Antiviral Activity of a New Class of Chemically Modified Antisense Oligonucleotides against Influenza A Virus

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Abstract—The paper reports the synthesis of a series of antisense oligonucleotides (aONs) directed against different segments of the influenza A virus genome (H1N1) and screening of their antiviral activity in the influenza A virus (H1N1)/MDCK cells and influenza A virus (H1N1)/A549 cells systems. The results of screening have shown that PB1-2AUG-R aON targeted towards the AUG codon region of the segment 2 of virus genome possessed the highest antiviral activity. The synthesized morpholino analog (PMO) and the new phosphoryl guanidine oligodeoxyribonucleotide (PGO) with the sequence of oligonucleotide PB1-2AUG-R provided a comparable biological effects: the influenza virus titer in MDCK cell culture was reduced by 15 times compared to the control when PGO was used in the concentration of 10 μ M and by 40 times when PMO was used in the concentration of 20 μ M. For the delivery of electrically neutral analogs of oligonucleotides (PGO and PMO), the perforation method proposed for PMO was used.

Keywords: influenza virus, pandemic influenza A virus (H1N1), antisense oligonucleotides, phosphoryl guanidines, morpholino oligonucleotides

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INTRODUCTION

Currently, according to WHO data, up to 20% of the population suffer from respiratory infections caused by influenza virus, depending on which viruses circulate in a particular influenza season. The challenges in the control of influenza infection are in direct connection with the specific features of the virus genome organization and functioning, in particular, the low efficiency of preventive immunization is mainly due to the extraordinarily high rate of virus evolution, which in its turn is partially accounted for by the segmented structure of the genome and its possibility for gene rearrangement [1–3].

To date, vaccination is considered the best way to protect against a virus infection. The new technologies which have emerged in the field of vaccine development have made it possible to obtain combined vaccines, vaccine vectors, DNA vaccines, etc, which are characterized by a lower frequency of adverse effects at the same time retaining the high efficiency of vaccina-

tion [4, 5]. However, due to the fact that influenza viruses change every year, and what is more, during the season, epidemics may be caused by different influenza virus types and subtypes, the use of influenza vaccines is inherently associated with certain problems which include, on one hand, the need to constantly vary vaccine composition, including new viral antigens in it according to the principle “new virus—new vaccine,” and, on the other hand, the need for annual revaccination in order to develop efficient immunity against new virus strains. Vaccination against the influenza virus is undoubtedly effective, but it is preventive in its nature and does not provide total protection against the disease, or against the complications caused by it. Currently, the vast majority of anti-influenza drugs are effective only against influenza A virus (but not B), have a wide range of contra-indications, and can lead to the emergence of new drug-resistant strains of the influenza virus [6]. Therefore, the development of preparations for prevention and treatment influenza infection remains very important problem.

Antisense oligonucleotides (aONs) possessing unique ability for specific interactions with biopolymers are a highly specific tool for regulation of gene expression [7–9]. One of the most important practical applications of antisense oligonucleotides in health care may be the control of infectious diseases, primar-

Abbreviations: aON, antisense oligonucleotide; PGO, phosphoryl guanidine oligonucleotide; PMO, morpholino oligonucleotide; PBS, phosphate buffered saline.

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ily, viral infections. The possibility of using aONs as antiviral agents was first demonstrated in principle in the works [10, 11].

The efficacy of antisense oligonucleotides largely depends on the selection of the optimal hybridization site within the target RNA molecule. However, the inhibitory activity of the oligonucleotide may not necessarily match the functional importance of the complementary RNA region. In addition, even a small shift in the oligonucleotide binding site may result in a decrease in, or even a complete loss of, their activity [12–14]. The results of the previously performed works have demonstrated that the use of antisense oligonucleotides and their derivatives to suppress influenza virus reproduction in the model systems *in vitro* is rather promising.

In the present work, we assessed the antiviral activity of the series of aONs directed against different regions of the influenza virus A/WSN/33/H1N1 genome by their ability to reduce virus titers in the cell culture. We have identified the leading aONs and demonstrated that the use of the modified oligodeoxynucleotides increases the antiviral activity of the compounds.

RESULTS AND DISCUSSION

The search and the development of the new highly active drugs that selectively suppress influenza A virus is an issue of a particularly urgent nature, since currently only a small number of drugs (adamantane derivatives inhibiting the M2 ion channel (Rimantadine and Amantadine) and neuraminidase inhibitors (Zanamivir and Oseltamivir)) are currently used in the clinic for the treatment of influenza infection.

One of the promising directions in the area of development of antiinfluenza pharmaceutical agents is design and synthesis of antisense oligonucleotides (aONs), the single-stranded DNA, or RNA molecules, or their analogs about 20 nt in length which are complementary to a certain sequence either in the influenza A virus genome itself (RNA(–)), or in the complementary RNA(+) copy synthesized inside the infected cell and serving as a template for the synthesis of viral proteins (mRNA). As a result of aON binding with the RNA target, RNA may be sterically blocked with subsequent suppression of the target protein expression, or, in the case of using aON of RNaseH substrates the RNA target may be enzymatically cleaved by RNase H. In other words, the effect of aONs consists in blocking the expression of viral proteins and suppression of virus replication.

