of the 10-23 type, namely, oligodeoxyribonucleotides with the 5'N_nR[GGCTAGCTACAACGA]N_n sequence, which consists of 15-mer catalytically active fragment (in box) flanked with RNA-recognizing sequences (Fig. 1). 10-23 DNAzymes can cleave target RNA at the minimal site 5'N_nR↓YN$_n$, where R = A or G and Y = U or C, with the most efficiently cleaved doublets being A↓U and G↓U [19]. 10-23 DNAzymes do not require cofactors and catalyze the RNA cleavage with the formation of 2',3'-cyclophosphate and 5'-hydroxyl groups (Fig. 1). The reaction rate ($k_{cat} \approx 1$ min^{-1}) for 10-23 DNAzyme is less than for native protein ribonucleases, e.g., RNAse A, by a factor of 10^4; however, the Michaelis constant ($K_m < 1$ nM) is less by a factor of 10^5 [19]. Thus, the specificity coefficient, which is the standard criterion of the enzymatic reaction efficiency, ($k_{cat}/K_m \approx 10^9$ M^{-1} min^{-1}) for 10-23 DNAzyme is even greater than for protein RNases.

The length of the substrate-binding regions of DNAzyme influences the specificity and stability of DNAzyme/RNA complexes and the rate of the DNAzyme release from complexes with products of the RNA cleavage. Consequently, the length of flanks should provide an optimal balance of these parameters and, in relation to the problem, can vary in six to ten or more nucleotide units. If necessary, the lengths of flanks can be asymmetrically varied depending on the particular target RNA structure or other experimental factors, but one must keep in mind that this can significantly affect the RNase activity of DNAzyme.

When cleaving high-molecular RNA targets (including viral RNAs), the main problem is related to their complex secondary and tertiary structure, i.e., with the steric accessibility of cleavage sites. This problem is usually solved experimentally by screening a large number of DNAzymes [20].

Taking into account the above considerations, we choose 10-23 DNAzymes with the following general structure: 5'N_8A[GGCTAGCTACAACGA]N_9. These DNAzymes are 33-mer oligonucleotides that contain a conserved catalytic domain (in box) and two substrate-recognizing 9-mer (in some cases 8/10-mer) flanks. DNAzymes are designed to cleave a target RNA at the AU or GU doublets with the formation of 2',3'-cyclophosphate of an unpaired purine nucleotide at the 3'-end of the resulting RNA fragment.

Study of Activity and Specificity of DNAzyme on Model RNA Target

10-23 DNAzyme and its modified derivatives addressed to the initiation translation region of the PB2 gene of influenza A/Puerto Rico/8/34 (H1N1) virus were previously studied in the works of Japanese authors [21, 22], who investigated the activity of DNAzyme called PB2Dz on a synthetic RNA target and its antiviral effect against influenza virus A/Puerto Rico/8/34 (H1N1) on the model of infected MDCK cells. Lipofectin and DOTAP were used as transfection agents. The authors showed a 50–100-fold decrease in the virus titer [22].

Table 1. Results of search for conserved regions and potential sites of their cleavage with 10-23 DNAzymes in segment 5 of RNA of influenza A virus[1]

(+/–) RNA strand	5'-sequence-3' of (+) or (–) strand of conserved region of RNA[1]	Length of a region	Positions in a segment[2]
(+)	AGCAAAAGCAGG G↓U AG A↓U A A↓U CACUCAC	28	1–28
(–)	GUGAGUG A↓U U A↓U CUACCCUGCUUUUGCU		
(+)	GAGUGACAUCAAC A↓U CAUGGCGUC	24	30–53
(–)	GACGCCAUG A↓U G↓U UGAUGUCACUC		
(+)	UAAUGAAGG A↓U CUU A↓U UUCUUCGGAGACAAUGCAGA	36	1491–1526
(–)	UCUGCAUU G↓U CUCCGAAGAA A↓U AAG A↓U CCUUCAUUA		
(+)	GAAAAAUACCCUU G↓U UUCUACU	22	1544–1565
(–)	AGUAGAAACAAGG G↓U AUUUUUC		

Notes: [1] Boxes and arrows designate potential cleavage sites.
[2] Numbering of nucleotides is given for the complementary (+) RNA copies.

