BIOCHEMISTRY AND BIOTECHNOLOGY

БИОХИМИЯ И БИОТЕХНОЛОГИЯ

ISSN 2686-7575 (Online)

https://doi.org/10.32362/2410-6593-2022-17-5-384-393

UDC 615.275.2

RESEARCH ARTICLE

Effect of antiviral siRNAs on the production of cytokines *in vitro*

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Abstract

Objectives. *To evaluate the dynamics of the expression level of IL-1β and IL-28β (IFN-λ3) genes as a result of complex knockdown of some cellular genes, whose expression products play an important role in the reproduction of the influenza virus.*

Methods. *Following the collection of virus-containing liquid and cell lysate within three days from the moment of transfection and infection, the intensity of viral reproduction was assessed using the cytopathic effect titration method. The concentration of viral ribonucleic acid (vRNA) and change in the expression of IL-1β and IL-28β (IFN-λ3) were determined by real-time reverse transcription quantitative polymerase chain reaction (real-time RT-qPCR). The nonparametric Mann–Whitney test was used to statistically calculate significant differences between groups.*

Results. *The use of each small interfering ribonucleic acid (siRNA) complex led to a decrease in viral reproduction on the first day at the multiplicity of infection (MOI) of 0.001. The use of complex A (FLT4.2 + Nup98.1) and D (FLT4.2 + Nup98.1 + Nup205) led to a decrease in viral titer by 2.8 lg$TCID_{50}$/mL and by 2.1 lg$TCID_{50}$/mL relative to the use of nonspecific L2 siRNA and viral control ($p \le 0.05$). Transfection of complexes B (Nup98.1 + Nup205) and C (FLT4.2 + Nup205) also reduced the viral titer by 1.5 lg$TCID_{50}$/mL and 1.8 lg$TCID_{50}$/mL relative to nonspecific L2 siRNA and viral control ($p \le 0.05$). When conducting real-time RT-qPCR, a significant decrease in the concentration of viral RNA was also noted. When using complexes B, C, and D, the concentration of vRNA decreased on the first day by 14.5, 4.1, and 15 times, respectively. On the second*

day, a decrease in vRNA was observed in cells with B and D complexes by 17.1 and 18.3 times (p ≤ 0.05). Along with a decrease in the viral titer and vRNA, an increase in the expression of the IL-1β and IL-28β genes was observed on the first day when using all siRNA complexes relative to nonspecific and viral controls (p ≤ 0.05). On the second day, an increase was also observed in cells with A and D complexes, while on the third day, there was an increase in the expression of these genes in cells with complex D (p ≤ 0.05).

Conclusions. *The use of siRNA complexes is shown to have a pronounced antiviral effect while simultaneously suppressing the activity of cellular genes (FLT4, Nup98 and Nup205). In parallel, the transfection of complexes that block the formation of expression products necessary for viral reproduction is demonstrated to lead to an increase in the level of expression of the IL-1β and IL-28β genes. These results indicate not only that the use of siRNA has antiviral activity, but also immunomodulatory activity, which can contribute to a more effective immune response of the body.*

Keywords: *RNA interference, IL-1β, influenza A virus, IFN-λ3, gene expression, siRNA, pro-inflammatory cytokines, IL-28β, viral RNA*

For citation: Pak A.V., Pashkov E.A., Abramova N.D., Poddubikov A.V., Nagieva F.G., Bogdanova E.A., Pashkov E.P., Svitich O.A., Zverev V.V. Effect of antiviral siRNAs on the production of cytokines *in vitro*. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2022;17(5):384–393 (Russ., Eng.). https://doi.org/10.32362/2410-6593-2022-17-5-384-393

НАУЧНАЯ СТАТЬЯ

Действие противовирусных миРНК на выработку цитокинов *in vitro*

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Аннотация

Цели. *Оценить динамику уровня экспрессии генов IL-1β и IL-28β (IFN-λ3) в результате комплексного нокдауна некоторых клеточных генов, чьи продукты экспрессии играют важную роль в репродукции вируса гриппа.*