The disadvantages of aONs are (1) their sensitivity to intracellular nucleases and (2) difficulties in aON delivery into the cell.

The most promising aON sequences were selected based on (a) the literature analysis (selection of the most efficient anti-influenza aONs described in liter-

ature) and (b) oligonucleotide design using the Influenza Research Database (<http://www.fludb.org>).

It is well known that influenza A virus genome is segmented and consists of eight segments of single-stranded RNA encoding 11 proteins (PB1, PB1-F2, PB2, PA, HA, NP, NA, M1, M2, NS1, and NS2). The search for aONs needed for our study was designed in such a way that the selected oligonucleotides were characterized not only by the high efficiency of action, but also were targeted to as many segments of the influenza A viral genome as possible. Suppression of the reproduction of the influenza virus can be achieved by blocking transcription, translation, or replication of viral RNAs carried out both by the virus-specific enzymes and host cell proteins. For this reason, both vRNAs representing segments of the viral genome, and mRNAs encoding viral proteins were used as the targets for antisense oligonucleotides. It has been traditionally believed that the most efficient suppression of virus development can be achieved through the action of derivatives of oligonucleotides on the mRNAs and genome segments encoding the protein components of the viral polymerase complex. Hence, in the majority of works devoted to the design and study of antiviral compounds based on oligonucleotide conjugates or oligonucleotide analogs, the oligonucleotides were directed either against mRNAs, or genome segments encoding PB1, PB2, and PA subunits of the polymerase complex. This target seems to us as the most promising, since the RNA polymerase plays the central role in the influenza virus life cycle.

In most works, the authors selected antisense nucleotide sequences considering the functional importance of the RNA region and the degree of their conservatism, which is critical when working with the influenza virus.

As a result of the analysis of the literature analysis [15–18], we chose 14 aON sequences possessing a sufficiently high antinfluenza activity according to the literature data, namely, 3 aONs were directed against the segment 1, encoding PB2, 7 aONs, against the segment 2 encoding PB1, 2 aONs, against the segment 3, encoding PA, and 2 aONs, against the segment 5, encoding NP (Table 1). The selected aONs can be roughly divided into three groups: (1) the oligonucleotides directed against the mRNA region including AUG codon (PB1-1AUG, PB1-2AUG, PB1-3AUG, PB2-1AUG, PB2-2AUG, PA1-AUG, and PA2-AUG); (2) the oligonucleotides directed against the loop region of the segment, PB2-loop (segment 1) and PB1-1loop, PB1-2loop, and PB1-3loop (segment 2); (3) the oligonucleotides directed against the noncoding conserved domains located at the start and at the end of each influenza A virus RNA segment [19], Flut (5'-terminal conservative region of segment 2) and NP (3'-terminal conservative region of segment 5).

In addition to aONs selected based on the literature data, we designed the aON series using the influenza

Table 1. Nucleotides sequences of the antisense oligonucleotides used in the work and the efficiency of antiviral activity

Code	Sequence 5'-3'	Target segment		Mean log ₁₀ (virus titer), logFFU/mL
			Reference	
Flut-F Flut-R	ccttggtttctact agtagaacaagg	5'-Conservative region	[17]	3.6 ± 0.2 3.1 ± 0.5
PB2-2AUG-R PB2-2AUG-F	cttttattctttccatattg caatatggaaagaataaaaag	Segment 1	[15, 16]	3.7 ± 0.5 3.9 ± 0.6
PB2-1AUG-F PB2-1AUG-R	tatattcaatatggaaagaa ttctttccatattgaatata		[16]	3.7 ± 0.1 3.5 ± 0.1
PB2-loop-F PB2-loop-R	ctgaatttcgtcaataggg ccctattgacgaaattcag		[16]	3.4 ± 0.4 3.5 ± 0.4
PB1-2AUG-R PB1-2AUG-F	gacatccattcaaattggtttg caaaccatttgaatggatgtc		[16, 18]	2.6 ± 0.5* 2.8 ± 0.4*
PB1-1AUG-R PB1-1AUG-F	cattcaaattggtttgctgc gcaggcaaaccatttgaatg	Segment 2	[18]	3.7 ± 0.4 3.7 ± 0.4
PB1-3AUG-F PB1-3AUG-R	aaccatttgaatggatgtca tgacatccattcaaattgggt		[18]	3.6 ± 0.2 2.9 ± 0.4*
PB1-2loop-F PB1-2loop-R	attaggcgtctccatcctga tcaggatggagacgccta		[16]	3.1 ± 0.5 3.1 ± 0.6
PB1-1loop-F PB1-1loop-R	atgttaagtactgtattaggc gcctaatacagtacttaacat		[16]	3.7 ± 0.2 3.6 ± 0.2
PB1-3loop-F PB1-3loop-R	atcctgaatcttggacaaaagag ctcttttgtccaagattcaggat		[16]	3.5 ± 0.1 3.8 ± 0.1
PA2-AUG-R PA2-AUG-F	caaaatcttccattttgaatc gattcaaaatggaagattttg		[15, 16]	3.6 ± 0.4 3.9 ± 0.3
PA1-AUG-F PA1-AUG-R	ctgattcaaaatggaagatt aatcttccattttgaatcag		[16]	3.6 ± 0.1 3.8 ± 0.1
HA-NP-R HA-NP-F	gaaaaacacccttgtttctact agtagaacaaggtgtttttc	Segment 4	Designed by the authors	3.4 ± 0.4 3.3 ± 0.4
NP-F NP-R	catcgaaatcatggcg cgccatgatttcgatg	Segment 5	[18]	3.4 ± 0.5 3.8 ± 0.4
NS-R NS-F	accctgcttttgct agcaaaagcagggt	Segment 8	[15]	3.8 ± 0.4 3.9 ± 0.3
NS1-1-F NS1-1-R	ggtgatgccccattccttgatc gatcaaggaatggggcatcacc		Designed by the authors	3.7 ± 0.5 3.5 ± 0.4
NS1-2-F NS1-2-R	ccgagatcagaagtccttaagag cacttagggacttctgatctcgg		Designed by the authors	3.6 ± 0.5 3.8 ± 0.6

