Synthesis of 1,2,3-triazolyl nucleoside analogues and their antiviral activity

Olga V. Andreeva · Bulat F. Garifullin · Vladimir V. Zarubaev · Alexander V. Slita · Iana L. Yesaulkova · Liliya F. Saifina · Marina M. Shulaeva · Maya G. Belenok · Vyacheslav E. Semenov · Vladimir E. Kataev

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Abstract

Based on the fact that a search for influenza antivirals among nucleoside analogues has drawn very little attention of chemists, the present study reports the synthesis of a series of 1,2,3-triazolyl nucleoside analogues in which a pyrimidine fragment is attached to the ribofuranosyl-1,2,3-triazol-4-yl moiety by a polymethylene linker of variable length. Target compounds were prepared by the Cu alkyne-azide cycloaddition (CuAAC) reaction. Derivatives of uracil, 6-methyluracil, 3,6-dimethyluracil, thymine and quinazolin-2,4-dione with ω-alkyne substituent at the N1 (or N5) atom and azido 2,3,5-tri-O-acetyl-D-β-ribofuranoside were used as components of the CuAAC reaction. All compounds synthesized were evaluated for antiviral activity against influenza virus A/PR/8/34/(H1N1) and coxsackievirus B3. The best values of IC50 (inhibiting concentration) and SI (selectivity index) were demonstrated by the lead compound 4i in which the 1,2,3-triazolylribofuranosyl fragment is attached to the N1 atom of the quinazoline-2,4-dione moiety by a butylene linker (IC50 = 30 μM, SI = 24) and compound 8n in which the 1,2,3-triazolylribofuranosyl fragment is attached directly to the N5 atom of the 6-methyluracil moiety (IC50 = 15 μM, SI = 5). According to theoretical calculations, the antiviral activity of the 1,2,3-triazolyl nucleoside analogues 4i and 8n against H1N1 (A/PR/8/34) influenza virus can be explained by their influence on the functioning of the polymerase acidic protein (PA) of RNA-dependent RNA polymerase (RdRP).

Graphic abstract

Keywords Nucleoside analogues · 1,2,3-Triazole · Influenza virus · Coxsackievirus · Click chemistry

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Vyacheslav E. Semenov
sve@iopc.ru

Extended author information available on the last page of the article

Abbreviations
PA Polymerase acidic protein
RdRP RNA-dependent RNA polymerase
PA-Nter N-terminal endonuclease domain
LBD Ligand-binding domain
Introduction

Naturally occurring nucleosides represent a unique scaffold for drug design due to their involvement in numerous biological processes as well as the fact that they serve as essential building blocks for DNA and RNA synthesis [1]. Nucleosides play important roles in the replication and transcription of genetic information and, as such, have been utilized for decades for antibacterial or antiviral therapeutics [2, 3]. In the early 1960s, the idea appeared that various nucleoside derivatives (or analogues) can also affect the biochemical processes in the cells of bacteria and viruses. Over the next 50 years, a diverse series of numerous nucleoside analogues have been synthesized and evaluated for their biological activities. Numerous modifications to the nucleosides included alterations to the sugar, nucleobase and glycosidic bond [2–5].

Overwhelming majority of synthesized nucleoside analogues displayed anticancer or antiviral activity. For example, Gemcitabine was approved for the treatment of breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer and bladder cancer, and Flouxuridine is an oncology drug to treat colorectal cancer [2–5]. Zalcitabine, Didanosine, Zidovudine, Stavudine, Carbovir and Entecavir were approved for the treatment of HIV/AIDS [2–6]. Trifluridine and Idoxuridine are used to treat herpes simplex virus [2, 4]. Entecavir was approved drug for treating hepatitis B virus (HBV) [2, 4]. Brivudine is an antiviral drug used in the treatment of herpes zoster (VZV) [2, 4, 7]. As to antiviral activity, the literature has provided just a few examples of nucleoside analogues which are influenza A virus inhibitors [8–13]. Among approved influenza drugs (Amantadine, Rimantadine, Zanamivir, Oseltamivir, Laninamivir octanoate, Peramivir, Favepiravir and Ribavirin [4]), there is only one nucleoside analogue, namely Ribavirin (1-β-D-ribofuranosyl-1,2,3-triazolidomethylyuracils) [4, 7, 14–17]. Ribavirin possesses non-interferon-inducing, broad-spectrum antiviral properties against a wide range of RNA viruses [7, 14–17]. These facts have drawn much attention of chemists and pharmacologists who began to synthesize Ribavirin derivatives for developing novel antiviral agents to treat various viral diseases including influenza [18–23]. Note that Ribavirin possesses the 1,2,4-triazole moiety. Triazole heterocyclic compounds are paid special attention due to their potential applications as medicinal agents since 1,2,4- and 1,2,3-triazole units which are not present in natural products are remarkably stable to metabolic transformations that allow to utilize them in various drugs [24, 25]. The introduction of “click chemistry” reactions using Cu alknye-azide cycloaddition (CuAAC) in medicinal chemistry has provided a great number of 1,2,3-triazolyl nucleoside analogues demonstrating various bioactivities. Nucleoside analogues containing substituted 1,2,3-triazole moieties at the C5’ position of the ribofuranose residue demonstrated significant anticancer activity against cancer cell lines A549, HT-29, MCF-7, A-375 and/or antibacterial and antifungal activities [26, 27]. Pyrimidine nucleoside analogues in which a 1,2,3-triazole ring was attached either directly to the C5 position of 2'-deoxyuridine or via a methylene unit were synthesized and exhibited both antiviral activity against herpes simplex viruses, varicella-zoster virus, human cytomegalovirus, vaccinia virus [28–31] and significant anticancer effects against cancer cell lines PC-3, MDA-MB-231, ACHN [28]. Recently, a series of nucleoside analogues in which the pyrimidine fragment was attached to the ribose moiety at the C1’ carbon via a 1,2,3-triazoly1 bridge has been synthesized [32–35]. No inhibitory activity against HCV virus was observed with any of these compounds, but several C5-substituted 1-β-D-ribofuranosyl-1,2,3-triazolidomethyluracils showed potent inhibitory activity against RNase A [34] and completely inhibited the angiogenic activity of hAng in vivo [35].

