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RESEARCH ARTICLE

Investigation of the anti-influenza activity of siRNA complexes against the cellular genes *FLT4*, *Nup98*, and *Nup205 in vitro*

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Abstract

Objectives. *Evaluation of changes in the viral activity of influenza A/WSN/33 after complex knockdown of combinations of cellular genes FLT4, Nup98 and Nup205 in human lung cell culture A549.*

Methods. *The work was carried out using the equipment of the Center for Collective Use of the I. Mechnikov Research Institute of Vaccines and Sera, Russia. The authors performed transfection of combinations of small interfering ribonucleic acid (siRNA) complexes that cause simultaneous disruption of the expression of cellular genes FLT4, Nup98, and Nup205. Within three days from the moment of transfection and infection, the supernatant fluid and cell lysate were taken for subsequent viral reproduction intensity determination using the titration method for cytopathic action. The dynamics of changes in the concentration of viral ribonucleic acid (vRNA) was determined by real-time reverse transcription polymerase chain reaction (real-time RT-PCR). The nonparametric Mann–Whitney test was used to calculate statistically significant differences between groups.*

Results. *Using all of the combinations of siRNA complexes, cell viability did not decrease below the threshold level of 70%. In cells treated with complex FLT4.2 + Nup98.1 + Nup205 at the multiplicity of infection (MOI) equal to 0.1, a significant decrease in viral reproduction by 1.5 lg was noted on the first day in relation to nonspecific and viral controls. The use of siRNA complexes at MOI 0.01 resulted in a more pronounced antiviral effect. The viral titer in cells treated with siRNA*

complexes FLT4.2 + Nup98.1 and Nup98.1 + Nup205 decreased by 1.5 lg on the first day. In cells treated with complexes FLT4.2 + Nup205 and FLT4.2 + Nup98.1 + Nup205, it decreased by 1.8 and 2.0 lg on the first day and by 1.8 and 2.5 lg on the second day, respectively, in relation to nonspecific and viral controls. When conducting real-time RT-PCR, a significant decrease in the concentration of vRNA was noted. At MOI 0.1, a 295, 55, and 63-fold decrease in the viral load was observed with the use of siRNA complexes FLT4.2 + Nup98.1, Nup98.1 + Nup205, and FLT4.2 + Nup98.1 + Nup205, respectively. On the second day, a decrease in vRNA was also observed in cells treated with complex A. A 415-fold decrease in vRNA on the third day was noted in cells treated with complex FLT4.2 + Nup205. At MOI 0.01, the concentration of vRNA decreased 9.5 times when using complex B relative to nonspecific and viral control.

Conclusions. *The study showed a pronounced antiviral effect of siRNA combinations while simultaneously suppressing the activity of cellular genes (FLT4, Nup98, and Nup205), whose expression products are playing important role in the viral reproduction process, and obtained original designs of siRNA complexes. The results obtained are of great importance for the creation of emergence prophylactic and therapeutic drugs, whose action is based on the mechanism of RNA interference.*

Keywords: *RNA interference, influenza A virus, gene expression, mRNA, small interfering RNA, viral RNA*

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НАУЧНАЯ СТАТЬЯ

Исследование противогриппозной активности комплексов миРНК против клеточных генов *FLT4*, *Nup98* и *Nup205* на модели *in vitro*

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Аннотация

Цели. *Оценка изменения вирусной активности гриппа A/WSN/33 после комплексного нокдауна комбинаций клеточных генов FLT4, Nup98 и Nup205 в культуре легочных клеток человека A549.*

Методы. *Работа выполнена с использованием оборудования центра коллективного пользования Научно-исследовательского института вакцин и сывороток им И.И. Мечникова (Россия). Авторами выполнялась трансфекция комбинаций комплексов миРНК, вызывающих одновременное нарушение экспрессии клеточных генов FLT4, Nup98 и Nup205. В течение трех дней с момента трансфекции и заражения проводился отбор надосадочной жидкости и клеточного лизата для последующего определения интенсивности вирусной репродукции по методу титрования по цитопатическому действию. Динамику изменения концентрации вирусной рибонуклеиновой кислоты (вРНК) определяли методом обратной транскрипции и полимеразной цепной реакции в режиме реального времени (ОТ-ПЦР-РВ). Для вычисления статистически значимых различий между группами использовали непараметрический критерий Манна-Уитни.*