F—forward—sequence corresponding to the nucleotide sequence of the (+)RNA of influenza A virus. R—reverse—sequence corresponding to the nucleotide sequence of the (–)RNA of influenza A virus. Virus titer in the cell culture incubated in the absence of antisense oligonucleotides was 3.8 ± 0.3 logFFU/mL. * $p < 0.05$.

Fig. 1. Kinetics of virus infection in the MDCK (a) and A549 (b) cells in vitro. Cells were infected with influenza virus for 1 h at 37°C in the presence of 5% CO₂ at the multiplicity of infection of 0.0001, 0.001, and 0.01 focus forming units (FFU/cell). After incubation, cells were washed from the virus with PBS, fresh infectious medium was added, and cells were incubated for 10, 18, and 26 h. Upon the end of incubation, the plates were subjected to a single freeze–thaw cycle, and virus titers were determined in the supernatant by the focus forming assay.

A virus genome sequences available in the Influenza Research Database focusing first of all on the aONs targeted to those viral RNA segments, which were not used in the published works. The work was carried out in two steps. At the first step, the secondary structure of the studied segment was analyzed using the on-line RNAstructure server (<http://rna.urmc.rochester.edu/RNAstructureWeb>) and regions with imperfect secondary structure which aONs may act upon with high probability were determined. At the second step, the optimal oligonucleotide sequences were selected using the Oligoanalyzer Tool Internet resource (<http://www.idtdna.com>), the selection criteria in this case being the minimum Gibbs energy and inability to form hairpins and dimers under physiological conditions. As a result, the following aONs were selected:

(1) HA-NP—aON directed against the noncoding 5'-terminal region of segment 4.

(2) NS1-1—aON directed against the 108–130 nt region of segment 8; according to El Hefnawi et al. [20] this region is highly conservative.

(3) NS1-2—this aON was designed based on the secondary structure of the conservative domain of segment 8 presented in [21].

In total, 17 aONs were synthesized 14 aONs selected during the literature analysis and 3 aONs proposed based on the analysis of the structure of viral RNAs. It should be mentioned that in the latter case, we synthesized not only the selected aON sequences, but also the complementary ones, which means that aONs were directed both against the (+) and (–) strands of the influenza A virus RNA. Thus, 34 aON was used in the study (Table 1).

In the present work, the laboratory influenza virus A/WSN/33 (H1N1) strain, which was kindly provided by Prof. N.V. Kaverin, Dr. Sci. (Ivanovskii Institute of Virology, Russian Academy of Medical Sciences, Moscow) was used. The antiviral properties of the syn-

thesized aONs were evaluated in the MDCK (epithelial cells of canine kidney) and A549 (human lung adenocarcinoma) cell cultures, which were obtained from the Collection of the Institute of Cytology, Russian Academy of Sciences (St. Petersburg).

To determine the conditions for in vitro experiments using aONs, we have identified the optimal influenza virus dose for cell infection and time of incubation of cells with aON. For this, MDCK and A549 cells were infected with the influenza A virus at the multiplicity of infection (MOI) of 0.0001, 0.001, or 0.01 focus forming units (FFU) per cell for 1 h at 37°C; cells were further washed with phosphate-buffered saline (PBS) to remove the virus, fresh infectious medium was added, and cells were incubated for 10, 18, and 26 h. At the end of incubation, the plates were subjected to a single freeze–thaw cycle, and virus titers were determined in the supernatant by a focus forming assay (FFA) (Fig. 1).