Методы. *Вируссодержащую жидкость и клеточный лизат отбирали в течение 3-х дней с момента трансфекции и заражения и оценивали интенсивность вирусной репродукции методами титрования по цитопатическому действию. Концентрацию вирусной рибонуклеиновой кислоты (вРНК) и изменение экспрессии IL-1β и IL-28β (IFN-λ3) 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. *Использование каждого комплекса малых интерферирующих РНК (миРНК) приводило к снижению вирусной репродукции на 1-е сутки при множественности заражения 0.001. Применение комплексов A (FLT4.2 + Nup98.1) и D (FLT4.2 + Nup98.1 + Nup205) приводило к снижению вирусного титра на 2.8 lgТЦД$_{50}$/мл и на 2.1 lgТЦД$_{50}$/мл относительно применения неспецифической миРНК L2 и вирусного контроля ($p \le 0.05$). В результате трансфекции комплексов B (Nup98.1 + Nup205) и C (FLT4.2 + Nup205) вирусный титр также снижался на 1.5 lgТЦД$_{50}$/мл и 1.8 lgТЦД$_{50}$/мл соответственно относительно неспецифической миРНК L2 и вирусного контроля ($p \le 0.05$). При проведении ОТ-ПЦР-РВ также было отмечено достоверное уменьшение концентрации вРНК. При использовании комплексов B, C и D концентрация вРНК снижалась на 1-е сутки в 14.5, 4.1 и 15.0 раз соответственно. На 2-е сутки в клетках с комплексами B и D наблюдалось уменьшение концентрации вРНК в 17.1 и 18.3 раз ($p \le 0.05$). Наряду со снижением вирусного титра и вРНК наблюдалось повышение экспрессии генов IL-1β и IL-28β на 1-е сутки при использовании всех комплексов миРНК относительно неспецифического и вирусного контроля ($p \le 0.05$). На 2-е сутки также наблюдалось повышение экспрессии в клетках с комплексами A и D, а на третьи – в клетках с комплексом D ($p \le 0.05$).*

Выводы. *Исследование показало, что применение комплексов миРНК приводит к выраженному противовирусному эффекту при одновременном подавлении активности клеточных генов (FLT4, Nup98 и Nup205). Параллельно с этим было выявлено, что при трансфекции комплексов, блокирующих образование продуктов экспрессии, необходимых для вирусной репродукции, повышается уровень экспрессии генов IL-1β и IL-28β. Данные результаты свидетельствуют о том, что используемые миРНК обладают не только противовирусной, но также и иммуномодулирующей активностью, что способствует более эффективному иммунному ответу организма.*

Ключевые слова: *РНК-интерференция, IL-1β, вирус гриппа А, IFN-λ3, экспрессия генов, миРНК, провоспалительные цитокины, IL-28β, вирусная РНК*

Для цитирования: Пак А.В., Пашков Е.А., Абрамова Н.Д., Поддубиков А.В., Нагиева Ф.Г., Богданова Е.А., Пашков Е.П., Свитич О.А., Зверев В.В. Действие противовирусных миРНК на выработку цитокинов *in vitro*. *Тонкие химические технологии*. 2022;17(5):384–393. https://doi.org/10.32362/2410-6593-2022-17-5-384-393

INTRODUCTION

The present work continues research on the creation of a universal platform for the rapid development of cost-effective and safe treatments for viral infections, which was launched in 2021 by a group of scientists from the I.I. Mechnikov Research Institute of Vaccines and Serums (Russia) [1, 2].

Today, respiratory viral infections have become one of the most pressing global problems, having severe social and economic consequences. For example, the COVID-19 pandemic caused by the SARS-CoV-2 virus has claimed the lives of more than 6.3 mln people worldwide since 2019[1], and influenza ended almost 650000 lives in 2021 alone[2]. Viral infections affect not

[1] https://coronavirus-graph.ru/mir, accessed June 20, 2022.

[2] https://www.euro.who.int/ru/media-centre/events/events/2021/10/flu-awareness-campaign-2021, accessed June 20, 2022.

only the respiratory, but also central nervous, genitourinary, cardiovascular and immune systems, as well as leading to the development of bacterial and fungal complications [3–6].

Influenza viruses have proteins with immunomodulatory properties, which can trigger secondary immunodeficiencies. Among these, the best-studied protein is NS-1 (nonstructural protein-1), one of whose main functions is to disrupt the functioning of interferon-mediated defense mechanisms of the body, reducing the production of pro-inflammatory cytokines known as interleukins, which in turn leads to a lack of immune response [7].

To date, there are a number of etiotropic, pathogenetic, symptomatic, and immunomodulatory drugs used for the treatment of influenza. However, achieving a full therapeutic effect from the use of these drugs is prevented by the emergence of new resistant forms of the influenza virus, the development of allergic reactions to drugs, and the need for their individual selection [8–11]. The question of the use of immunomodulatory drugs also remains open, since the effect of their use is limited, and in some cases can lead to serious consequences for the patient himself [12–14]. The use of anti-influenza drugs also has certain limitations [15]. To overcome these problems, the design and development of fundamentally new antiviral drugs is required. One of the promising new technologies for creating specific antiviral drugs is based on the mechanism of RNA interference [16–18].