Результаты. *При использовании всех комбинаций комплексов малых интерферирующих РНК (миРНК) жизнеспособность клеток не снижалась ниже порогового уровня в 70%. В клетках, обработанных комплексом FLT4.2 + Nup98.1 + Nup205 при множественности заражения (Multiplicity of infection, MOI) 0.1 достоверное снижение вирусной репродукции на 1.5 lg отмечалось на первые сутки по отношению к неспецифическому и вирусному контролям. Использование комплексов миРНК при MOI 0.01 приводило к более выраженному противовирусному эффекту. Вирусный титр в клетках, обработанных комплексами миРНК FLT4.2 + Nup98.1 и Nup98.1 + Nup205 снижался на первые сутки на 1.5 lg. В клетках, обработанных комплексами FLT4.2 + Nup205 и FLT4.2 + Nup98.1 + Nup205 снижался на 1.8 и 2 lg на первые сутки и на 1.8 и 2.5 lg на вторые сутки соответственно по отношению к неспецифическому и вирусному контролям. При проведении ОТ-ПЦР-РВ отмечено достоверное снижение концентрации вирусной РНК. При MOI 0.1 снижение вирусной в 295, 55 и 63 раза отмечался при использовании комплексов миРНК FLT4.2 + Nup98.1, Nup98.1 + Nup205 и FLT4.2 + Nup98.1 + Nup205 соответственно. На вторые сутки снижение вирусной РНК также отмечалось в клетках, обработанных комплексом FLT4.2 + Nup98.1. Снижение вРНК на третьи сутки в 415 раз отмечалось в клетках, обработанных комплексом FLT4.2 + Nup205. При MOI 0.01 концентрация вРНК снизилась в 9.5 раз при использовании комплекса Nup98.1 + Nup205 относительно неспецифического и вирусного контроля.*

Выводы. *В ходе исследования был показан выраженный противовирусный эффект комбинаций миРНК при одновременном подавлении активности клеточных генов (FLT4, Nup98 и Nup205), чьи продукты экспрессии играют важное участие в процессе вирусной репродукции, а также получены оригинальные конструкции комплексов миРНК. Полученные результаты имеют важное значение для создания препаратов для экстренной профилактики и терапии, чье действие основано на механизме РНК-интерференции.*

Ключевые слова: *РНК-интерференция, вирус гриппа А, экспрессия генов, матричная РНК, малые интерферирующие РНК, вирусная РНК*

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INTRODUCTION

The influenza virus is the cause of the most common anthroponotic infections affecting the upper respiratory tract. According to the World Health Organization, in 2021, up to 1.2 billion of new cases of influenza infection, up to 5 million cases of severe illness, and up to 650000 deaths were observed worldwide[1]. Influenza A virus, which has high clinical significance and significant pandemic potential, poses an increased threat to global health [1]. Additionally, influenza complications can affect organ systems such as the central nervous,

[1] https://www.euro.who.int/ru/media-centre/events/events/2021/10/flu-awareness-campaign-2021

genitourinary, and cardiovascular systems. The risk of developing bacterial and fungal post-influenza complications is also no exception [2–5].

The continued threat of new epidemics and pandemics demonstrates that the progress made in the development of health infrastructure, even in the most developed countries, does not guarantee the protection of the population from newly emerging infections [6]. It is known that during outbreaks of bacterial infections, the answer to such challenges is sought in the development of new variants of antibacterial drugs. In the case of viral infections, today there are practically no approaches for the emergency development and creation of drugs. Some examples of the successful solution of this problem (human immunodeficiency virus protease inhibitor *Lopinavir* for the treatment of human immunodeficiency virus (HIV) infection; inhibitors of non-structural protein 5B—*Sofosbuvir*, *Dasabuvir*—for the treatment of infection caused by the virus hepatitis C) show that the development of emergency targeted antiviral drugs takes a long time, and the high cost of development makes them inaccessible for widespread use [7–9].