The above brief review of the literature reveals that all natural nucleosides and their analogues, including 1,2,3-triazole ones, evaluated primarily for their anticancer and antiviral activities. As a result, many nucleoside analogues were approved as drugs for the treatment of HIV/AIDS, HBV, HCV, HSV [2–7]. Unfortunately, the search for influenza drugs among nucleoside analogues has drawn very little attention of chemists and pharmacologists. This is despite the fact that influenza virus infection constitutes a significant health problem in need of more effective therapies [8]. The worldwide spread of drug-resistant influenza strains poses an urgent need for novel antiviral drugs, particularly with a different mechanism of action [10]. In this regard, 1,2,3-triazole nucleoside analogues are a promising scaffold for creating new anti-influenza drugs. Herein, we report the synthesis of a series of 1,2,3-triazolyl nucleoside analogues using the Cu alknye-azide cycloaddition (CuAAC) reaction and evaluation of their ability to inhibit the in vitro growth of influenza virus A/PR/8/34 (H1N1) and coxsackievirus B3. In these compounds the pyrimidine fragment is attached to the ribofuranosyl-1,2,3-triazol-4-yl moiety via polymethylene linker of variable length.

Results and discussion

Synthetic chemistry

At first, we focused on the synthesis of alkyne components of the CuAAC reaction, namely pyrimidine derivatives 1a–c, 2a–c, 3a–c, 4a–c, 6a–c with ω-alkyne substituents at the N1 atom. They were prepared according to
the method reported previously [36]. Starting pyrimidines uracil (1), 6-methyluracil (2), thymine (3) and condensed uracil derivative, namely quinazoline-2,4-dione (4), were reacted with an excess of hexamethyldisilazane (HMDS) in toluene in the presence of H$_2$SO$_4$ at reflux for 8 h to afford bissilylated derivatives 5 which were then engaged in the alkylation with ω-iodo-alk-α-yynes without purification (Scheme 1). Alkyne derivatives of uracil (1b,c; 2b,c), thymine (3b,c) and quinazolin-2,4-dione (4a–c) were obtained in 33–64% yield. As we have reported [36], in the case of 6-methyluracil 2 the reaction of its bissilylated derivative 5 with a propargyl bromide unexpectedly led to the mixture of N1- and N3-substituted products. Therefore, N1-propargyl derivatives of uracil 1, 6-methyluracil 2, thymine 3, as well as N1-ω-alkynyl derivatives of 3,6-dimethyluracil 6 (compounds 1a, 2a, 3a, 6a–c, respectively) were

Scheme 1 Synthesis of pyrimidine derivatives containing an ω-alkyne substituent at the N1 or the C5 position of the pyrimidine ring
synthesized by the alkylation of monosodium salts 7 [36] (Scheme 1).

6-Methyluracil derivatives 8g,h,i with an ω-alkyne substituent at the C5 atom were prepared in three steps by analogy with procedures earlier described [37, 38]. Initially, ethylcetatoacetate was alkylated in a solution of sodium in ethanol by treatment with (a) propargyl bromide to prepare ethyl 2-acetyl-4-penta noate 8a; (b) 6-iodo-pent-1-ynoate to prepare ethyl 2-acetyl-6-heptynoate 8b; (c) 6-iodo-hex-1-ynoate to prepare ethyl 2-acetyl-7-octynoate 8c (Scheme 1). Then, β-keto esters 8a, 8b, 8c were condensed with thiourea to give 6-methyl-5-(ω-alkyne)-2-thio-(1H,3H)pyrimidine-4-ones. These thiopyrimidines were next hydrolyzed with chloroacetic acid in EtOH/H2O to afford target 6-methyl-5-(ω-alkyne)pyrimidine-2,4(1H,3H)-diones in good yields (85%, 65%, 80%, respectively).

The azide component of the CuAAC reaction, namely azido ribofuranosyl 9c, was prepared based on the published procedure [36] (Scheme 2). D-ribose was converted into methyl β-ribofuranoside 9a by reacting with methanol and subsequent acetylation. The methoxyl group of the obtained monosaccharide 9a was replaced by an acetoxy group which then was converted to an azido one by the reaction of 9b with trimethylsilyl azide (TMSN3). The target compound 9c was obtained in 95% yield.

Coupling the alkylene components 1a–c, 2a–c, 3a–c, 4a–c, 6a–c, 8 with the azido component 9c was accomplished by a CuAAC reaction in according with a procedure already described [36] (Scheme 3). The 1,2,3-triazolyl analogues 1–4, 6, 8 with acetyl protection were obtained in good yields (60–96%). The 1H NMR spectra of these compounds showed a singlet within the range 7.50–7.97 ppm corresponding to the triazolyl proton C5-H [29–32, 34, 36]. The signals within the range 142.9–147.8 ppm and 120.1–123.5 ppm in the 13C NMR spectra were certainly assigned to the triazolyl carbons C4 and C5, respectively [29–32, 34, 36]. The anomic protons of 1,2,3-triazolyl analogues 1–4, 6, 8 resonated in the 1H NMR spectra as doublets within the range 5.98–6.20 ppm with vicinal coupling constants of 3.5–4.2 Hz that confirmed β-orientation of the glycoside bonds in a full agreement with the literature [39]. Removal of the acetyl protection of 1–4, 6, 8 provided the target 1,2,3-triazolyl nucleoside analogues 1–4, 6, 8 with free hydroxy groups in good yields (76–98%) (Scheme 3).

**Biological evaluation. Antiviral activity**

The in vitro antiviral activity of the compounds synthesized was evaluated regarding A/Puerto Rico/8/34 (H1N1) strain of influenza virus and coxsackievirus B3. The resulting data expressed as virus-inhibiting activity (IC50), cytotoxicity (CC50) as well as selectivity index (SI), which is the ratio CC50/IC50, are presented in Tables 1 and 2. The analysis of Table 1 leads to the following four important conclusions. First of all, 1,2,3-triazolyl derivatives of natural nucleosides uridine (1) and thymidine (3) lack antiviral activity against influenza virus A/PR/8/34(H1N1). On the contrary, the antiviral activity was demonstrated by 1,2,3-triazolyl nucleoside analogues in which nucleic base has been replaced with 6-methyluracil (compounds 2h, 2i, 8j, 8k, 8m, 8n) or quinazoline-2,4-dione (compounds 4g, 4i). The high antiviral activity (IC50 = 30 µM), low cytotoxicity (CC50 > 719 µM) and the high selectivity index (SI) value of 24 were shown by 1,2,3-triazole nucleoside analogue 4i possessing the quinazoline-2,4-dione moiety instead of a nucleic base. Secondly, the antiviral activity depends both on the nature of the pyrimidine moiety and the length of the linker coupling it with the 1,2,3-triazolylribofuranosyl fragment. Thus, in a series of the derivatives of 6-methyluracil 2g, 2h, 2i and quinazoline-2,4-dione 4g, 4h, 4i the best antiviral activity was exhibited by compounds 2h, 2i and 4i having propylene and butylene linker, respectively. Among the derivatives of 6-methyluracil 2g, 2h, 2i, compound 2g with methylene linker appeared to be inactive (no virus inhibition was achieved even at highest concentrations used), while among the derivatives of quinazoline-2,4-dione 4g, 4h, 4i, compound 4g with the same methylene linker showed a good activity (IC50 = 42 µM). Thirdly, the antiviral activity depends on the position of attachment of the 1,2,3-triazolylribofuranosyl fragment to the pyrimidine moiety. Thus, if compound 2i in which the 1,2,3-triazolyl-ribofuranosyl fragment was attached to the 6-methyluracil moiety at the N1 atom via butylene linker showed a good activity (CC50 = 311 µM, CC50 = 48 µM, SI = 6), then compound 8o in which the same fragment was attached to the