Previously, we have demonstrated a pronounced antiviral effect from the use of small interfering RNAs (siRNAs) directed to one, two, or more cellular genes simultaneously, whose expression products are important in viral reproduction. However, in earlier works, we did not evaluate changes in the expression of some pro-inflammatory cytokines role in the formation of antiviral immunity [1, 2, 19]. *IL-1β* is involved in increased expression of the MCP-1 and MCP-3 genes and functional maturation of tissue macrophages and dendritic cells [20, 21]. This leads to increased inflammatory response and activation of an efficient antigen presentation system. *IFN-λ3*, which are formed earlier than other types of interferons, demonstrate a powerful protective function at early stages of infection. The use of siRNAs in relation to cellular genes involved in the reproduction of the influenza virus can reduce viral activity *in vitro* and promote a more effective immune response [18].

Based on the foregoing, the aim of the present study is to evaluate the dynamics of the expression level of *IL-1β* and *IL-28β* (*IFN-λ3*) genes as a result of complex knockdown of some cellular genes, whose expression products play an important role in the reproduction of the influenza virus.

MATERIALS AND METHODS

Methods used in the present work included selection of siRNAs, oligonucleotides, sequences of siRNAs used, information about the influenza A/WSN/33 (**H1N1**) virus used, cell cultures, method for assessing the cytotoxicity of siRNA complexes, method for transfection of siRNA cells with subsequent infection, siRNA complexes used. The used virus titration method on the end point of cytopathic action was as presented in our earlier studies [1, 2, 19]. The expression of *IL-1β* and *IFN-λ3* genes was studied by real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

Detection of viral RNA

Total RNA was isolated from the cell lysate using the ExtractRNA kit (*Evrogen*, Russia). The OT-1 reagent kit (*Syntol*, Russia) was used to set up the reverse transcription reaction. Changes in the concentration of viral RNA (vRNA) were monitored using quantitative real-time RT-PCR with a set of primers and probes for the influenza A M gene [22]. To assess the expression of *IL-1β* and *IFN-λ3*, real-time RT-PCR and the expression evaluation criterion $2^{-\Delta\Delta Ct}$ were used.

For real-time PCR, a set of reagents in the presence of EVA Green dye and reference dye ROX (*Syntol*) and a 2.5-fold reaction mixture for real-time PCR (*Syntol*) were used. 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 and 5 min (1 cycle), 62°C and 40 s, 95°C and 15 s (40 cycles). Primers and probes were synthesized by *Syntol* and presented in [2].

Statistical data processing

The statistical significance of the results obtained was determined using the Mann–Whitney U test. The difference was considered significant at a statistical significance level of $0.01 \leq p \leq 0.05$. Reliability indicators were calculated using the Minitab software[3].

[3] https://www.minitab.com/en-us/, accessed June 08, 2022.

RESULTS

Assessment of cytotoxicity

Previously, similar siRNA sequences were used in a study to evaluate the antiviral effect against the influenza virus. Detailed results of the cytotoxicity evaluation are presented in the study [2].

Influence of siRNA complexes on virus titer

To assess the effectiveness of the antiviral action of siRNA in reducing viral activity, titration of the virus-containing liquid was performed on a Madin-Darby Canine Kidney cell culture, which was taken at 24, 48, and 72 h from the moment of transfection of the siRNA complexes into the A549 cell culture. In contrast to our previous study [1], in this work, the multiplicity of infection (MOI) was 0.001. It was found that the use of all siRNA complexes at a given MOI directed to cellular genes leads to a significant decrease in viral reproduction on the first day after infection. The obtained data shown in Fig. 1 indicate the ability of siRNA to reduce viral activity *in vitro*. Upon transfection of the A complex directed to the *FLT4* + *Nup98* genes, a significant decrease in the viral titer was observed compared to the nonspecific control by 2.8 $lgTCID_{50}$/mL ($p \leq 0.05$). Upon transfection of the B complex (*Nup98* + Nup205 genes), the corresponding increase was 1.5 $lgTCID_{50}$/mL ($p \leq 0.05$). The use of complexes C (*FLT4* + *Nup205* genes) and D (*FLT4* + *Nup98* + *Nup205* genes) led to a significant decrease in viral titer compared with nonspecific control by 1.8 and 2.1 $lgTCID_{50}$/mL, respectively ($p \leq 0.05$).