In parallel with this, the use of many anti-influenza drugs aimed at the therapy and prevention of this infection does not bring the desired result due to the fact that new viral strains resistant to these drugs are detected annually [10]. Modern vaccines also do not guarantee complete disease protection, since they do not always cause a sufficient immune response, against which the acquired immunity lasts only 6 months [11]. It should be borne in mind that influenza vaccines must be recycled every year, since new influenza virus strains appear every year, which reduces the effectiveness of previously created vaccines. In addition, vaccination is difficult for people who are allergic to egg white, as well as for people with immunodeficiency [12–15]. In summary, the creation of a universal platform for the rapid development of cost-effective and safe therapies for viral infections is of obvious relevance for ensuring human safety since this will allow creating approaches to control the circulation of influenza viruses pathogenic to humans.

Ribonucleic acid (RNA) interference (RNAi, RNAi) is a sequence of regulatory reactions in eukaryotic cells caused by a foreign double-stranded RNA molecule. The mechanism of RNA interference is the separation of exogenous double-stranded RNA into small sequences by *Dicer* endonuclease, which are small interfering RNAs (siRNAs). After that, siRNA binds to the RNA-induced gene shutdown complex (RNA-induced silencing complex or *RISC*), which includes three proteins: Argonaut-2 (*Ago2*), the cellular protein activator of protein kinase R or protein activator of the interferon-induced protein kinase (*PACT*), and the transactivation response element RNA-binding protein (*TRBP*). The resulting complex degrades the target matrix RNA (mRNA) [16, 17].

To date, there is a trend towards the creation of drugs based on the RNA interference mechanism. *Patisiran* and *Givosiran*, which are used in the treatment of genetically determined diseases—amyloid polyneuropathy and acute hepatic porphyria—have already received approval for clinical use [18, 19]. There are also a number of antiviral drugs in various stages of clinical trials for the treatment of hepatitis C, respiratory syncytial virus (RSV) infection, and HIV infection [20, 22].

It should be kept in mind that one of the main factors that reduces the antiviral activity of RNA interference is the ability to "escape" from siRNAs specific to viral genes [23]. In view of this, the most important feature of the applied approach based on RNA interference inducers, which makes it possible to avoid the emergence of resistance of the virus to therapy, is the simultaneity of the therapeutic effect and the multiple targets of the destructive effect of synthetic oligonucleotides on the host cell transcripts, which are vital for the reproduction of the virus.

Since the antiviral effect of a single knockdown of cellular genes using siRNA was previously shown [24, 25], the purpose of this study is to experimentally substantiate and evaluate the effectiveness of the simultaneous knockdown of two or more cellular genes (*FLT4*, *Nup98*, and *Nup205*) in order to reduce the reproduction of the influenza A/WSN/33 virus (H1N1) in A549 cell culture.

MATERIALS AND METHODS

siRNA

The selection of siRNAs was carried out using the siDirect 2.0 resource. Oligoribonucleotides (*Syntol*, Russia) were diluted with water to a concentration of 100 pmol/μL. Next, complementary oligonucleotides (*Syntol*, Russia) were mixed, incubated in a thermostat at 60°C for 1 min, then cooled to room temperature. The prepared RNA duplexes were stored at −80°C. All work with finished duplexes was carried out using a cold tripod. The sequences of the siRNAs used are presented in Table 1. As a nonspecific control, *siL2* siRNA was used, which is specific to the firefly luciferase gene and does not affect the life cycle of A549 cells.

Table 1. siRNA sequences used in the work

siRNA	Sequence
FLT4.2	UGAAGUUCUGUUGAAAAAG**dAdC** CUUUUUCAACAGAACUUCA**dCdA**
Nup98.1	AGUCUUUGUUUCAGAAAGC**dGdC** GCUUUCUGAAACAAAGACU**dCdA**
Nup205	UCAAAAUCUUAUCAAGAAG**dGdT** CUUCUUGAUAAGAUUUUGA**dAdG**
siL2 (nonspecific siRNA)	UUUCCGUCAUCGUCUUUCC**dTdT** GGAAAGACGAUGACGGAAA**dTdT**

Virus

Influenza A/WSN/33 (H1N1) virus (*St. Jude's Children's Research Hospital*, USA) was used in the work. Cultivation and determination of the virus titer was carried out on a cell culture Madin-Darby Canine Kidney (MDCK).