It has been demonstrated that for both cell lines, the optimum MOI in the aON experiments was 0.001 FFU. In this case, virus titer did not exceed 10⁴ FFU/mL (Fig. 1). Higher virus titers observed at MOI = 0.01 FFU appeared to be unacceptable since at these titers, viral infection develops too quickly which does not allow to reliably determine the effects of the analyzed aONs; for the initial screening we used non-modified oligodeoxyribonucleotides, which are sensitive to cellular nucleases, this characteristic significantly reduce their antiviral activity. At the same time, MOI = 0.0001 FFU also appeared to be unsuitable because of the extremely low virus titers observed in this case. The optimum time point for sample collection and freezing at MOI = 0.001 FFU was 18 h post infection (here and below, hpi). Under these viral infection conditions, virus titer in the supernatant approaches to its maximum values yet does not reach it (Fig. 1).

Screening of the synthesized aONs for their ability to suppress influenza A virus reproduction in vitro revealed that the majority of them either reduced virus titers to 0.5 log FFU/mL or have no effect on the viral replication (Table 1). The observed low antiviral activity of the aONs may be associated with the efficiency of their delivery into the infected cells. In these experiments, Lipofectamine® 2000 was used for aON delivery into the cell which is a “gold standard” for the delivery of double-stranded DNA and siRNA mainly in nanomolar concentrations, but not in micromolar level used in our experiments. It should also be noted that the aONs described in the literature, the sequences of which were selected for screening were chemically modified. The most effective antiviral activity was observed for the PB1-2AUG-R, PB1-2AUG-F, and PB1-3AUG-R aONs directed to segment 2 (Table 2, shown in bold). Incubation of cells with these aONs decreased virus titer in the culture medium to 2.6 ± 0.5 , 2.8 ± 0.4 , and 2.9 ± 0.4 log FFU/mL, respectively, while virus titer in the control, the cells infected with the virus, without treatment, was 3.8 ± 0.3 log FFU/mL. These aONs are promising candidates for the introduction of chemical modifications.

The use of unmodified antisense oligonucleotides in vivo is significantly restricted by their high sensitivity to cellular nucleases [22]. This problem can be solved by using oligonucleotides containing various modifications of the sugar-phosphate backbone, namely, phosphorothioate, PNA, LNA, PMO, and phosphoryl guanidine (PGO) oligonucleotides. The direct comparison of the antiviral activity of nonmodified and phosphorothioate oligodeoxynucleotides

Table 2. Antiviral activity of the morpholino aON in vitro

	$\log_{10}$ (virus titer), logFFU/mL
Control*	4.3 ± 0.2
PMO-PB1-2AUG-R, 20 μ M	$2.7 \pm 0.2^{***}$

* Control—virus-infected cells without aON treatment.

*** $p < 0.001$

revealed that morpholino oligonucleotides have more pronounced antiviral efficacy and lower toxicity in vitro.

To increase the efficiency of antiviral activity, two types of aON were synthesized and tested, namely, the morpholino oligomer (PMO) and phosphoryl guanidine oligodeoxyribonucleotide (PGO), which are electroneutral analogues of oligonucleotides (Fig. 2).

These analogs are electrically neutral, therefore, their intracellular delivery is problematic when using classical transfectants based on cationic liposomes, such as Lipofectamine. To date, several approaches to electroneutral aON transfection have been developed, the most well-known of which are electroporation [23], direct microinjection [24], osmotic transfection [25], the use of streptococcal hemolytic endotoxin [25], aON conjugation with peptides [26], dendrimers [27], complementary DNA strands, and ethoxylated polyethyleneimine [28], and a number of other techniques.

To deliver PMO into the cells, we used the perforation technique using a cell scraper, specially developed by Partridge et al. [29] for morpholino oligonucleotides delivery as well as electroporation. In the case of electroporation, the cell death rate reached 40% even in the absence of virus infection and the obtained results were not reproducible (data not published). So

Fig. 2. The structures of the 2'-deoxy PGO (a) and PMO (b) aON analogs used in the study as the nuclease-resistant aONs.

Table 3. The influence of transfection approaches of PGO-PB1-2AUG-R on the antiviral activity in vitro

Delivery method		$\log_{10}(\text{virus titer}), \log\text{FFU/mL}$
Lipofectamine	Control*	3.8 ± 0.2
	PGO-PB1-2AUG-R, 5 μM	4.3 ± 0.5
	PGO-PB1-2AUG-R, 10 μM	4.1 ± 0.2
Perforation technique	Control*	4.9 ± 0.1
	PGO-PB1-2AUG-R, 5 μM	4.3 ± 0.3
	PGO-PB1-2AUG-R, 10 μM	$3.7 \pm 0.1^{**}$
Endo-Porter	Control*	2.9 ± 0.4
	PGO-PB1-2AUG-R, 5 μM	2.8 ± 0.4
	PGO-PB1-2AUG-R, 10 μM	2.4 ± 0.3

* Control—virus-infected cells without aON treatment.

** $p < 0.05$.

in the experiments we settled on the perforation method. Cell death did not exceed 10% in the absence of virus infection. This approach is based on detachment of cell monolayer from the plate bottom using the cell scraper in the presence of the aON solution of desired concentration.