In our work, we used DNAzyme PB2Dz [21, 22] as a control sample. PB2Dz was designed for the specific A/Puerto Rico/8/34 (H1N1) strain and was complementary to a rather variable part. We have adjusted its RNA-recognizing flanks by replacing two nucleotides to a working strain of avian influenza virus A/chicken/Kurgan/05/2005 (H5N1). Resulting DNAzyme 5'-TTCTCTCCAGGCTAGCTACAACGAATTGAATAT-3' is designated as dzPB2-28+ (1 in Table 2).

First, we studied the efficiency of the action of dzPB2-28+ on a synthetic RNA target radioactively labeled 25-mer oligoribonucleotide 5'[^{32}P]-AAUAUAUU CAAU A↓UG GAGAGAAUAA, which corresponds to the initiation translation region (in box) in mRNA of the PB2 gene of avian influenza virus A/chicken/Kurgan/05/2005(H5N1). Figure 2 shows an electrophoregram of the reaction mixtures and the kinetic curve obtained during the interaction of dzPB2-28+ and the target RNA in a molar ratio of 1 : 1. It can seen that, after just 10 min, the cleavage of the target occurs at more than 85%, and RNA is cleaved almost quantitatively in 2 h.

We then estimated the specificity of the cleavage of the synthetic RNA target with DNAzyme. During the interaction of RNA target with dzPB2-28+ in a molar ratio of 1 : 10, the highly specific cleavage of RNA occurs at the expected site of AU between unpaired A and paired U (Fig. 3).

Thus, the synthesized DNAzyme dzPB2-28+ retains the ability of the prototype DNAzyme PB2Dz [21, 22] to efficiently and site-specifically cleave the RNA target in the complementary complex.

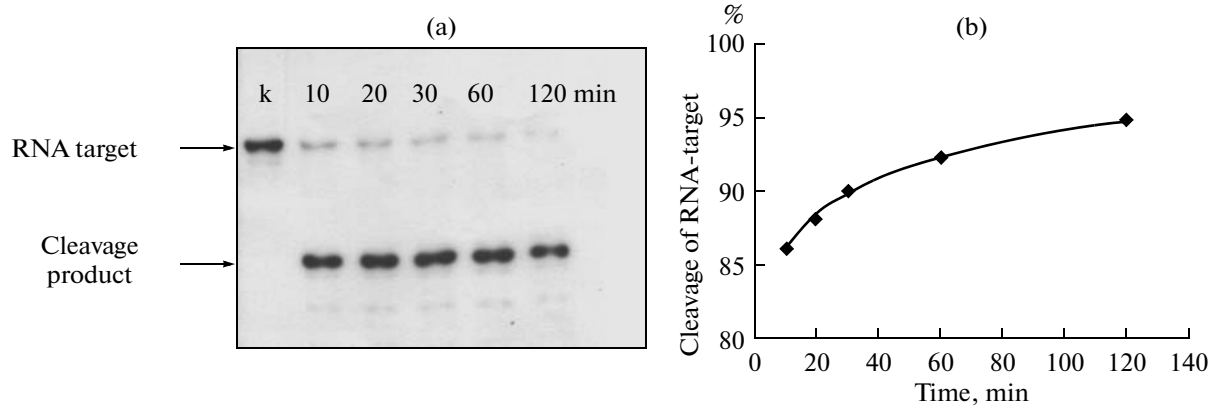

Fig. 2. Gel electrophoresis (a) and kinetic curve (b) of the reaction mixture after the action of DNAzyme dzPB2a-28+ (1 in Table 2) on model synthetic [^{32}P]RNA target corresponding to the initiation of the translation region of the PB2 gene of avian influenza virus A/chicken/Kurgan/05/2005(H5N1). Reaction was carried out in buffer containing 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5) at 37°C at concentrations of DNAzyme and RNA target being 10 μM.