Influence of siRNA on vRNA concentration

Figure 2 shows the change in *in vitro* vRNA concentration resulting from siRNA transfection as assessed using real-time RT-PCR. At MOI = 0.001, the use of B, C, and D complexes led to a significant decrease in vRNA on the first day as compared with nonspecific control by 14.5, 4.1, and 15 times, respectively ($p \leq 0.05$). On the second day, a decrease in vRNA was observed when using B and D complexes by 17.1 and 18.3 times, respectively ($p \leq 0.05$).

Expression dynamics of IL-1β and IFN-λ3

Expression of *IL-1β* and *IFN-λ3* was assessed using real-time RT-PCR and the expression evaluation criterion $2^{-\Delta\Delta Ct}$. Figure 3 shows the results of the evaluation of the expression of *IL-1β*. At MOI = 0.001, a significant increase in *IL-1β* expression by 18% relative to nonspecific control was observed on day 1 when complex A was used. When transfecting B, C, and D complexes, a significant increase in *IL-1β* expression was also

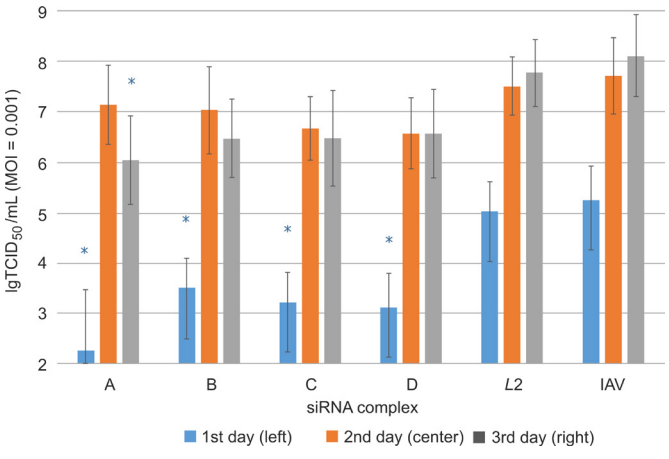

Fig. 1. Influence of siRNAs complexes (A: *FLT4* + *Nup98*; B: *Nup98* + *Nup205*; C: *FLT4* + *Nup205*; D: *FLT4* + *Nup98* + *Nup205*) directed to the *FLT4, Nup98*, and *Nup205* genes on the reproduction of the influenza virus ($p \leq 0.05$). IAV—influenza A virus. The ordinate indicates the change in virus titer in $lgTCD_{50}$/mL. The abscissa shows siRNA complexes.

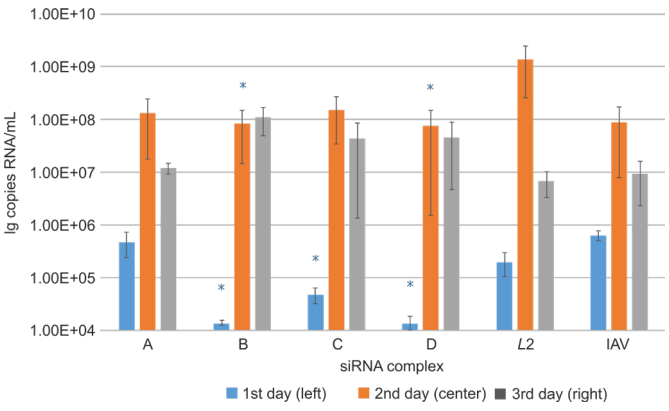

Fig. 2. Effect of siRNA complexes (A: *FLT4* + *Nup98*; B: *Nup98* + *Nup205*; C: *FLT4* + *Nup205*; D: *FLT4* + *Nup98* + *Nup205*) on the concentration of viral RNA. (On the graph, the data are given in log_{10}, in the text the decrease is indicated in the number of times) ($p \leq 0.05$). IAV—influenza A virus. The ordinate indicates the change for vRNA in log_{10}. The abscissa shows siRNA complexes.

noted on days 10, 17, and 25%, respectively ($p \leq 0.05$). On the second day, the expression level of *IL-1β* increased in cells transfected with A and D complexes by 118 and 90% ($p \leq 0.05$) as compared with the nonspecific control, which also exceeded the expression level in uninfected cells by 45 and 17%, respectively. On the third day, an increase in *IL-1β* expression by 47% was noted in cells transfected with complex D ($p \leq 0.05$). Figure 4 shows data on the change in the expression of *IFN-λ3* within three days from the moment of transfection and infection. A significant increase inexpression relative to nonspecific control was noted only on the second day when using A and C complexes by 10 and 24%, respectively ($p \leq 0.05$).