Cell culture

Cocker spaniel kidney cells MDCK (*Institut Pasteur*, France) and human lung adenocarcinoma cells A549 (*ATCC*, USA) were used in the work. MDCK cells were grown in MEM medium (*PanEco*, Russia) containing 5% Gibco fetal bovine serum (ESC) (*Fisher Scientific*, New Zealand), 40 μg/mL gentamicin (*PanEco*, Russia), and 300 μg/mL L-glutamine (*PanEco*, Russia) at 37°C in a CO_2 incubator. A549 cells were grown in DMEM medium (*PanEco*, Russia) containing 5% ESC, gentamicin 40 μg/mL, and L-glutamine 300 μg/mL at 37°C in a CO_2 incubator.

MTT test

The survival of A549 cells treated with siRNA complexes was assessed using the methylthiazolyltetrazolium bromide (MTT) test. On days 1, 2, and 3 after transfection, 20 μL of MTT solution at a concentration of 5 mg/mL (*PanEco*, Russia) was added to the wells with cells of a 96-well plate and incubated at 37°C in an atmosphere of 5% CO_2 for 2 h. Next, the culture liquid was taken and added to the wells, 100 μL of isopropanol (*Sigma-Aldrich*, USA) in each well. Using a plate spectrophotometer (*Varioscan*, *Thermo Fisher Scientific*, USA), the optical density of each well was determined at 530 nm, considering the background values at 620 nm.

Transfection of siRNA cells followed by infection

For transfection of siRNA complexes, A549 cells were seeded in 24-well plates at a seeding concentration of 1:3. After the formation of 80% cell monolayer, the cells were washed with phosphate-buffered saline and serum-free Opti-MEM medium (*Thermo Fisher Scientific*). Next, a mixture of Lipofectamin 2000 (*Thermo Fisher Scientific*) and Opti-MEM was added to the siRNA solution in Opti-MEM medium and incubated at room temperature for 20 min. The total concentration of each of the four siRNA complexes required for gene knockdown was 20 pmol/μL per well. The compositions of siRNA complexes and their sequences are listed in Tables 1, 2, and 3, respectively. After incubation, the complexes were added to the cells. *siL2* siRNA was used as a nonspecific control. The cells were then incubated at 37°C in a CO_2 incubator. After 4 h, the culture medium was removed from all wells, except for the negative control. Then, 0.5 mL of virus-containing liquid with a multiplicity of infection (MOI) of 0.1 and 0.01, consisting of DMEM medium, 0.001% tosyl phenylalanyl chloromethyl ketone (TPCK) (*Sigma-Aldrich*, Germany), and 40 μg/mL gentamicin was added. After that, the cells were again placed in a CO_2 incubator. Over the next three days, supernatant samples were taken for subsequent titration and a cell lysate was taken to assess the viral RNA (vRNA) concentration dynamics by real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

vRNA detection

vRNA was isolated from the cell lysate using a Ribosorb kit (*Helicon*, Russia). The OT-1 reagent kit (*Syntol*, Russia) was used to set up the reverse transcription reaction. Changes in the concentration

Table 2. Complex siRNA used in the work

Complex siRNA	Composition of complex siRNA
Complex A	*FLT4.2 + Nup98.1*
Complex B	*Nup98.1 + Nup205*
Complex C	*FLT4.2 + Nup205*
Complex D	*FLT4.2 + Nup98.1 + Nup205*

Table 3. Primers for real-time RT-PCR of the influenza A virus (IAV) M-gene

Primer	Sequence
IAV M F:	GGAATGGCTAAAGACAAGACCAAT
IAV M R:	GGGCATTTTGGACAAAGCGTCTAC
IAV M Pr: FAM	AGTCCTCGCTCACTGGGCACGGTG-BHQ1

of vRNA were monitored by quantitative real-time RT-PCR with a set of primers and probes for the M gene of the influenza A virus (IAV) [26]. Real-time polymerase chain reaction (PCR was performed using a set of reagents for real-time PCR in the presence of EVA Green dye and ROX reference dye (*Syntol*, Russia). The working concentration of primers and probes was 10 pmol/μL and 5 pmol/μL, respectively. The real-time PCR reaction was carried out in a DT-96 amplifier (*DNA technology*, Russia). The temperature-time regime was 95°C—5 min (1 cycle); 62°C—40 s, 95°C—15 s (40 cycles). Primers and probes (*Synthol*, Russia) are presented in Table 3.