Table 2. Structure of synthesized DNAzymes and titers of avian influenza virus A/chicken/Kurgan/05/2005(H5 N1) in control MDCK cells and after incubation with DNAzymes for 3 days after infection (virus titers are in decimal logarithms of 50% tissue cytopathic doses in 1 mL)

No.	Designation[1]	5'-3' sequence of DNAzyme[2]	Virus titer, log$TCID_{50}$/mL
	Control	–	6.5
1.	dzPB2-28+	TTCTCTCCA GGCTAGCTACAACGA ATTGAATAT	4.7
2.	dzPB2-175+	TTGCCATCA GGCTAGCTACAACGA CCATTTCAT	5.5
3.	dzPB2-179–	AATGGATGA GGCTAGCTACAACGA GGCAATGAA	5.5
4.	dzPB2-406–	AGGTTAAAA GGCTAGCTACAACGA ATGGAACCT	4.0
5.	dzPB2-2254+	CAGTAAGTA GGCTAGCTACAACGA GCTAGAGTC	5.5
6.	dzPB2-2287+	CCATCCGAA GGCTAGCTACAACGA TCTTTTGGT	4.0
7.	dzPB2-2294–	GAATTCGGA GGCTAGCTACAACGA GGCCATCAA	4.2
8.	dzPB2-2273–	ACAGCCAGA GGCTAGCTACAACGA AGCGACCAA	5.0
9.	dzPB2-2255–	ACTCTAGCA GGCTAGCTACAACGA ACTTACTGA	4.0
10.	dzPB2-2329–	TAAAAACGA GGCTAGCTACAACGA CTTGTTTCT	5.5
11.	dzPB1-19+	CCATTCAAA GGCTAGCTACAACGA GGTTTGCCT	5.5
12.	dzPB1-25+	TGACATCCA GGCTAGCTACAACGA TCAAATGGT	5.5
13.	dzPB1-30–	TTGAATGGA GGCTAGCTACAACGA GTCAATCCG	5.5
14.	dzPB1-20–	GGCAAACCA GGCTAGCTACAACGA TTGAATGGA	4.0
15.	dzPB1-2260+	TGGAACAGA GGCTAGCTACAACGA CTTCATGAT	4.0
16.	dzPB1-2270–	TCTGTTCCA GGCTAGCTACAACGA TTGAAGA	4.5
17.	dzPB1-2255–	CTGAGATCA GGCTAGCTACAACGA GAAGATCTG	5.5
18.	dzPA-43–	TTTGTGCGA GGCTAGCTACAACGA AATGCTTCA	4.0
19.	dzPA-719+	CCATCCACA GGCTAGCTACAACGA AGGCTCTAA	5.5
20.	dzPA-725+	TCGAATCCA GGCTAGCTACAACGA CCACATAGG	4.5
21.	dzPA-736–	GGATTCGAA GGCTAGCTACAACGA CGAACGGCT	4.0
22.	dzPA-726–	CTATGTGGA GGCTAGCTACAACGA GGATTCGAA	4.5
23.	dzPA-2092+	ACTCCTCAA GGCTAGCTACAACGA TGCTTCATA	5.0
24.	dzPA-2093–	ATGAAGCAA GGCTAGCTACAACGA TGAGGAGTG	5.5
25.	dzPA-2218+	AACAAGGTA GGCTAGCTACAACGA TTTTTTGGA	5.5
26.	dzPA-2221–	AAAAAAGTA GGCTAGCTACAACGA CTTGTTTCT	4.5
27.	dzNP-13+	GATTATCTA GGCTAGCTACAACGA CCTGCTTTT	5.5
28.	dzNP-17+	GAGTGATTA GGCTAGCTACAACGA CTACCCTGC	3.5
29.	dzNP-18–	CAGGGTAGA GGCTAGCTACAACGA AATCACTCA	3.7
30.	dzNP-43+	ACGCCATGA GGCTAGCTACAACGA GTTGATGTC	5.5
31.	dzNP-44–	ACATCAACA GGCTAGCTACAACGA CATGGCGTC	3.5
32.	dzNP-42–	TGACATCAA GGCTAGCTACAACGA ATCATGGCG	3.5
33.	dzNP-1500+	GAAATAAGA GGCTAGCTACAACGA CCTTCATTA	3.2
34.	dzNP-1505+	CCGAAGAAA GGCTAGCTACAACGA AAGATCCTT	4.0

Table 2. (Contd.)