The study of the antiviral activity of the PMO analog of the leading aON, PMO-PB1-2AUG-R, using perforation technique for intracellular delivery, revealed that modification of aON significantly increased antiviral activity in vitro—investigated aON at 20 μM was shown to reduce virus titer by 40 times compared to control (Table 2).

PGOs represent a new class of electrically neutral nucleic acid analogs [30, 31]. As in the case of phosphorothioate oligonucleotides, the backbone modification restricted only to phosphate moieties of oligomer and introduced by standard amidophosphite method on the oxidation step of attached unit. Therefore, it is possible to obtain various backbone types by using commercially available monomer sets (deoxy-, 2'-OMe-, LNA, 2'-F, etc.) and different reagent on the oxidation step to obtain different types of internucleotide phosphate groups (phosphodiester, phosphorothioate and phosphoryl guanidine).

In the present work, we focused on completely modified oligodeoxyribonucleotide derivative, in which each internucleotide bond contains a tetraalkyl-substituted guanidine-1,3-dimethyl imidazolidine-2-imine residue. It has been demonstrated that oligonucleotides containing phosphoryl guanidine groups are able to form stable duplexes with natural nucleic acids without changing their structure [32].

We have synthesized the PGO analog of the most active PB1-2AUG-R aON (further referred to as PGO-PB1-2AUG-R) (Fig. 2). We compared three approaches for PGO-PB1-2AUG-R delivery: using Lipofectamine, using the commercially available

agent based on the Endo-Porter peptide representing a specially designed porter for uncharged nucleic acid analogs, and the perforation technique using the cell scraper, which was developed for the delivery of PMO analogs [29] (Table 3).

Predictably, Lipofectamine was shown to not deliver PGO-PB1-2AUG-R into the cells, as indicated by the absence of the antiviral effect (Table 3). A decrease in the virus titer when Endo-Porter was used was observed only for the PGO-PB1-2AUG-R concentration of 10 μM and amounted to 3.2 times compared to the corresponding control. The best result was obtained using the perforation approach. In this case, PGO-PB1-2AUG-R showed a pronounced antiviral activity, at the concentrations of 5 and 10 μM it significantly reduced virus titers by 4 and 15 times compared to the control (Table 3).

Thus, it has been shown that modified electroneutral aONs have a greater antiviral activity compared to unmodified ones, which indicates the importance of the nucleus resistance property of the used class of oligonucleotides. The phosphoryl guanidine analog PGO-PB1-2AUG-R (at the concentration of 10 μM) reduced the virus titer by 1.5 times, while the morpholino analog (at the concentration of 20 μM), by 4 times compared to the unmodified PB1-2AUG-R (at the concentration of 20 μM).

It seemed obtained results revealed similar antiviral effects of nonmodified aON and PGO. However, it should be taken into account that the nonmodified oligonucleotide was delivered into the cells using Lipofectamine 2000, which ensures transfection of 100% of cells along with the high percentage of aON accumulation in the cell. In the case of PGO, its delivery into the cells was carried out by perforation technique, which does not involve the usage of special transfection agent. According to published data, in this case, no more than 10–15% of the amount of oli-

gonucleotide delivered using a transfectant enters the cell. The fact that similar antiviral effects were observed for the two aONs even under these conditions and at a two times lower concentrations (20 μ M for the nonmodified aON and 10 μ M for PGO) indicates that PGO may be used as a platform for development of effective antiviral agents.

Apart from the differences in the concentrations used, the higher activity of the PMO can probably be associated with the choice of the delivery method. The perforation technique was designed specifically for morpholino derivatives and, hence, may not be sufficiently effective in the case of 2'-deoxy PGO.

It has been recently demonstrated in a number of works that the efficiency of the antiviral activity of aONs varies considerably and depends on the selected target influenza virus genome region, model strain, and to a large extent on the delivery method. In the study performed by Gabriel et al. [33], the authors tested PMO conjugated with arginine-rich peptides (P-PMO), complementary to the translation start region of the PB1 mRNA, or to the 3'-terminal region of NP of the highly pathogenic mouse-adapted influenza virus strain A SC35M (H7N7). MDCK cells were incubated with P-PMO for 5 h prior to the infection with the virus at MOI = 0.001, and 48 h later, virus titers significantly decreased, namely, incubation with P-PMO reduced the virus titer by 3 log, while when P-PMO-PB1 was used, virus titer was reduced by 6 log.

However, in the work performed by Lenartowicz et al. [34], the efficiency of 2-*O*-methyl and LNA aONs complementary to the region of segment 8 of the virus RNA of the influenza virus strain A/California/04/2009 (H1N1) was considerably lower, with virus titers 18 h after the virus infection at MOI = 0.001 being reduced by 10 times compared to the control. Therefore, apart from the abovementioned parameters, efficiency of antiviral activity is affected by the type of modification introduced into the sugar-phosphate backbone of the oligonucleotide. Unlike the nucleic acid analogs with electroneutral backbone with "conservative" structure such as PNA and PMO, PGO can be synthesized in a standard automated DNA/RNA synthesizer, while using a variety of commercially available monomers and modifiers it is possible to obtain a high diversity of PGO backbone variants including gapmer and chimeric constructs.