DISCUSSION

The present work evaluates the effect of siRNAs on the induction of *IL-1β* and *IFN-λ3* production and the concomitant decrease in viral activity. A series of experiments was carried out to assess changes in the expression of the level of *IL-1β* and *IFN-λ3* during suppression of the expression of cellular genes *FLT4*, *Nup98* and *Nup205*, which are important for the reproduction of the influenza virus using siRNA. To evaluate the effectiveness of cytokine expression and decrease in viral activity, two mutually-compatible methodological approaches were used: virus titration by cytopathic effect and real-time RT-PCR. The use of siRNA was shown to lead to a pronounced antiviral effect: the obtained data indicated a dependent relationship between a decrease in viral titer, a change in the amount of vRNA, and increased levels of *IL-1β* and *IFN-λ3*. Earlier results demonstrated the low cytotoxicity of siRNA complexes, allowing significant disturbances in cell vital activity following knockdown of one or several genes to be avoided [2].

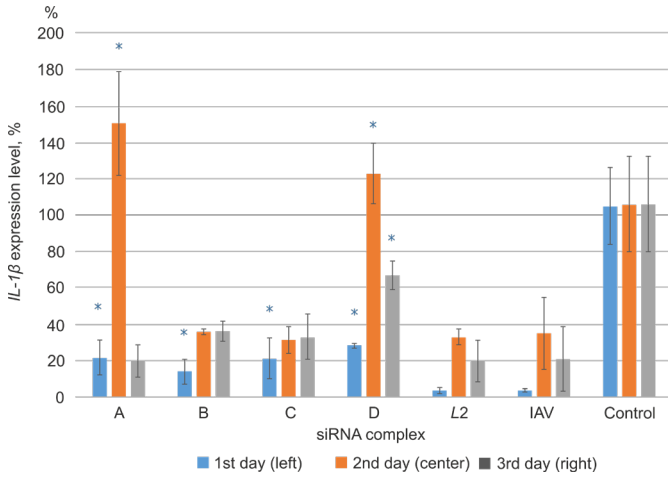

Fig. 3. Effect of siRNA complexes (A: *FLT4* + *Nup98*; B: *Nup98* + *Nup205*; C: *FLT4* + *Nup205*; D: *FLT4* + *Nup98* + *Nup205*) on changes in *IL-1β* expression ($p \leq 0.05$). IAV—influenza A virus. The ordinate shows the change in the expression level of *IL-1β*. The abscissa shows siRNA complexes.

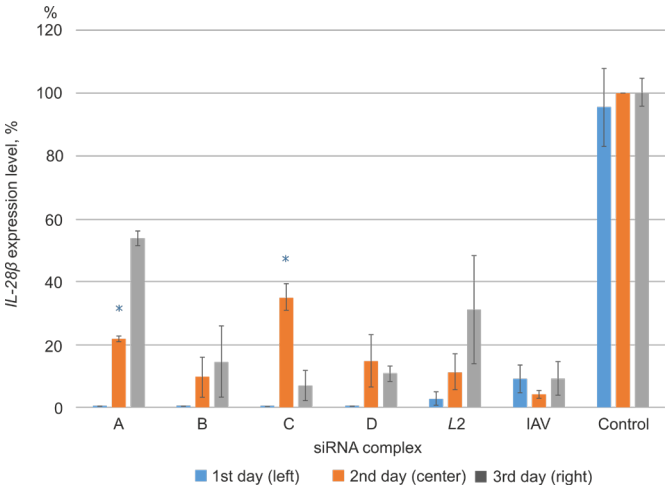

Fig. 4. Effect of siRNA complexes (A: *FLT4* + *Nup98*; B: *Nup98* + *Nup205*; C: *FLT4* + *Nup205*; D: *FLT4* + *Nup98* + *Nup205*) on changes in *IFN-λ3* expression ($p \leq 0.05$). IAV—influenza A virus. The ordinate shows the change in the expression level of *IFN-λ3*. The abscissa shows siRNA complexes.