No.	Designation[1]	5'-3' sequence of DNAzyme[2]	Virus titer, log $TCID_{50}$/mL
	Control	–	6.5
35.	dzNP-1506–	AGGATCTTA GGCTAGCTACAACGA TTCTTCGGA	4.5
36.	dzNP-1501–	AATGAAGGA GGCTAGCTACAACGA CTTATTTCT	4.5
37.	dzNP-1557+	AGTAGAAA GGCTAGCTACAACGA AAGGGTATTT	6.5
38.	dzNP-1551–	GAAAAATA GGCTAGCTACAACGA CCTTGTTTCT	5.5
39.	dzM-44+	ACGTTTCGA GGCTAGCTACAACGA CTCGGTTAG	5.5
40.	dzM-168–	GGCTAAAGA GGCTAGCTACAACGA AAGACCAAT	6.5
41.	dzM-227+	CACTGGGCA GGCTAGCTACAACGA GGTGAGCGT	4.0
42.	dzM-252+	AAGCGTCTA GGCTAGCTACAACGA GCTGCAGTC	4.0
43.	dzM-257–	CAGCGTAGA GGCTAGCTACAACGA GCTTTGTCC	4.5
44.	dzM-225–	TCACGCTCA GGCTAGCTACAACGA CGTGCCCAG	4.0
45.	dzM-579–	CCAGCACTA GGCTAGCTACAACGA AGCTAAGGC	5.0
46.	dzM-1012–	AGTAAAAAA GGCTAGCTACAACGA TACCTTGTT	3.5

Notes: [1] PB2, PB1, PA, NP and M are RNA segments; figure represents the number of a nucleotide (or complementary to it) in full-sized (+) RNA segment (as well as in cDNA), at the 3'-end of which the cleavage occurs; +/– designate (+) or (–) strand of viral RNA cleaved with DNAzyme.)

[2] Boxes show catalytic fragment of DNAzymes.

Choice of Regions in Influenza A Virus RNA and Their Sites of Cleavage with Virus-Specific DNAzymes

As mentioned above, the goal of this study is the development of universal DNAzymes aimed at influenza A virus strains of H5N1, H3N2, and H1N1 subtypes. The task is complicated by the fact that the genetic material of the virus consists of eight RNA segments and, when a body is infected by two or more strains, segments of the viral genome can be mixed in any combination. The second reason for the variability of influenza A viruses are adaptive mutations of RNA during replication. Thus, deoxyribozymes should be aimed at the most functionally significant segments of the viral genome or, more precisely, their conserved regions.

A summarized analysis of more than 36000 protein sequences encoded by various strains of influenza A viruses isolated over the past 30 years was carried out [23]. These data show that, in the case of heterogeneous and variable surface glycoproteins, i.e., hemagglutinin (HA) and neuraminidase (NA), which determine the virus subtype, the level of conservatism is expectedly low. The most conserved are subunits of RNA-dependent RNA polymerase (PB2, PB1, and PA), nucleoprotein NP, and matrix protein M1. These proteins are crucial for viral replication. Thus, it is obvious that the most promising targets for the action of DNAzymes are rather extended invariable regions of five segments of viral RNAs and/or their transcripts, which encode PB2, PB1, PA, NP, and M1.

To reveal general conserved regions, we used the following strains: A/turkey/Suzdalka/12/05(H5N1), A/goose/Guangdong/1/96(H5N1), A/chicken/Kurgan/05/2005(H5N1), A/Hong Kong/1/68(H3N2), A/New York/392/2004(H3N2), A/Brisbane/10/2007(H3N2), A/Puerto Rico/8/34(H1N1), A/New Caledonia/20/1999(H1N1), and A/California/04/2009(H1N1). These strains are usual laboratory standards or they are chosen for reasons of their relevance in the past few years and the availability of full genomic sequences. As a small, such a sample, in our opinion, was quite sufficient to meet the challenges of this work.

After the alignment of sequences of five RNA segments of the mentioned influenza A viruses strains, we identified 24 conserved regions with lengths of more than 19 nucleotides, which were promising in terms of designing virus-specific oligonucleotide reagents, such as antisense oligonucleotides, small interfering RNA, and RNA-cleaving nucleozymes.