Virus titration at the endpoint of the cytopathic effect

The viral titer was determined by the extreme point of the visual manifestation of the cytopathic effect in the MDCK cell culture. MDCK cells were seeded into 96-well plates at an inoculum concentration of $1 \cdot 10^4/cm^2$. Two days later, the nutrient medium was removed from the wells, 10-fold serial dilutions of the viral material were added in a maintenance medium without trypsin, and incubated for four days in a CO_2 incubator at 37°C. On the fourth day, the titration results were visually recorded under a microscope for the presence of a specific cytopathic effect for the influenza virus (change, deformation, detachment of dead cells from the bottom of the well). Viral titer was calculated from [27] and expressed as the decimal logarithm of 50% tissue cytopathic doses in mL ($lgTCD_{50/mL}$).

Statistical data processing

The statistical significance of the results obtained was determined using the Mann–Whitney test. The difference was considered significant at $p \leq 0.01$ and $p \leq 0.05$.

RESULTS

Effect of siRNA complexes on the survival of transfected cells

The survival rate of A549 cells transfected with siRNA was assessed for three days. By analogy with [28], the survival threshold was set at 70%. After 24 h, the viability of cells treated with complexes C and D decreased by 15%–17%. On the second day, the survival rate of cells treated with the same complexes did not practically change, however, the toxicity of complexes A and B for cells was 24% and 21%, respectively. On the third day, cell survival rates practically did not change. The survival rate of nontransfected cells was taken as 100%. Survival values were normalized to the mean absorbance of nontransfected cells at each respective time interval after transfection. The data obtained is presented in Table 4.

Effect of siRNA complexes on virus titer

In order to assess the viral activity dynamics, titration of the virus-containing liquid was carried out on MDCK cells, which was taken within three days from the moment the siRNA complexes were

introduced into the A549 cell cultures. The data shown in Fig. 1 indicates the ability of siRNA complexes to reduce the reproduction of the influenza virus *in vitro*. Figure 1a shows the decrease in viral titer at MOI = 0.1. It was found that at this MOI value, the use of the siRNA complex directed to the *FLT4*, *Nup98*, and *Nup205* genes led to a significant decrease in viral reproduction by 1.5 $lgTCD_{50/mL}$ on the first day compared to *siL2* siRNA. In nontransfected cell culture, virus titers increased over time, reaching peak values at 48 and 72 h. The same was noted in cells transfected with nonspecific *siL2* siRNA. Figure 1b shows that at MOI = 0.01, the viral titer in cells treated with the A and B complexes significantly decreased on the first day by 1.5 $lgTCD_{50/mL}$ relative to control ($p < 0.05$). The use of the C and D complexes led to a significant decrease in viral titer by 1.8 and 2.0 $lgTCD_{50/mL}$ on the first day and by 1.8 and 2.5 $lgTCD_{50/mL}$ on the second day ($p < 0.05$), respectively, according to the controls compared.

Influence of siRNAs on the vRNA concentration

Figure 2 shows the effect of siRNA on vRNA concentration *in vitro*. To assess the change in the concentration of vRNA, real-time RT-PCR was performed. Figure 2a shows that at MOI = 0.1, the use of the A, B, and D complexes led to a significant decrease in vRNA on the first day compared to *siL2* siRNA at 295, 55 and 63 times, respectively ($p < 0.05$). On the second day, a 205-fold decrease in vRNA was observed in cells transfected with the A complex ($p < 0.05$). When using the C complex, a 415-fold decrease in vRNA was noted on the third day ($p < 0.05$). Figure 2b shows that the concentration of vRNA in cells with MOI = 0.01 decreased by 9.5 times on the first day when using the B complex ($p < 0.05$) compared with nonspecific control.