![Scheme 2](image-url)
6-methyluracil moiety at the C5 atom was completely inactive (IC\textsubscript{50} > 787 µM). Fourthly, 1,2,3-triazolyl nucleoside analogues based on 3,6-dimethyluracil in which both nitrogen atoms of the pyrimidine fragment are alkylated (compounds 6g, 6h, 6i) completely devoid of antiviral activity against influenza virus A H1N1. Summarizing the data presented in Table 1, one can see that the 1,2,3-triazole nucleoside analogue 4i seems to be the most promising one among others combining high antiviral potency (IC\textsubscript{50} = 30 µM) and very low cytotoxicity (CC\textsubscript{50} > 719 µM) with the high selectivity index (SI) of 24. Nucleoside 8n, although possessing more higher cytotoxicity (CC\textsubscript{50} = 79 µM), can also be considered a lead compound because it had the highest antiviral activity among the studied ones (IC\textsubscript{50} = 15 µM).

It is interesting to compare antiviral activity of the lead compound 4i and antiviral activity of the reference compounds which differ according to both their structure and viral targets. Rimantadine blocks the transport of H\textsuperscript{+} ions through the M2 protein channels, Oseltamivir is a highly selective inhibitor of influenza A and B virus neuraminidases and Ribavirin in the triphosphate form efficiently inhibits the RNA polymerase of those viruses [4]. And as for the structure, Ribavirin is the only drug approved for the treatment of influenza viruses which is a nucleoside analogue and its structure is the closest to the structure of the compounds synthesized in this study. Table 1 demonstrates that the IC\textsubscript{50} value of the lead compound 4i is very close to the IC\textsubscript{50} value of Ribavirin that can be an indirect indication that 4i affects the same viral target as Ribavirin does.

The results of an in vitro study of cytotoxic and antiviral properties of several synthesized compounds against Coxsackie B3 virus are presented in Table 2. It can be seen that almost all the compounds studied were inactive against the Coxsackie B3 virus. The only exception is the 1,2,3-triazolyl pyrimidine analogue 8k in which the 1,2,3-triazolyribofuranosyl residue is attached via propylene linker to the C5 atom of the 6-methyluracil moiety. This lead compound showed
Table 1  Antiviral activity against H1N1 A/Puerto Rico/8/34 influenza virus and cytotoxicity of the synthesized 1,2,3-triazolyl nucleoside analogues

| Compound | Structure | IC$_{50}$ (µM) | CC$_{50}$ (µM) | SI$^a$
|----------|-----------|----------------|----------------|-----|
| 4g       | ![structure](image) | > 880          | > 880          | 1   |
| 1h       | ![structure](image) | > 850          | > 850          | 1   |
| 1l       | ![structure](image) | > 817          | 343 ± 41       | 2   |
| 2g       | ![structure](image) | > 885          | > 885          | 1   |
| 2h       | ![structure](image) | 254 ± 19       | 43 ± 5         | 6   |
| 3l       | ![structure](image) | 311 ± 27       | 48 ± 6         | 6   |
| 3g       | ![structure](image) | > 885          | 590 ± 50       | 2   |
| 3h       | ![structure](image) | > 817          | 354 ± 39       | 2   |
| 3l       | ![structure](image) | > 787          | 278 ± 31       | 3   |
| 4g       | ![structure](image) | 132 ± 9        | 42 ± 5         | 3   |
| 4h       | ![structure](image) | > 744          | 212 ± 25       | 4   |
| 4l       | ![structure](image) | > 719          | 30 ± 4         | 24  |
| 6g       | ![structure](image) | > 850          | > 850          | 1   |
| 6h       | ![structure](image) | > 756          | 483 ± 52       | 2   |
| 6l       | ![structure](image) | > 730          | > 730          | 1   |
| 8j       | ![structure](image) | 77 ± 5         | > 27           | 3   |
| 8k       | ![structure](image) | 104 ± 8        | 67 ± 8         | 2   |
| 8l       | ![structure](image) | > 592          | > 592          | 1   |
| 8m       | ![structure](image) | 132 ± 7        | 97 ± 10        | 1   |
| 8n       | ![structure](image) | 79 ± 6         | 15 ± 3         | 5   |
| 8o       | ![structure](image) | > 787          | > 787          | 1   |
| Rimantadine | ![structure](image) | 340 ± 16       | 77 ± 8         | 4   |
| Oseltamivir carboxylate | ![structure](image) | > 200          | 0.3            | > 667 |
| Ribavirin | ![structure](image) | 94 ± 48        | 31 ± 9         | 3   |
Table 1 (continued)  

| Compound | Structure | CC<sub>50</sub>[μM] | IC<sub>50</sub>[μM] | SI<sup>c</sup> |
|----------|-----------|---------------------|---------------------|-------------|
| 1g       | ![Structure](1g.png) | >1173               | >1173               | 1           |
| 1i       | ![Structure](1i.png) | >1089               | >1089               | 1           |
| 2g       | ![Structure](2g.png) | >1180               | >1180               | 1           |
| 2h       | ![Structure](2h.png) | >1090               | >1090               | 1           |
| 2i       | ![Structure](2i.png) | 656 ± 33            | 106 ± 11            | 6           |
| 4g       | ![Structure](4g.png) | 166 ± 12            | 133 ± 15            | 1           |
| 4h       | ![Structure](4h.png) | 101 ± 8             | >82                 | 1           |
| 4i       | ![Structure](4i.png) | 101 ± 9             | >79                 | 1           |
| 8j       | ![Structure](8j.png) | 77 ± 5              | >27                 | 3           |
| 8k       | ![Structure](8k.png) | 144 ± 11            | 9 ± 2               | 16          |
| 8l       | ![Structure](8l.png) | 313 ± 23            | >197 ± 21           | 2           |
| 8m       | ![Structure](8m.png) | 184 ± 13            | >148                | 1           |
| 8n       | ![Structure](8n.png) | 140 ± 10            | >34                 | 4           |
| 8o       | ![Structure](8o.png) | 525 ± 31            | >263                | 2           |
| Pleconaril | ![Structure](Pleconaril.png) | >1000               | 21.6                | 46          |
| Ribavirin | ![Structure](Ribavirin.png) | >1000               | >1000               | -           |

<sup>a</sup>CC<sub>50</sub> is the median cytotoxic concentration, i.e., the concentration causing 50% cell death

<sup>b</sup>IC<sub>50</sub> is the concentration causing 50% inhibition of virus replication

<sup>c</sup>SI is the selectivity index, which is the CC<sub>50</sub>/IC<sub>50</sub> ratio

Table 2  Antiviral activity against Coxsackie B3 virus and cytotoxicity of synthesized 1,2,3-triazolyl nucleoside analogues
both high antiviral activity (IC$_{50}$ = 9 μM) and good selectivity index (SI = 16).