As an example, we show the result of the search of conserved regions and potential sites of their cleavage with 10-23 DNAzymes with the sequence of $5'N_8A$ GGCTAGCTACAACGA N_9 for segment 5 of influenza A virus encoding nucleoprotein NP (Table 1). Despite the expected high overall level of nucleotide sequence identity, only four conserved regions in (+)

and (–) strands with lengths of 22–36 nucleotides that contain 15 potential cleavage sites are of practical interest in terms of designing virus-specific DNAzymes. A similar analysis of segments 1, 2, 3, and 7, which encode subunits of RNA-dependent RNA polymerase and matrix protein M1, revealed approximately 70 more possible cleavage sites.

The further choice of DNAzyme structures was determined by criteria such as the minimization of the formation of homodimeric or hairpin structures and the most central position within the conserved target site of viral RNA (if there are several cleavage sites within one conserved region). As a result, 45 deoxyribozymes of 33 nucleotides in lengths specific to both (+) and (–) strands of invariable regions in RNA segments of influenza A virus of H5N1, H3N2, and H1N1 strains were synthesized in addition to control DNAzyme dzPB2-28+ (Table 2).

Study of Antivirus Activity of DNAzymes on Infected MDCK Cells

The antiviral activity of the synthesized virus-specific DNAzymes was studied on the model MDCK cells infected with the high-pathogenic strain of avian influenza virus A/chicken/Kurgan/05/2005(H5N1). The concentration of DNAzymes was 10 μM. To transport DNAzymes into cells, we used lipofectin, a widely used transfection agent for the delivery of oligonucleotide/plasmid DNAs in eukaryotic cells. Cytopathic action was registered in the cell monolayer by an invert microscope 3 days after transfection, followed by the evaluation of the virus titer in the culture medium by the hemagglutination reaction with 0.5% rooster erythrocytes [17].

The results of screening of the antiviral activity of the synthesized DNAzymes are presented in Table 2 in $\log TCID_{50}$/mL (logarithms of 50% tissue cytopathic doses in 1 mL) without the addition (virus control) and with the addition of the DNAzyme sample to infected cells. In addition, Fig. 4 shows a histogram of the neutralization indices, i.e., the ratios of $TCID_{50}$/mL in the virus control to this value in the experiment with DNAzyme. Most DNAzymes demonstrated antiviral activity to a greater or lesser extent. Seventeen DNAzymes that, under experimental conditions, lead to the reduction of cytopathic virus activity by no less than by 2.5 $\log TCID_{50}$/mL (i.e. neutralization index was 300 or more) were particularly noteworthy; five of them inhibited the virus titer by three orders of magnitude. It should be noted that four out of the five most active DNAzymes (dzNP-17+, dzNP-44–, dzNP-42–, and dzNP-1500+) were specific to the (+) or (–) RNA of segment 5 of virus genome encoding nucleoprotein NP. This protein plays a central role in the migration of viral RNAs to nucleus of infected cells and is required for subsequent replication and assembly of the virus [24–26]. The fifth of the most effective DNAzymes (dzM-1012–) was specific to (–) RNA of segment 7 of the viral genome, which encodes the matrix and membrane proteins.