When the virus was titrated against cytopathic effect, each siRNA complex was shown to lead to a decrease in viral activity on the first day following infection. Table 1 shows that at MOI = 0.001, the viral titer in cells treated with A and D complexes decreased by 2.8 $lgTCID_{50}$/mL and by 2.1 $lgTCID_{50}$/mL, respectively, relative to nonspecific *L2* siRNA ($p \leq 0.05$). Upon transfection of B and C complexes, the respective decreases in viral titer were 1.5 $lgTCID_{50}$/mL and 1.8 $lgTCID_{50}$/mL as compared with the nonspecific control *L2* ($p \leq 0.05$).

According to the results of RT-PCR, there was a decrease in the amount of vRNA in the cells treated with the complexes as compared to nonspecific and viral controls. The use of complexes B, C, and D led to a significant decrease in vRNA on the first day compared to si*L2* siRNA by factors of 14.5, 4.1, and 15, respectively ($p \leq 0.05$). When using the B and D complexes, the vRNA concentration on the

second day was observed to decrease by 17.1 and 18.3 times, respectively ($p \leq 0.05$). It is significant that, when using complex A, despite a pronounced observed decrease in viral titer, there was no decrease in the concentration of vRNA. This result is likely due to the combination of complex A directed to the *FLT4* and *Nup98* genes leading to partial synthesis of vRNA. Nevertheless, there was limited assembly and release of the virion from the cell, while the remaining complexes, apparently, completely blocked the synthesis of vRNA, the assembly and release of the virion. Similar results are noted in the work of J. Piasecka *et al*., where the antiviral effect of siRNAs is also assessed [23].

Expression of *IL-1β* and *IFN-λ3* was assessed using real-time RT-PCR and the expression evaluation criterion $2^{-\Delta\Delta Ct}$. Figures 3 and 4 show data on the dynamics of *IL-1β* and *IFN-λ3* expression within three days from the moment of transfection and infection. The most effective increase in *IL-1β* expression is observed when using A and D complexes. In relation to the nonspecific control *L2* ($p \leq 0.05$), the increase in expression on the first and second days following transfection was 18/118% for complex A and 25/90% for complex D, respectively, as well as exceeding the expression level in uninfected cells by 45% and 17% on the second day. On the third day, an increased expression was also noted when the complex D was used by 47%. When using B and C complexes, an increase in expression was noted only on the first day by 10% and 17%, respectively ($p \leq 0.05$). When assessing the increase in the expression level of *IFN-λ3*, an increase was observed only on the second day when using A and C complexes by 10% and 24%, respectively, relative to the nonspecific control ($p \leq 0.05$). This result of a heterogeneous increase in the expression of *IL-1β* and *IFN-λ3* is apparently due to different siRNA nucleotide sequences differently inducing the production of pro-inflammatory cytokines and interferons through Toll-like receptors.

CONCLUSIONS

The challenge of developing medicines for the prevention and treatment of highly contagious respiratory infections is currently of particular relevance. It is necessary that such medicinal substances be safe, non-toxic for the patient, as well as having a low spectrum of contraindications. In addition, such drugs should offer a clear therapeutic and prophylactic effect despite the drug resistance of the pathogen. The present study provides evidence that the simultaneous knockdown of several cellular genes that play an important role in viral reproduction by means of siRNA complexes significantly reduced influenza viral activity *in vitro*. Despite the ability of the influenza virus to exert an immunosuppressive effect, a pronounced decrease in vRNA and increase in the expression level of IL-1β and IFN-λ3 was observed. The results indicate that siRNAs used in the work have not only antiviral but also immunomodulatory activity, which contributes to a more effective immune response. Additionally, these results represent the development of principles for the rapid design and creation of specific antiviral agents designed to protect against existing and emerging pathogenic viruses, ensure the anti-epidemic safety of various population groups, and effectively respond to the emergence of pandemics and possible cases of bioterrorism.

Acknowledgments

The study was carried out using the scientific equipment of the Center for Collective Use of the I. Mechnikov Research Institute of Vaccines and Sera, and supported by the Ministry of Science and Higher Education of the Russian Federation, Agreement No. 075-15-2021-676 dated July 28, 2021.

Authors' contributions

A.V. Pak, E.A. Pashkov, N.D. Abramova – conducting the experiments;

E.A. Pashkov, E.A. Bogdanova – writing the text of the article and the analysis of the obtained results;

A.V. Poddubikov, F.G. Nagieva – scientific editing;

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

The authors declare no obvious and potential conflicts of interest related to the publication of this article.

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The article was submitted: July 19, 2022; approved after reviewing: August 30, 2022; accepted for publication: September 20, 2022.

Translated from Russian into English by H. Moshkov
Edited for English language and spelling by Thomas Beavitt