Therefore, PGO may be used as a platform for the development of effective antisense agents. In the case of PGO-PB1-2AUG-R based on the 2'-deoxy PGO backbone in contrast to the aONs based on PMO it is possible to obtain aONs with higher efficiency of penetration into the cell and/or higher biological effect varying the parts of the antisense agent backbone without changing its nucleotide sequence.

EXPERIMENTAL

Virus and cells. MDCK canine kidney epithelial cell culture and A549 human lung adenocarcinoma cells obtained from the Collection of the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg) were used. Cells were cultured in DMEM medium in the presence of 10% fetal bovine serum, antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin), and antimycotic amphotericin (0.25 μ g/mL).

The laboratory influenza virus strain A/WSN/33 (H1N1) kindly provided by Prof. N.V. Kaverin (Ivanovskii Institute of Virology of the Russian Academy of Medical Sciences, Moscow) was used as a model virus. Influenza virus was cultured according to technique described in [35].

MDCK and A549 cells were infected with influenza A virus in IMDM medium in the presence of the indicated concentrations of the antibiotics, antimycotic, and TPCK-trypsin (2 μ g/mL) hereinafter, the infectious medium).

Synthesis of antisense oligonucleotides. The oligonucleotides were synthesized on automatic synthesizer ASM800 (Biosset, Russia) according to the standard protocol of the 2-cyanoethyl phosphoramidite method using the standard commercial deoxyribonucleoside monomers and the corresponding porous glass (Glen Research, United States). Oligonucleotides containing phosphoryl guanidine units were synthesized using the protocol previously described in the works [30, 31].

Native oligonucleotides and phosphoryl guanidine analogs (PGO) were purified by RP-HPLC performed in the Agilent 1200 series high-pressure liquid chromatograph (Agilent, United States) on the column (4.6 $\times$ 150 mm) containing the Eclipse XDB-C18 sorbent (5 μ m) (Agilent, United States) in the 0–90% linear acetonitrile concentration gradient in 0.02 M triethylammonium acetate solution for 30 min, with a flow rate of 1.5 mL/min.

Morpholino oligonucleotides with neutral phosphorodiamidate internucleotide bond (PMO) were synthesized according to the method described in the patent [36]. To do this, first the corresponding activated morpholino nucleoside monomers (T, C, A, and G) and aminomethyl polystyrene resin with disulfide linker (modification rate 629 μ mol/g) as a solid phase carrier were obtained. Nonautomated synthesis of oligomers on the resin was performed in 1-mL spin columns (20 μ m) placed in the 2-mL tubes (Receiver Columns 740522, Macherey-Nagel).

Analytical HPLC was carried out in the Milikhrom A-02 chromatograph (Econova, Russia) using the reverse-phase column, 2 $\times$ 75 mm, ProntoSIL 120-5C18 AQ (Bischoff, Leonberg, Germany).

The patented solid phase synthesis method does not include the capping stage, thus the use of this tech-

nique results in the pool of fractions mostly containing the target product. Preparative HPLC was performed on the column packed with the Q Sepharose™ Fast Flow resin (GE Healthcare, Sweden, $V = 2.5 \times 10$ cm). The product was eluted in the concentration gradient of B solution in A solution ($0 \rightarrow 100\%$, 2×150 mL, A—0.28% ammonia, and B—1 M NaCl in 0.28% ammonia). The product of subsequent detritylation was purified by solid phase extraction on Polyoprep C18, 50–100 μ (Macherey-Nagel, Germany) (3 mL).

To determine the amount of the PMO obtained, the optical density of the solution in 50% methanol was measured ($\lambda = 260$ nm, spectrophotometer UV-1800 (Shimadzu, Japan)). The oligomer amount was calculated adjusted for dilution, extinction coefficient, and molecular weight.

Molecular weights of oligonucleotides and oligonucleotide analogs were determined by mass spectrometry (with electrospray ionization) in Agilent G6410A LCMS/MS mass spectrometer (United States). Native oligonucleotide samples were prepared by dissolving them in 20 mM triethylammonium acetate in 60% aqueous acetonitrile solution to a concentration of 0.1 mM and volume 10 μ L. The analysis was performed using 80% aqueous acetonitrile as an eluent with the flow rate of 0.1 mL/min in the negative ion mode. To analyze uncharged PGO and PMO analogs, the samples were prepared by dissolving them in 20 mM formic acid in 60% aqueous acetonitrile to a concentration of 0.1 mM and volume 10 μ L. The analysis was performed in the positive ion mode.