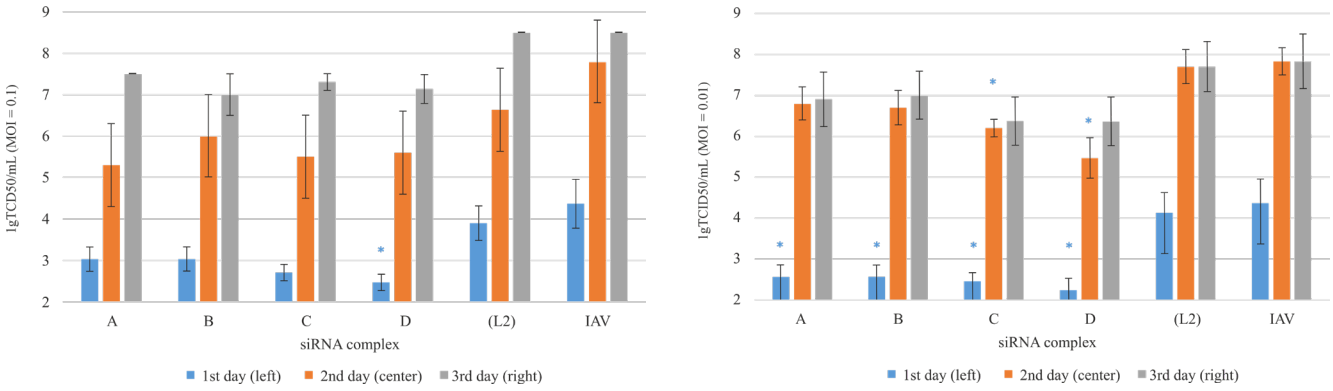
DISCUSSION

This work is a continuation of studies on the evaluation of the antiviral activity of single knockdowns of the above cellular genes by means of siRNA, carried out by the authors earlier [21, 22]. A series of experiments was carried out to evaluate the efficiency of simultaneous knockdown of several cellular genes using siRNA complexes directed to the *FLT4*, *Nup98*, and *Nup205* genes. A pronounced antiviral effect of siRNAs directed simultaneously to several mRNAs of these genes was shown, and data were obtained indicating a correlation between a decrease in cellular gene expression and a decrease in viral reproduction. To assess the effectiveness of siRNA complexes, two methodological approaches were used: virus titration by the cytopathic effect and real-time RT-PCR, which were consistent with each other. In addition to the effective gene expression reduction, an important criterion for the use of siRNAs or their complexes is their low effect on the vital activity of cells as a result of knockdown of one or more target genes.

It was found that siRNA compositions directed simultaneously to the *FLT4*, *Nup98*, and *Nup205* genes did not reduce cell viability below the

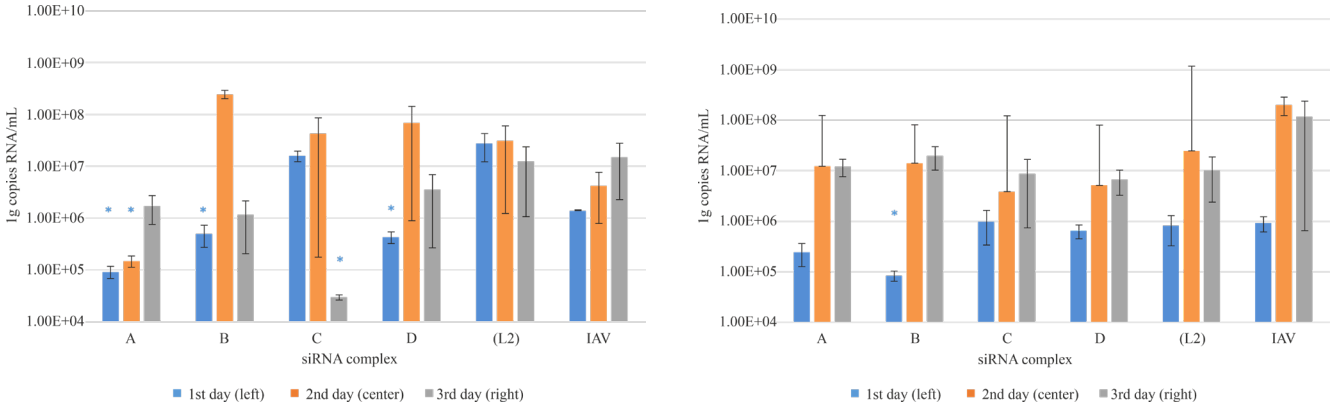
Table 4. Cell survival after siRNA transfection in %