The analysis of Table 2 enables to note the following two interesting features. Firstly, it was namely compound 8k with protected hydroxy groups of sugar residue showed maximum antiviral activity against coxsackievirus B3. In our opinion, this is the first case when antiviral activity was exhibited by a nucleoside analogue with protected hydroxy groups of the sugar residue. Secondly, Ribavirin having the structure similar to that of compound 8k, in contrast to 8k, appeared to be inactive against this enterovirus. At the same time, compound 8k showed antiviral activity against coxsackie B3 virus which was three times higher than that of Pleconaril having the structure significantly different from that of 8k. As to Pleconaril, it prevents the interaction of Coxsackievirus genome with surface glycoproteins ICAM-1 of cells of the human immune system thus inhibiting the viral replication [40]. Does this mean that compound 8k affect the same target of Coxsakie B3 virus? The question is certainly interesting and requires a special research.

Molecular docking

Current researches on developing new antiviral drugs focus on a limited number of potential targets [41]. Most approved antivirals target surface proteins of influenza A virus [41] such as the M2 ion channel, neuraminidase and hemagglutinin which allow the virus to interact with the cell surface. However, the future belongs to antivirals inhibiting proteins which drive the virus replication cycle [42] such as viral polymerase, non-structural proteins and nucleoproteins. Great hopes are pinned on new inhibitors of RNA-dependent RNA polymerase (RdRp) [43] because of its critical role in virus replication and high degree of sequence conservation in influenza A and B viruses, particularly in the active sites for RNA binding, cleavage or elongation [44]. The RdRp consists of three separate polypeptides called polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA) [45]. If these proteins will be inhibited or their mutual interaction will be disrupted, the RNA-dependent RNA polymerase will not be able to function and virus replication will be stopped [43, 45]. It has been shown that the antiviral activity of nucleoside and nucleobase analogues can be due to interactions with the fragment of PA containing the N-terminal endonuclease domain (PA-Nter) of RdRp [43, 46]. This is also true for compounds having similar structural motif with the lead compound 4i, e.g., BMS-183355, BMS-183021 [47] and Ribavirin (RBV) [4, 7, 14–17]. Therefore, we decided to test the ability of the lead compounds 4i, 8n as well as RBV which was chosen as one of the reference compounds to bind to RNA-dependent RNA polymerase (RdRp) in molecular docking simulations. As a potential target we chose the ligand-binding domain (LBD) of acidic polymerase (PA) of RNA-dependent RNA polymerase. The PA-Nter domain has a cation-dependent endonuclease active site core, the catalytic residues His41, Glu80, Asp108 and Glu119 being conserved among all influenza A subtypes and strains [42]. In addition to synthesized compounds 4i, 8n and reference compound RBV, the ability of their 5′-triphosphate (TP) derivatives 4i-TP, 8n-TP and RBV-TP to bind to the LBD has been also evaluated, because nucleoside analogues undergo an intracellular phosphorylation and inhibit RdRp in a 5′-triphosphate form [48–50].

We have evaluated the binding of 4i, 8n and Ribavirin (RBV) as well as their 5′-triphosphate derivatives 4i-TP, 8n-TP and RBV-TP to the PA-Nter endonuclease domain (PDB code 4AWK). The positions of the optimized docking models of compounds 4i, 8n, 4i-PPP, 8n-PPP demonstrating the best binding energy in the PA-Nter active site are shown in Fig. 1. According to the docking simulations, all ligands are located deep in the cavity of the active site of PA-Nter, being in the same amino acid environment. Compounds 4i, 8n and RBV do not show a similar binding motif in LBD PA-Nter. The lead compound 4i is retained in the PA-Nter cavity by hydrogen bonding of the C5′-OH group with the amino acid residues Arg82, Glu23 and by the π–π interaction between the quinazoline-2,4-dione moiety and Tyr24. The electrostatic interaction of the amino acid residues Ala20, Ile38 and His41 with the polar part of the quinazoline-2,4-dione moiety also contributes to the binding. However, the lead compound 8n binds to the protein cavity mainly due to nonspecific interactions, and the only electrostatic interaction is observed with the Glu23. RBV binding occurs due to the hydrogen bond of the C5′-OH group with Tyr130 and the π–π interaction of the 1,2,4-triazole ring of RBV with the imidazole moiety of His41, as well as hydrophobic interactions of RBV with Ala20, Ile120 and Val122. At the same time, 4i-TP and 8n-TP bind to the PA-Nter cavity in a similar manner due to the hydrogen bonds of their heterocyclic moieties with the Glu80′ residue and the triphosphate anion moiety with the Lys134 residue. Besides, 8n-TP forms additional hydrogen bonds with the Glu23 and Leu106 residues of the protein molecule and 4i-TP binds by electrostatic interactions with amino acid residues Glu23, Tyr24 and Lys137. The binding pattern of RBV-TP and triphosphates 4i-TP and 8n-TP
Fig. 1 Molecular docking simulations and two-dimensional interaction map of the optimized docking model of compounds 4i (a), 4i-TP (b), 8n (c), 8n-TP (d) RBV (e), RBV-TP (f) in the PA-Nter (PDB code 4AWK) active site obtained in the lowest-energy conformations.
Fig. 1 (continued)
in the PA-Nter cavity differs significantly. The RBV-TP molecule interacts with the protein through the hydrogen bonds of its heterocyclic moiety with Pro107 and the sugar residue with Glu119.