Cell Transfection with aON

Using lipofectamine. MDCK cells in IMDM medium (with serum, without antibiotic) were inoculated into 48-well plates in a concentration of 40 000 cells/well and volume 200 μ L/well. The plates were incubated at 37°C in the presence of 5% CO₂ for 24 h.

aONs were diluted with Opti-MEM medium to the concentration of 120 μ M. Lipofectamine 2000 (Invitrogen, United States) was diluted with Opti-MEM medium at the rate of 2 μ L of lipofectamine per well, incubated for 5 min at room temperature, and mixed with the aON solution at the 1 : 1 volume ratio. The resulting solution was incubated at room temperature for 20 min. Further, 100 μ L of DMEM (without antibiotic and serum) were added to the well, then 50 μ L of the lipofectamine + aON solution, so that the final concentration of ODN was 20 μ M, and the obtained mixture was incubated at 37°C in the presence of 5% CO₂ for 4 h. Each aON transfection was performed in three replications. After incubation, the supernatant was removed, and cells were infected with the virus at the corresponding multiplicity of infection for 1 h under the standard conditions. After the incubation, virus inoculate was removed and 250 μ L of the infectious medium was added to the wells without washing

cells, and incubation was performed at 37°C in the presence of 5% CO₂ for 18 h. Then, the supernatant was collected, and virus titers in it were determined by focus forming assay.

Using the perforation technique. A549 cells were inoculated into 24-well plates to the concentration of 10⁵ cells/well in DMEM medium containing 10% serum and incubated for 24 h under the standard conditions. After incubation, cells were washed with PBS; 250 μ L of aON solution of the appropriate concentration was added to the cells, and cells were mechanically detached using a cell scraper. Cell were then incubated for 10 min under the standard conditions, and after the incubation, serum was added to the wells to the final concentration of 10% (v/v). The plates were further incubated at 37°C in the presence of 5% CO₂ for 4 h. After incubation, the supernatant was removed, and cells were infected with the virus at the corresponding multiplicity of infection for 1 h under the standard conditions. After incubation, viral inoculate was removed and 250 μ L of the infectious medium was added to the wells without washing cells, and incubation was performed at 37°C in the presence of 5% CO₂ for 18 h. Then, the supernatant was collected, and virus titers were determined by focus forming assay.

Using endo-porter. A549 cells were placed into 24-well plates in a concentration of 10⁵ cells/well in DMEM medium containing 10% serum and incubated for 24 h under the standard conditions. After incubation, the number of cells in the wells was calculated, and cells were infected with the virus at the corresponding multiplicity of infection for 1 h under the standard conditions. Then, cells were washed with PBS to remove the residual virus, aON solution with the analyzed concentration containing Endo-Porter (6 μ L/mL) was added, and cells were incubated for 18 h. The supernatant was further collected, and virus titers were determined by focus forming assay.

Virus titer measurement in the focus forming assay. To determine virus titers by focus forming assay, cells were grown in 96-well plates in DMEM medium containing 10% fetal bovine serum and 2 mM glutamine till they formed a monolayer. The medium was then removed, cells were washed with the infectious medium and infected with different virus dilutions. After incubation at 37°C for 24 h, the medium was removed, and cells were washed with PBS and fixed with cooled acetone for 10–15 min. After fixing was finished, cells were washed with PBS, and treated with the monoclonal antibodies against the influenza A virus NP protein (Chemicon, United States) at 37°C for 30 min. After incubation with antibodies, cells were washed again with PBS, and goat antibodies against mouse IgG conjugated with horse-radish peroxidase (Sigma, United States) were added. The presence of peroxidase in the complexes located on the surface of the infected cells was revealed using the enzymatic reaction with the chromogenic substrate 3-amino-9-

ethylcarbazole (AEC) (Sigma). Virus-infected stained cells (foci) were counted in the LOMO inverted microscope (St. Petersburg).

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COMPLIANCE WITH ETHICAL STANDARDS

No experimentation involving human or animals was part of the present work.

Conflict of Interests

Authors declare that they have no conflict of interest.