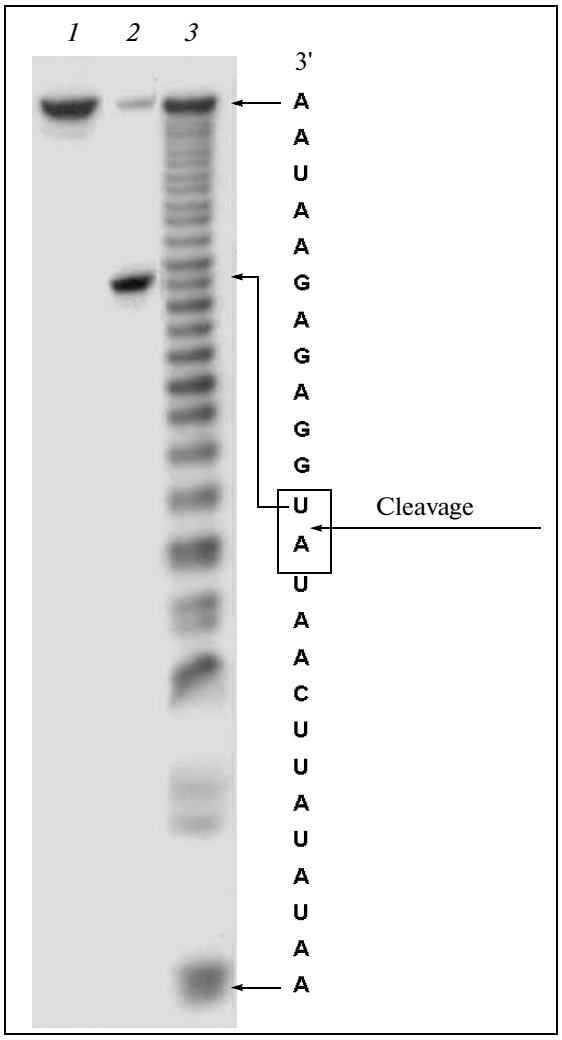

Fig. 3. Electrophoregram of reaction mixture after action of DNAzyme dzPB2a-28+ on model synthetic [^{32}P]RNA target. Reaction was carried out in buffer containing 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5) at 37°C at concentrations of DNAzyme and RNA target being 10 μM and 1 μM, respectively. Initial [^{32}P]RNA target in a buffer (lane 1); RNA target + DNAzyme (lane 2); partial alkaline hydrolysis of RNA target (lane 3). RNA structure and its cleavage site are shown on the right.

As expected, control DNAzyme dzPB2-28+ (a modified version of PB2Dz [21, 22]) also showed antiviral activity, i.e., a 50-fold reduction in virus titer was achieved under experimental conditions.

DISCUSSION

The threat of influenza, which seems to be a familiar seasonal infection, is actually much more serious because of the high variability of influenza viruses, the

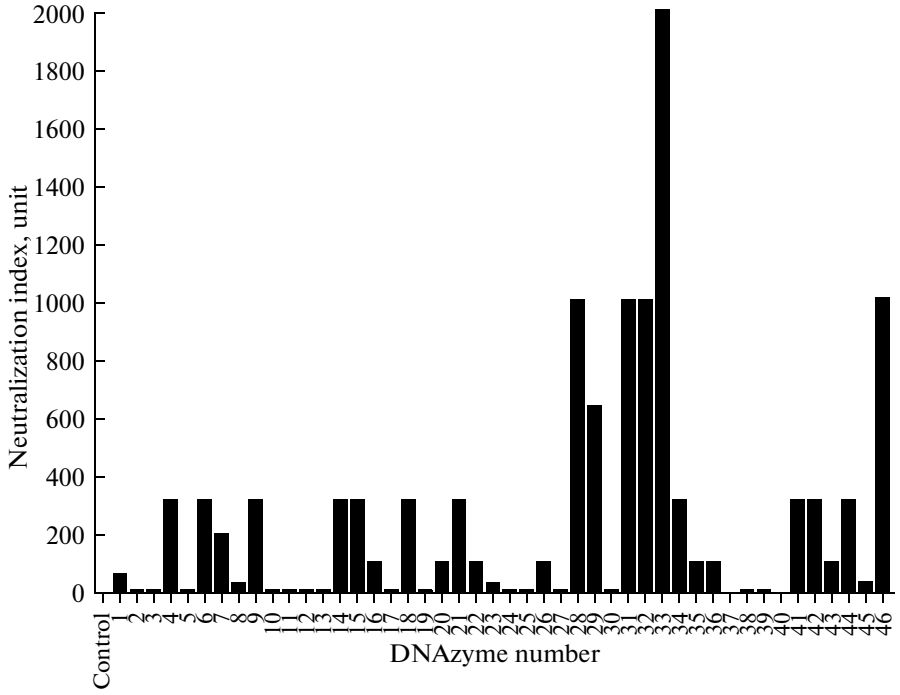

Fig. 4. Results of screening antiviral activity of DNAzyms on model MDCK cells infected with avian influenza virus A/chicken/Kurgan/05/2005(H5N1). Lipofectin was used as transfection agent. Concentration of DNAzymes was 10 μM. Histogram is presented in values of neutralization indices (ratios of $TCID_{50}$/mL in the virus control to this value in the experiment with DNAzyme). Numbering of DNAzymes on abscissa corresponds to their numbers in Table 2.