The lead compounds 4i and 8n were found to bind equally well to the PA-Nter active site with a high binding energy (−8.1 and −7.9 kcal/mol, respectively), while RBV binding is worser (−6.1 kcal/mol) (Table 3). At the same time, their 5′-triphosphate derivatives 4i-TP, 8n-TP and RBV-TP bind to the LBD PA-Nter better than their precursors demonstrating binding energies −8.3, −8.5 and −8.0 kcal/mol, respectively. It should be noted that if there is no a correlation between the antiviral activity and the binding energy of the ligand–protein complex for the lead compounds 4i, 8n and reference compound RBV, then for their 5′-triphosphate derivatives 4i-TP, 8n-TP and RBV-TP such a correlation is clearly visible in Table 3. The IC_{50} values decrease in the series of 5′-triphosphates RBV-TP > 4i-TP > 8n-TP from 32 to 15 μM, that is, the antiviral activity increases in this row, and in the same direction the binding energy increases from 8.0 to 8.5 kcal/mol, that is, the binding of these 5′-triphosphates with the active site of the protein becomes more stronger when going from RBV-TP to 8n-TP. Since Ribavirin inhibits RNA-dependent RNA polymerase (RdRp) in the 5′-triphosphate form [4, 48, 49], the observed correlation testifies that the cause of the antiviral activity of the lead compounds 4i and 8n against influenza virus A/PR/8/34 can also be associated with the inhibition of RdRp by their 5′-triphosphate derivatives.

**Conclusion**

In summary, a series of 1,2,3-triazolyl nucleoside analogues have been synthesized using “click” chemistry methodology. It has been observed that 1,2,3-triazolyl derivatives of natural nucleosides uridine (1g, 1h, 1i) and thymidine (3g, 3h, 3i) lack antiviral activity against influenza virus A/PR/8/34/(H1N1). On the contrary, the antiviral activity was demonstrated by 1,2,3-triazolyl nucleoside analogues in which the nucleic bases were replaced by 6-methyluracil (compounds 2h, 2i, 2j, 8k, 8m, 8n) and quinazoline-2,4-dione (compounds 4g, 4h, 4i) moieties. The best values of IC_{50} and SI were demonstrated by the lead compound 4i in which the 1,2,3-triazolylribofuranosyl fragment is attached to the N1 atom of the quinazoline-2,4-dione moiety via butylene linker (IC_{50} = 30 μM, CC_{50} > 719 μM, SI = 24). Compound 8n in which the 1,2,3-triazolylribofuranosyl fragment is

| Compound | S | IC_{50} [μM] | E_{bind} [kcal/mol] |
|----------|---|-------------|-------------------|
| Ribavirin (RBV) | | 32 ± 9 | 6.1 |
| 4i | | 30 ± 4 | 8.1 |
| 8n | | 15 ± 3 | 7.9 |
| RBV-TP | | 32 ± 9 | 8.0 |
| 4i-TP | | 30 ± 4 | 8.3 |
| 8n-TP | | 15 ± 3 | 8.5 |
attached to the N5 atom of the uracil moiety via propylene linker showed the highest antiviral activity among the studied compounds (IC50 = 15 µM) but more higher cytotoxicity (CC50 = 79 µM) and very low selectivity index (SI = 5). It should be noted that alkylation of the N3 atom of the 6-methyluracil moiety in the 1,2,3-triazole nucleoside analogues, that is, going from compounds 2g, 2h, 2i to compounds 6g, 6h, 6i leads to the complete loss of antiviral activity against influenza virus A H1N1. Almost all the compounds studied appeared to be inactive against the coxsackievirus B3. The only exception is the 1,2,3-triazolyl pyrimidine analogue 8k in which the 1,2,3-triazolyl ribofuranosyl residue is attached via propylene linker to the C5 atom of the 6-methyluracil moiety. This lead compound showed both high antiviral activity (IC50 = 9 µM) and a good selectivity index (SI = 16). According to theoretical calculations, the antiviral activity of the 1,2,3-triazolyl nucleoside analogues 4i, 8n against H1N1 (A/PR/8/34) influenza virus can be explained by their influence on the functioning of the polymerase acidic protein (PA) of RNA-dependent RNA polymerase (RdRp).

**Starting ω-alkyne substituted pyrimidines**

Pyrimidine derivatives 1a–c, 2a–c, 3a–c, 4a,c, 6a–c with ω-alkyne substituents at the N1 atom were prepared as described earlier [36]. Spectral data of 1b,c, 2a–c, 3b,c, 4a,c, 6a–c were in keeping with published ones [36]. Spectral data of 1a, 3a agreed with the literature [51]. Pyrimidine derivatives 8d, 8e, 8g were synthesized according to the known protocols [37, 38]. Spectral parameters of 8e and 8g agreed with those presented in Refs. [37, 38], respectively.

**N1-(Pent-4′-yn-1′-yl)quinazoline-2,4-dione (4b)** Concentrated sulfuric acid (0.15 mL) was added under stirring at room temperature to a suspension of quinazoline-2,4(1H,3H)-dione (3 g, 19.0 mol) and hexamethyldisilazane (HMDS) (6.86 g, 47.0 mmol) in toluene (100 mL). The mixture was refluxed for 4 h, the solvent and excessive HMDS were distilled off, and a solution of 5-iodo-1-pentyne (5.53 g, 28.5 mol) in DMF (3 mL) were added in the residue. The mixture was stirred at 110–120 °C for 12 h. The solvent was distilled off, chloroform (150 mL) was added to the residue, and the mixture was filtered. The filtrate was concentrated to 15–20 mL, and the residue was subjected to column chromatography on silica gel using first petroleum ether, second petroleum ether-ethyl acetate (1.5:1) and then ethyl acetate as eluent. Compound 4b was isolated from the fraction of ethyl acetate as a cream-colored powder. Yield: 3.54 g (82%); mp: 140 °C. 1H NMR (400 MHz, CDCl3): δ 8.42 (s, 1H, NH), 8.23 (dd, 1H, J = 1.8, 7.9 Hz, ArH), 7.74–7.68 (m, 1H, ArH), 7.36–7.27 (m, 2H, ArH), 4.24 (t, 2H, J = 7.7 Hz, CH2-11), 2.41–2.33 (m, 2H, CH2-12), 2.07 (t, 1H, J = 2.8 Hz, CH-15), 2.02–1.93 (m, 2H, CH2-13), 1.45 (s, 1H, NH), 2.15–2.11 (m, 2H, CH2-9), 2.07–2.03 (m, 2H, CH2-8). 13C NMR (100 MHz, CDCl3): δ 161.89 (C-4), 150.33 (C-2), 140.94 (C-9), 135.55 (C-10), 128.99, 123.08, 116.15, 113.88 (C-5, C-6, C-7, C-8), 82.85 (C-14), 69.50 (C-15), 41.95 (C-11), 25.72 (C-13), 16.03 (C-12). MALDI-MS calcd. for C13H12N2O2: [M+H]+ 251.1. Anal. calcd. for C13H12N2O2, %: C, 68.41; H, 5.30; N, 12.34. Spec-ral data of 4b were in keeping with published ones [36]. Spectral data of 3a was prepared as described earlier [36]. Spectral data of 1a, 3a, 4a,c, 6a–c were in keeping with published ones [36]. Spectral data of 1a, 3a agreed with the literature [51]. Pyrimidine derivatives 8d, 8e, 8g were synthesized according to the known protocols [37, 38]. Spectral parameters of 8e and 8g agreed with those presented in Refs. [37, 38], respectively.