REFERENCES

- Drake, J.W. and Holland, J.J., *Proc. Natl. Acad. Sci. U. S. A.*, 1999, vol. 96, pp. 13910–13913.
- Domingo, E., *Virology*, 2000, vol. 270, pp. 251–253.
- Ferguson, N.M. and Anderson, R.M., *Nat. Med.*, 2002, vol. 8, pp. 562–563.
- Palese, P., Basler, C.F., and Garcia-Sastre, A., *Nat. Med.*, 2002, vol. 8, pp. 927–928.
- Wareing, M.D. and Tannock, G.A., *Curr. Opin. Pulm. Med.*, 2002, vol. 8, pp. 209–213.
- Luscher-Mattli, M., *Arch. Virol.*, 2000, vol. 145, pp. 2233–2248.
- Kole, R., Krainer, A., and Altman, S., *Nat. Rev. Drug Discov.*, 2012, vol. 11, pp. 125–140.
- Crooke, S.T., *Methods Enzymol.*, 2000, vol. 313, pp. 3–45.
- Agrawal, S. and Kandimalla, E.R., *Curr. Cancer. Drug Targets*, 2001, vol. 1, pp. 197–209.
- Zamechnik, P. and Stephenson, M., *Proc. Natl. Acad. Sci. U. S. A.*, 1978, vol. 75, pp. 280–284.
- Stephenson, M. and Zamechnik, P., *Proc. Natl. Acad. Sci. U. S. A.*, 1978, vol. 75, pp. 285–288.
- Hatta, T., Nakagawa, Y., Takai, K., Nakada, S., Yokota, T., and Takaku, H., *Biochem. Biophys. Res. Commun.*, 1996, vol. 223, pp. 341–346.
- Hatta, T., Takai, K., Nakada, S., Yokota, T., and Takaku, H., *Biochem. Biophys. Res. Commun.*, 1997, vol. 232, pp. 545–549.
- Abe, T., Hatta, T., Takai, K., Nakashima, H., Yokota, T., and Takaku, H., *Nucleosides Nucleotides*, 1998, vol. 17, pp. 471–478.
- Duan, M., Zhou, Z., Lin, R.X., Yang, J., Xia, X.Z., and Wang, S.Q., *Antivir. Ther.*, 2008, vol. 13, no. 1, pp. 109–114.
- Ge, Q., Pastey, M., Kobasa, D., Puthavathana, P., Lupfer, C., Bestwick, R.K., Iversen, P.L., Chen, J., and Stein, D.A., *Antimicrob. Agents Chemother.*, 2006, vol. 50, no. 11, pp. 3724–3733.
- Leiter, J.M., Agrawal, S., Palese, P., and Zamechnik, P.C., *Proc. Natl. Acad. Sci. U. S. A.*, 1, vol. 87, no. 9, pp. 3430–3434.
- Abe, T., Mizuta, T., Hatta, T., Miyano-Kurosaki, N., Fujiwara, M., Takai, K., Shigeta, S., Yokota, T., and Takaku, H., *Eur. J. Pharm. Sci.*, 2001, vol. 13, no. 1, pp. 61–69.
- Neumann, G. and Kawaoka, Y., *Nat. Rev. Microbiol.*, 2015, vol. 13, no. 1, pp. 28–41.
- ElHefnawi, M., Alaidi, O., Mohamed, N., Kamar, M., El-Azab, I., Zada, S., and Siam, R., *Virol. J.*, 2011, vol. 8, p. 44.
- Priore, S.F., Kierzek, E., Kierzek, R., Baman, J.R., Moss, W.N., Dela-Moss, L.I., and Turner, D.H., *PLoS One*, 2013, vol. 8, no. 9. e70615.
- Dirin, M. and Winkler, J., *Expert Opin. Biol. Ther.*, 2013, vol. 13, no. 6, pp. 875–888.
- Peng, H., Wu, Y., and Zhang, Y., *PLoS One*, 2012, vol. 7, no. 8. e43748.
- Kos, R., Tucker, R.P., Hall, R., Duong, T.D., and Erickson, C.A., *Dev. Dyn.*, 2003, vol. 226, no. 3, pp. 470–477.
- Ghosh, C. and Iversen, P.L., *Antisense Nucleic Acid Drug Dev.*, 2000, vol. 10, no. 4, pp. 263–274.
- Moulton, H.M., *Curr. Pharm. Des.*, 2013, vol. 19, no. 16, pp. 2963–2969.
- Li, Y.F. and Morcos, P.A., *Bioconjugate Chem.*, 2008, no. 7, pp. 1464–1470.
- Morcos, P.A., *Genes*, no. 3, pp. 94–102.
- Partridge, M., Vincent, A., Matthews, P., Puma, J., Stein, D., and Summerton, J., *Antisense Nucleic Acid Drug Dev.*, 1996, vol. 6, no. 3, pp. 169–175.
- Kupryushkin, M.S., Pyshnyi, D.V., and Stetsenko, D.A., *Acta Naturae*, 2014, vol. 6, no. 4, p. 23.
- Stetsenko, D.A., Kupryushkin, M.S., and Pyshnyi, D.V., Modified oligonucleotides and methods for their synthesis, Patent no. WO2016028187A1, 2014.
- Dmitrienko, E., Naumova, O., Fomin, B., Kupryushkin, M., Volkova, A., Amirkhanov, N., Semenov, D., Pyshnaya, I., and Pyshnyi, D., *Nanomedicine*, 2016, no. 16, pp. 2073–2082.
- Gabriel, G., Nordmann, A., Stein, D.A., Iversen, P.L., and Klenk, H.D., *J. Gen. Virol.*, 2008, vol. 89, pp. 939–948.
- Lenartowicz, E., Nogales, A., Kierzek, E., Kierzek, R., Martinez-Sobrido, L., and Douglas, H., *Nucleic Acid Ther.*, 2016, vol. 26, no. 5, pp. 277–285.
- Vydelenie virusov grippa v kletochnykh kul'turakh i kurinykh embrionakh i ikh identifikatsiya. metodicheskie rekomendatsii* (Isolation of Influenza Viruses in Cell Cultures and Chicken Embryos and Their Identification: Guidelines), 2006, no. 0100/4430-06-34.
- Weller, D.D., et al., Oligonucleotide analogs having cationic intersubunit linkages, US Patent no. 7943762 B2, 2011.

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