siRNA complex	1st day	2nd day	3rd day
Complex A	98	76	75
Complex B	97	79	73
Complex C	85	84	86
Complex D	83	78	81
siL2 (nonspecific)	98	84	95
K-(nontransfect.)	100	100	100



a b

Fig. 1. (a) Multiplicity of infection (MOI) = 0.1; (b) MOI = 0.01. Influence of siRNAs complexes (A – *FLT4.2* + *Nup98.1*; B – *Nup98.1* + *Nup205*; C – *FLT4.2* + *Nup205*; D – *FLT4.1* + *Nup98.1* + *Nup205*) directed to the *FLT4*, *Nup98*, and *Nup205* genes on the reproduction of the influenza virus (on the graph, the data are given in $\log_{10}$).



a b

Fig. 2. (a) Multiplicity of infection (MOI) = 0.1; (b) MOI = 0.01. The effect of siRNA complexes (A, B, C, and D) on the concentration of vRNA (on the graph, the data are given in $\log_{10}$).

threshold level of 70%, similarly to [28]. When titrating the virus by the cytopathic effect, the following decrease in viral activity was noted. At MOI = 0.1, a significant decrease in viral reproduction by 1.5 $lgTCD_{50/mL}$ was noted only when the D complex was used on the first day with respect to nonspecific *siL2* siRNA. The best result was noted at a multiplicity of infection of 0.01. Table 2 shows that at MOI = 0.01, the viral titer in cells treated with the A and B complexes significantly decreased on the first day by 1.5 $lgTCD_{50/mL}$ relative to the control ($p < 0.05$). The use of the C and D complexes led to the significant decrease in viral titer by 1.8 and 2.0 $lgTCD_{50/mL}$ on the first day and by 1.8 and 2.5 $lgTCD_{50/mL}$ on the second day ($p < 0.05$), respectively, compared with controls. According to the results of real-time RT-PCR, there was a decrease in the amount of vRNA in the cells treated with complexes compared to controls. At MOI = 0.1, the use of the A, B, and D complexes led to a significant decrease in vRNA on the first day compared to *siL2* siRNA by factors of 295, 55, and 63, respectively ($p < 0.05$). On the second day, a similar effect was noted in cells treated with the A complex. When using the B complex, a 415-fold decrease in vRNA was noted on the third day. Table 4 shows that the concentration of vRNA in cells with MOI = 0.01 decreased on the first day by 9.5 times when the B complex was used ($p < 0.05$) compared with the nonspecific control. It should be noted that the accumulation of vRNA is apparently associated with the fact that a partial synthesis of vRNA was carried out, but there was no assembly of the virion. Against this background, the accumulation of vRNA occurred *in vitro*. Similar results were shown in the paper [29].

CONCLUSIONS

Today, the issue of creating safe and effective drugs for the treatment and prevention of influenza and its complications is of great importance. In the present study, the data were obtained that the simultaneous knockdown of several cellular genes that play important roles in the process of viral endocytosis and nuclear import/export of vRNA using the siRNA complexes significantly and effectively reduced the reproduction of the influenza virus *in vitro*. Effective suppression of viral reproduction was noted when using the siRNA complex directed to all three genes at once. This indicates that the violation of viral reproduction simultaneously at different stages leads to the great effect and, as a result, to a decrease in viral activity. The results obtained make it possible to recommend siRNAs directed to cellular genes for research as potential drugs for emergency prevention and treatment of influenza in an animal model of infection. In parallel, the results obtained contribute to the development of principles for the rapid design and development of specific and effective antiviral siRNAs that can be used to develop protection means against viruses belonging to different taxonomic groups. This technology should become highly universal and, in the future, can enter the system of rapid response to the emergence of new pathogenic viruses, pandemics, and biological terrorism.

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Authors' contributions

E.A. Pashkov, M.O. Korotysheva, A.V. Pak, and ***Zaiceva T.A.*** – conducting the experiments;

E.A. Pashkov, E.P. Bystritskaya, Yu.E. Dronina, and ***V.K. Solntseva*** – writing the text of the article and the analysis of the obtained results;

E.B. Fayzuloev, A.V. Poddubikov, and ***A.V. Sidorov*** – scientific editing;

E.P. Pashkov, A.S. Bykov, O.A. Svitich, and ***V.V. Zverev*** – idea of the study, summary, and general management.

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