**Starting ω-alkyne substituted pyrimidines**

Pyrimidine derivatives 1a–c, 2a–c, 3a–c, 4a,c, 6a–c with ω-alkyne substituents at the N1 atom were prepared as described earlier [36]. Spectral data of 1b,c, 2a–c, 3b,c, 4a,c, 6a–c were in keeping with published ones [36]. Spectral data of 1a, 3a agreed with the literature [51]. Pyrimidine derivatives 8d, 8e, 8g were synthesized according to the known protocols [37, 38]. Spectral parameters of 8e and 8g agreed with those presented in Refs. [37, 38], respectively.

**Experimental**

**Chemicals and instruments**

The 1H NMR spectra were recorded on 400 MHz and 600 MHz Brucker Advance. 13C NMR spectra were obtained in the above instruments operating at 100.6 MHz. Melting points were obtained on an Electrothermal IA 9000 instrument (Electrothermal, Great Britain). Mass spectra (MALDI) were recorded in a positive ion mode on a Bruker Ultraflex III TOF/TOF mass spectrometer for 10−3 mg/ml solutions. The ESI MS measurements were performed using an AmazonX ion trap mass spectrometer (Bruker Daltonic GmbH, Germany) in positive mode in the mass range of 70–3000. The ESI MS measurements were performed using an AmazonX ion trap mass spectrometer (Bruker Daltonic GmbH, Germany) in positive mode in the mass range of 70–3000. The capillary voltage was 4000 V, nitrogen drying gas 10 L min−1, desolvation temperature 250 °C. A methanol/water solution (70:30) was used as a mobile phase at a flow rate of 0.2 mL/min by binary pump (Agilent 1260 chromatograph, USA). The sample was dissolved in methanol to a concentration of 10−6 g L−1. Flash chromatography was performed on silica gel 60 (40–63 µm, Buchi, Sepacore). Thin-layer chromatography was performed on silica gel (Sorbfil, Russia). Spots of compounds were visualized by using ultraviolet fluorescence under a short wavelength (254 nm) followed by heating the plates (at ca. 150 °C) for 5 min in an oven. All reactions sensitive to air and/or moisture were carried out under argon atmosphere with anhydrous solvents. Anhydrous solvents were purified and dried (where appropriate) according to standard procedures.

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147.92 (C-6), 107.97 (C-5), 84.30 (C-10), 71.05 (C-11), 27.28 (C-7), 23.16 (C-9), 17.34 (C-8), 15.73 (CH₂). MALDI-MS calc. for C₉H₁₂N₂O₃: [M+H]⁺ 193.1, [M+Na]⁺ 215.1, [M+K]⁺ 231.1. found: [M+H]⁺ 193.2, [M+Na]⁺ 215.2, [M+K]⁺ 231.2. Anal. calc. for C₉H₁₂N₂O₃: %: C 64.07; H 6.84; N 13.58; found, %: C 64.59; H 6.87; N 13.58. M 210.27.

**Ethyl 2-acetyl-7-octynoate (8c)** Acetoxy ester (20 g, 153.8 mol) was added dropwise with stirring to a solution of sodium (3.54 g, 153.9 mol) in 200 ml of ethanol at 0–5 °C, the mixture then was treated with 5-iodo-hex-5-yn-1-yne (13.12 g, 160.0 mol) at temperature not above than 10 °C. The reaction mixture was stirred for 2.5 h at room temperature and then refluxed for 10 h. The solvent was removed, and the residue was treated with chloroform and filtered. Distillation of the dried MgSO₄ chloroform solution gave 8c as a colorless oil. Yield: 21.00 g (65%); bp: 192.22. 1H NMR (400 MHz, CDCl₃): δ 4.11 (q, J = 7.2 Hz, CH-12), 2.27–2.21 (m, 2H, CH₂-10), 2.20–2.13 (m, 2H, CH₂-9), 1.50–1.41 (m, 4H, CH₂-8, CH₂-7). 13C NMR (100 MHz, DMSO-d₆): δ 173.76 (C-4), 164.24 (C-11), 153.80 (C-2), 148.15 (C-6), 114.42 (C-5), 84.25 (C-11), 70.92 (C-12), 27.50 (C-7), 26.98 (C-10), 23.25 (C-9), 17.40 (C-8), 15.53 (CH₃). MALDI-MS calc. for C₁₂H₁₈O₃: [M+H]⁺ 207.1, [M+Na]⁺ 229.1, [M+K]⁺ 245.1; found: [M+H]⁺ 207.1, [M+Na]⁺ 229.1, [M+K]⁺ 245.1. Anal. calc. for C₁₂H₁₈O₃: %: C 64.06; H 6.84; N 13.58; found, %: C 64.15; H 8.67; N 13.50. M 206.25.

2,4-(1H,3H)‑pyrimidine (8f) The mixture of 8c (7.27 g, 34.6 mol) and K₂CO₃ (4.77 g, 34.6 mol) in 150 ml of methanol was refluxed for 15 h. The solvent was distilled off, the residue was insoluble in water, and the solution was acidified to approximately pH 2–3. The precipitate was recrystallized from methanol to afford compound 8f. Yield: 1.88 g (80%); mp: 203 °C. 1H NMR (600 MHz, DMSO-d₆): δ 10.83 (s, 1H, NH), 10.55 (s, 1H, NH), 2.67 (m, 1H, CH-12), 2.23–2.13 (m, 4H, CH₂-7, CH₂-10), 2.04 (s, 3H, CH₃), 1.45–1.37 (m, 4H, CH₂-8, CH₂-9). 13C NMR (100 MHz, DMSO-d₆): δ 164.24 (C-4), 150.64 (C-2), 147.57 (C-6), 108.60 (C-5), 84.36 (C-11), 70.96 (C-12), 27.54 (C-7), 23.21 (C-10), 17.43 (C-9, C-8), 15.74 (CH₃). MALDI-MS calc. for C₁₁H₁₄N₂OS: [M+H]⁺ 261.1; found: [M+H]⁺ 261.1. Anal. calc. for C₁₁H₁₄N₂OS: %: C 68.54; H 8.63; found, %: C 68.59; H 8.70. M 192.22.