limited effectiveness of seasonal vaccines and imperfect therapy. Hence, there is a need for further work on the search for new therapeutic agents. Ongoing approaches to the development of effective agents that suppress the replication of the influenza virus are described in the summary monograph [27]. It has been shown that there are high expectations associated, in particular, with DNAzymes, which are capable of the highly specific catalysis of the cleavage of functionally important viral RNAs. Some works have been aimed at creating new chemically modified DNAzymes in order to increase their catalytic activity, stability toward cellular exo-and endonucleases, the change of cleavage sites, etc., as well as developing methods for evaluating antiviral activity in vitro and in vivo [22, 27–29]. However, as noted [27, p. 138], the most conserved sequences in the influenza virus genome of all known subtypes have not yet been selected. The choice of these regions may contribute to the creation of therapeutic drugs, the activity of which will be less dependent on mutations.

The aim of this study was to design DNAzymes addressed to the general conserved regions in RNA of influenza A virus of subtypes H5N1, H3N2, and H1N1, which have become important in recent years, and a comparative study of their ability to inhibit viral reproduction based on the example of highly pathogenic avian influenza virus of subtype H5N1.

At first, to determine the most universal DNAzymes against several influenza A viruses, we used the published data [23], where the authors gave a global analysis of protein sequences encoded by strains of human and avian influenza A viruses (subtypes H1N1, H1N2, H3N2, H5N1, etc.) isolated over the last 30 years. As expected, the conservativeness is not detected in case of heterogenous and variable surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which determine subtypes of influenza A virus. The subunits of the RNA-dependent RNA polymerase (PB2, PB1 and PA), nucleoprotein NP, and matrix protein M1, which are the most functionally important for viral replication, are the most conserved.

The mentioned RNA segments were aligned, and 24 conserved blocks with the length of more than 19 nucleotides were revealed. These regions were further taken into account to design virus-specific deoxyribozymes. The deoxyribonucleotide of the 10-23 structure was chosen as the basis to cleave the $5'N_nR{\downarrow}YN_n$ fragment in the RNA sequence.

An example of the search results for potential cleavage sites in RNA segment 5 of influenza A virus with 10-23 DNAzymes is presented in Table 1. In the same way, about 80 potential cleavage sites were revealed in the (+) and (–) strands of conserved regions of segments 1, 2, 3, and 7 in genomes of influenza A viruses. The next step of choosing DNAzymes

was determined by criteria such as the minimization of the formation of homodimeric or hairpin structures and a most central position within the conserved target site of viral RNA (if there are several cleavage sites within one conserved region). As a result, 46 deoxyribozymes (along with control dzPB2-28+) with lengths of 33 nucleotides were synthesized, which were specific to both the (+) and (–) strands of full-sized regions (including nontranslated 5'- and 3'-sequences) in RNA segments of influenza A virus of H5N1, H3N2, and H1N1 strains (Table 2).

The main experimental result was the screening of the antiviral activity of the synthesized DNAzymes aimed at genomic sequences of influenza A virus on model MDCK cells infected with avian influenza virus A/chicken/Kurgan/05/2005(H5N1). Most DNAzymes demonstrate antiviral activity to some extent (Tables 2 and 4). Seventeen DNAzymes are of particular interest, since, under experimental conditions, they lead to a reduction in the cytopathic virus activity by a factor of 300; five of them inhibited the virus titer by three orders of magnitude. It is interesting that four of the most active DNAzymes were specific to (+) or (–) RNA of segment 5 of virus genome, which encodes nucleoprotein NP. The role of this protein was considered above [24–26]. The fifth preparation (dzM-1012–) was specific to (–) RNA of segment 7, which encodes the matrix and membrane proteins. Thus, we revealed and synthesized a number of virus-specific DNAzymes that have pronounced antiviral activity. These DNAzyms are promising in terms of the preparation of anti-flu drugs on their basis.

In conclusion, it should be noted that similar gene-targeted drugs can be quickly redirected to any newly emerged virus strain (including drug-resistant mutant forms), while the search for new effective low molecular weight compounds usually takes years.

ACKNOWLEDGMENTS

The work was supported by Federal Target Program, grant no. 02.512.12.2038 and analytical departmental target program of Ministry of Education and Science of RF "Development of Scientific Potential of Higher Education," grant no. 2.1.1/5642.

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