**Target 1,2,3-triazolyl nucleosides analogues**

N₁-[(1-2,3,5′-tri-O-acetyl-β-D-ribofuranosyl)-1H-1,2,3-triazol-4-yl]propyl]-2,4(1H,3H)-quinoxalinedione (4e) A white foam, yield: 0.38 g (83%). 1H NMR (400 MHz, CDCl₃): δ 8.92 (s, 1H, CH-4), 8.20 (dd, 1H, J = 7.8, 1.5 Hz, ArH.), 7.73–7.67 (m, 1H, ArH), 7.67 (s, 1H, CH-5′), 7.31–7.23 (m, 2H, ArH), 6.16 (d, 1H, J = 3.6 Hz, CH-1′), 5.82 (dd, 1H, J = 5.2, 3.9 Hz, CH-3′), 5.62 (t, 1H, J = 5.2 Hz, CH-2′), 4.49–4.44 (m, 1H, CH-4′), 4.41 (dd, 1H, J = 12.1, 3.2 Hz, CH-5′a), 4.27–4.18 (m, 3H, CH-5′b, CH₂-11), 2.88 (t, 2H, J = 7.1 Hz, CH₂-13), 2.23–2.15 (m, 2H, CH₂-12), 2.12 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.07 (s, 3H, OAc). 13C NMR (100 MHz, CDCl₃): δ 173.76 (C-12), 164.24 (C-11), 153.80 (C-2), 148.15 (C-6), 114.42 (C-5), 84.25 (C-11), 70.92 (C-12), 27.50 (C-7), 26.98 (C-10), 23.25 (C-9), 17.40 (C-8), 15.53 (CH₃). MALDI-MS calc. for C₂₅H₂₇N₅O₉: [M+H]⁺ 530.1, [M+Na]⁺ 552.1, [M+K]⁺ 568.1; found: [M+H]⁺ 530.1, [M+Na]⁺ 552.1, [M+K]⁺ 568.0. Anal. calc. for C₂₅H₂₇N₅O₉: %: C 54.44; H 5.14; N 13.23; found, %: C 54.41; H 5.19, N 13.26. M 529.50.

N₁-[(1-β-D-ribofuranosyl)-1H-1,2,3-triazol-4-yl]propyl]-2,4(1H,3H)-quinoxalinedione (4d) A white foam, yield: 0.14 g (92%). 1H NMR (400 MHz, DMSO-d₆): δ 11.49 (s, 1H, NH), 8.04 (s, 1H, CH-5′), 7.98 (dd, 1H, J = 7.9, 1.5 Hz, ArH.), 7.75–7.70 (m, 1H, ArH), 7.39 (d, 1H, J = 8.4 Hz, ArH), 7.26 (t, 1H, J = 7.5 Hz, ArH), 5.86 (d, 1H,

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6-Methyl-5-[(1-2′,3′,5′-tri-O-acetyl-β-D-ribofuranosyl)-1-
H-1,2,3-triazol-4-yl]methyl]-2,4(1H,3H)-pyrimidine-
dione (8l)

A white foam, yield: 0.41 g (75%). 1H NMR (600 MHz, DMSO-d6): δ 9.93 (s, 1H, NH), 9.17 (s, 1H, NH), 7.58 (s, 1H, CH-5′), 6.13 (d, 1H, J = 3.8 Hz, CH-1′), 5.81–5.77 (m, 1H, CH-3′), 5.63 (t, 1H, J = 5.4 Hz, CH-2′), 4.48–4.39 (m, 2H, CH-4′, CH-5′a), 4.23 (dd, 1H, J = 11.6, 4.1 Hz, CH-5′b), 2.77 (t, 2H, J = 7.5 Hz, CH2-10), 2.38 (t, 2H, J = 7.7 Hz, CH2-7), 2.16 (s, 3H, CH3), 2.12 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.08 (s, 3H, OAc), 1.76–1.67 (m, 2H, CH2-9), 1.54–1.45 (m, 2H, CH2-8). 13C NMR (100 MHz, CDCl3): δ 170.52, 169.48, 169.30 (COCH3), 164.25 (C-4), 151.66 (C-2), 148.29 (C-6), 147.11 (C-4′), 120.16 (C-5′), 111.22 (C-5), 89.94 (C-1′), 80.65 (C-4′), 74.35 (C-3′), 70.81 (C-2′), 63.07 (C-5′), 28.72 (C-10), 27.91 (C-7), 24.99, 24.10 (C-9, C-8), 20.71, 20.48, 20.41 (COCH3), 16.80 (CH3). MALDI-MS calcd. for C21H29N5O9: [M+H]+ 507.4, [M+Na]+ 530.4; found: [M+H]+ 507.4, [M+Na]+ 530.4. Anal. calcd. for C21H29N5O9, %: C 52.07; H 5.76; N 13.80; found, %: C 52.01; H 5.79, N 13.85. M 507.4.

6-Methyl-5-[(1-β-D-ribofuranosyl)-1H-1,2,3-triazol-4-y]-
yl]methyl]-2,4(1H,3H)-pyrimidine-dione (8m)

A white powder, yield: 0.1 g (83%). mp: 259–260 °C. 1H NMR (600 MHz, DMSO-d6): δ 7.94 (s, 1H, CH-5′), 5.83 (d, 1H, J = 4.7 Hz, CH-1′), 5.57 (br s, 1H, OH), 5.28 (br s, 1H, OH), 5.05 (br s, 1H, OH), 4.35–4.29 (m, 1H, CH-3′), 4.11–4.04 (m, 2H, CH2-9), 3.96–3.91 (m, 1H, CH-4′), 3.6 (2H, CH2-7), 3.58–3.43 (m, 2H, CH2-5′), 2.11 22 (3H, CH3). 13C NMR (100 MHz, DMSO-d6): δ 164.45 (C-4), 151.09 (C-2), 149.86 (C-6), 146.01 (C-4′), 120.95 (C-5′), 106.88 (C-5), 92.07 (C-1′), 85.89 (C-4′), 75.09 (C-3′), 70.57 (C-2′), 61.60 (C-5′), 20.61 (C-7), 16.51 (CH3). MALDI-MS calcd. for C15H17N5O6: [M+Na]+ 362.1; found: [M+Na]+ 362.2. Anal. calcd. for C15H17N5O6, %: C 46.02; H 5.05; N 20.64; found, %: C 45.99; H 4.99, N 15.09. M 349.30.

6-Methyl-5-[(1-β-D-ribofuranosyl)-1H-1,2,3-triazol-4-
yl]propyl]-2,4(1H,3H)-pyrimidine-dione (8n)

A white powder, yield: 0.36 g (78%); mp: 191–193 °C. 1H NMR (600 MHz, CD3OD): δ 8.08 (s, 1H, CH-5′), 6.00 (d, 1H, J = 3.8 Hz, CH-1′), 4.47 (t, 1H, J = 4.2 Hz, CH-3′), 4.29 (t, 1H, J = 4.7 Hz, CH-2′), 4.13–4.10 (m, 1H, CH-4′), 3.81 (dd, 1H, J = 12.4, 3.1 Hz, CH-5′a), 3.68 (dd, 1H, J = 12.3, 4.1 Hz, CH-5′b), 2.74 (t, 2H, J = 7.3 Hz, CH2-9), 2.40 (t, 2H, J = 7.6 Hz, CH2-7), 2.11 (s, 3H, CH3), 1.84–1.78 (m, 2H, CH2-8). 13C NMR (100 MHz, CD3OD): δ 167.01 (C-4′), 152.94 (C-2′), 150.37 (C-6), 148.92 (C-4′), 122.24 (C-5′), 110.97 (C-5), 94.58 (C-1′), 87.25 (C-4′), 77.12 (C-3′), 71.96 (C-2′), 62.91 (C-5′), 39.25 (C-9), 25.86 (C-7), 24.86 (C-8), 24.76 (C-9), 24.16 (C-8), 23.16 (C-7), 22.16 (C-8), 21.16 (C-9), 19.86 (C-10), 18.46 (C-11), 18.16 (C-12, C-13). MALDI-MS calcd. for C23H29N5O10: [M+H]+ 560.4, [M+Na]+ 582.4; found: [M+H]+ 560.8, [M+Na]+ 582.4. Anal. calcd. for C23H29N5O10, %: C 52.07; H 5.76; N 13.80; found, %: C 52.01; H 5.79, N 13.85. M 560.4.
16.39 (CH₃). ESI-MS calcd. for C₁₅H₁₄N₄O₆: [M+H]+ 368.2, [M+Na]+ 390.2; found: [M+H]+ 368.2, [M+Na]+ 390.2. Anal. calcd. for C₁₅H₁₄N₄O₆, %: C 49.04; H 7.56; N 19.06; found, %: C 49.00; H 5.79, N 19.09. M 367.36.

6-Methyl-5-[1-β-D-ribofuranosyl]–1H-1,2,3-triazol-4-y]butyl]-2,4(1H,3H)-pyrimidinedione (8o) A white powder, yield: 0.11 g (76%); mp: 179–181 °C. ¹H NMR (400 MHz, CD₃OD): δ 167.02 (C-4), 152.96 (C-2), 150.10 (C-6), 144.22 (C-10), 122.01 (C-5), 111.42 (C-5′), 77.06 (C-2′), 71.98 (C-3′), 62.97 (C-5′a), 30.09 (C-10), 29.29 (C-7), 26.03, 25.02 (C-9, C-8), 16.41 (CH₃). ESI-MS calcd. for C₁₆H₂₃N₅O₆: [M+H]+ 382.2; found: [M+H]+ 382.2. Anal. calcd. for C₁₆H₂₃N₅O₆, %: C 50.39; H 5.76; N 18.34.

Molecular docking was carried out using the AutoDock 4.2 Vina software and AutoDock Tools (ADT 1.5.6) [52]. The standard 3D structures of 4i, 8n, RBV, 4i-TP, 8n-TP and RBV-TP were constructed using the HyperChem 8.0 [55] and converted into a pdb file by Open Babel [56], and a cubic grid box of 16 x 16 x 18 Å (x, y, z) with a spacing of 1.000 Å and grid maps were generated. The docking parameters were used as the default settings. The enzyme–ligand interactions were detected using ADT 1.5.6 and have been presented as 2D diagrams.

Biology

Cytotoxicity assay

MDCK (ATCC CCL-34) and Vero (ATCC CCL-81) cells initially obtained from American Type Culture Collection (Rockville, MD, USA) were seeded into 96-well plates and incubated for 24 h at 36 °C at 5% CO₂ until confluent monolayer is formed. Threefold dilutions (400–4 μg/mL) were prepared on Eagle’s minimal essential medium (MEM) from the compounds under investigation, added to the cells and incubated for 24 h at 36 °C at 5% CO₂. The cell monolayer was washed twice with saline (0.9% NaCl), and 100 μL of MTT solution [3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide], 0.5 μg/mL in MEM, was added into each well. The plates were incubated for 1 h at 36 °C, then the medium was removed and formazan pellets were dissolved in dimethyl sulfoxide (0.1 mL per well). The optical density in the wells was measured on a spectrophotometer Thermo Multiskan FC at the wavelength of 540 nm. The results obtained were used for calculating the concentration of the compound resulting in death of 50% cells in the culture (CC₅₀) using GraphPad Prism software employing the four-parameter logistic curve model. The values of CC₅₀ were then converted from μg/mL to μM.

Cell protection assay

The compounds in appropriate concentrations were added to cells (0.1 mL per well). Cells were further infected with either A/Puerto Rico/8/34 (H1N1) influenza virus (for MDCK cells) or Coxackie B3 virus (for Vero cells) (m.o.i 0.01 in both cases) and incubated for 48 h at 36 °C at 5% CO₂. After that, cell viability was assessed by MTT test (see above). The cytoprotective activity of compounds was considered as their ability to increase the values of OD comparing to control wells (with virus only, no drugs). Based on the results obtained, the values of IC₅₀, i.e., concentration of compounds that result in 50% cells protection, were calculated using GraphPad Prism software.

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Affiliations

Olga V. Andreeva\textsuperscript{1} · Bulat F. Garifullin\textsuperscript{1} · Vladimir V. Zarubaev\textsuperscript{2} · Alexander V. Slita\textsuperscript{2} · Iana L. Yesaulkova\textsuperscript{2} · Liliya F. Saifina\textsuperscript{1} · Marina M. Shulaeva\textsuperscript{1} · Maya G. Belenok\textsuperscript{1} · Vyacheslav E. Semenov\textsuperscript{1,2} · Vladimir E. Kataev\textsuperscript{1}

\textsuperscript{1} Arbuzov Institute of Organic and Physical Chemistry, FRC Kazan Scientific Center, Russian Academy of Sciences, Arbuzov Str., 8, Kazan, Russian Federation 420088

\textsuperscript{2} Pasteur Institute of Epidemiology and Microbiology, Mira Str., 14, Saint Petersburg, Russian Federation 197101