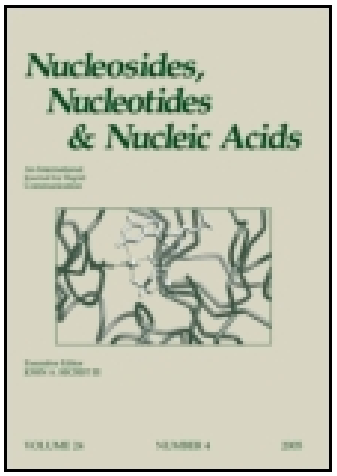


Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lncn20

Synthesis and Comparative Study of Anti-Adenoviral Activity of 6-Azacytidine and Its Analogues

Inna Alexeeva[a], Lydia Nosach[b], Larisa Palchykovska[a], Lyubov Usenko[a] & Olga Povnitsa[b]

[a] Institute of Molecular Biology and Genetics, National Academy of Sciences, Kyiv, Ukraine

[b] D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences, Kyiv, Ukraine

Published online: 13 Jul 2015.

To cite this article: Inna Alexeeva, Lydia Nosach, Larisa Palchykovska, Lyubov Usenko & Olga Povnitsa (2015) Synthesis and Comparative Study of Anti-Adenoviral Activity of 6-Azacytidine and Its Analogues, Nucleosides, Nucleotides and Nucleic Acids, 34:8, 565-578, DOI: 10.1080/15257770.2015.1034363

To link to this article: http://dx.doi.org/10.1080/15257770.2015.1034363

Nucleosides, Nucleotides and Nucleic Acids, 34:565–578, 2015

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770.2015.1034363

SYNTHESIS AND COMPARATIVE STUDY OF ANTI-ADENOVIRAL ACTIVITY OF 6-AZACYTIDINE AND ITS ANALOGUES

Inna Alexeeva,[1] Lydia Nosach,[2] Larisa Palchykovska,[1] Lyubov Usenko,[1] and Olga Povnitsa[2]

[1]*Institute of Molecular Biology and Genetics, National Academy of Sciences, Kyiv, Ukraine*
[2]*D.K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences, Kyiv, Ukraine*

□ *This paper presents the results of synthesis and study of cytotoxicity and the anti-adenoviral activity of new* N4*-derivatives of 6-azacytidine and its* α*-L-glycopyranosyl analogues obtained by the simplified one-pot version of the silyl condensation method. The resulting acylated 4-methylmercapto-1,2,4-triazin-3(2H)-one glycosides then underwent the amination and/or ammonolysis to provide 6-azacytidine glycoside analogues (**2–6**, **12**, **15**, **17**) and compounds with modifications at both base and sugar fragments (**11**, **15**). The evaluation of cytotoxicity and antiviral activity of new compounds against AdV5 showed high selectivity indexes for* N4*-methyl-6-azacytidine (**2**) and* N,O*-tetraacetyl-6-azacytidine (**8**). High anti-adenoviral activity of* N4*-methyl-6-azacytidine as well as very low cytotoxicity may suggest its further investigation as potential compound for the therapy of AdV infection.*

Keywords 6-azacytidine; nucleoside analogues; human adenovirus; antiviral agents

INTRODUCTION

Human adenoviruses (AdV) are ubiquitous infectious DNA viruses possessing a broad spectrum of pathogenicity. More than 50 human AdV serotypes have been identified that are responsible for respiratory, gastrointestinal, and ocular diseases. AdV are able to persist in humans for a long time in the latent state and can be reactivated by various factors, especially grave problems they cause in immunocompromised hosts by the development of generalized AdV infection. Disseminated AdV infections frequently lead to a lethal outcome.

Currently known efficient inhibitors of AdV reproduction are represented by various classes of synthetic and natural compounds. In particular, compounds inhibiting AdV adsorption, virus DNA replication, synthesis

Received 23 March 2014; accepted 23 March 2015.
Address correspondence to Dr. Inna Alexeeva, Institute of Molecular Biology and Genetics, National Academy of Sciences, 150 Zabolotnoho Str. 03680, Kyiv, Ukraine. E-mail: i.v.alexeeva@imbg.org.ua

of structural viral proteins, and proteolytic processing of some structural polypeptides were found. Some of the discussed compounds that demonstrated anti-adenoviral activity *in vitro* and *in vivo* could be potent antiviral drugs.[1–4] Nevertheless, only two nucleoside analogues, namely cydofovir and ribavirin, are currently used in clinical practice for the treatment of patients with life-threatening AdV infections.[3] Some side effects of these drugs have limited their application in the treatment of AdV diseases.

6-Azacytidine (2-β-D-ribofuranosyl-5-amino-1,2,4-triazin-3(2H)-one, 6-azaCyd), a structural analogue of natural cytidine, was synthesized by the Czech–Ukrainian team,[5] and studied first as a potential cancerostatic.[6,7] More recent research on antiviral properties of 6-azaCyd has demonstrated its high activity relative to human AdV *in vitro* and *in vivo* systems.[8–13] In cell cultures, the anti-adenoviral effect of 6-azaCyd was expressed by inhibiting the formation of virus-specific intranuclear DNA-containing inclusion bodies, the synthesis of viral proteins and infectious virus.[12,14]

The application of 6-azaCyd on a model of disseminated AdV infection in newborn Syrian hamsters led to significant decrease of virus titer and shortening of the time of virus clearance in organs.[11,12]

This work describes the synthesis and comparative results of the studies of anti-adenoviral activity of novel and already known 6-azaCyd structural analogues (N-α-L-glycosides 6-azacytosine, its N^4-derivatives, and *seco*-6-aza-nucleosides). The goal of the present study was to find the most effective inhibitor against AdV in its parental nucleoside form and to determine separate molecular fragment that enhances the anti-AdV activity.

RESULTS AND DISCUSSION

Chemistry

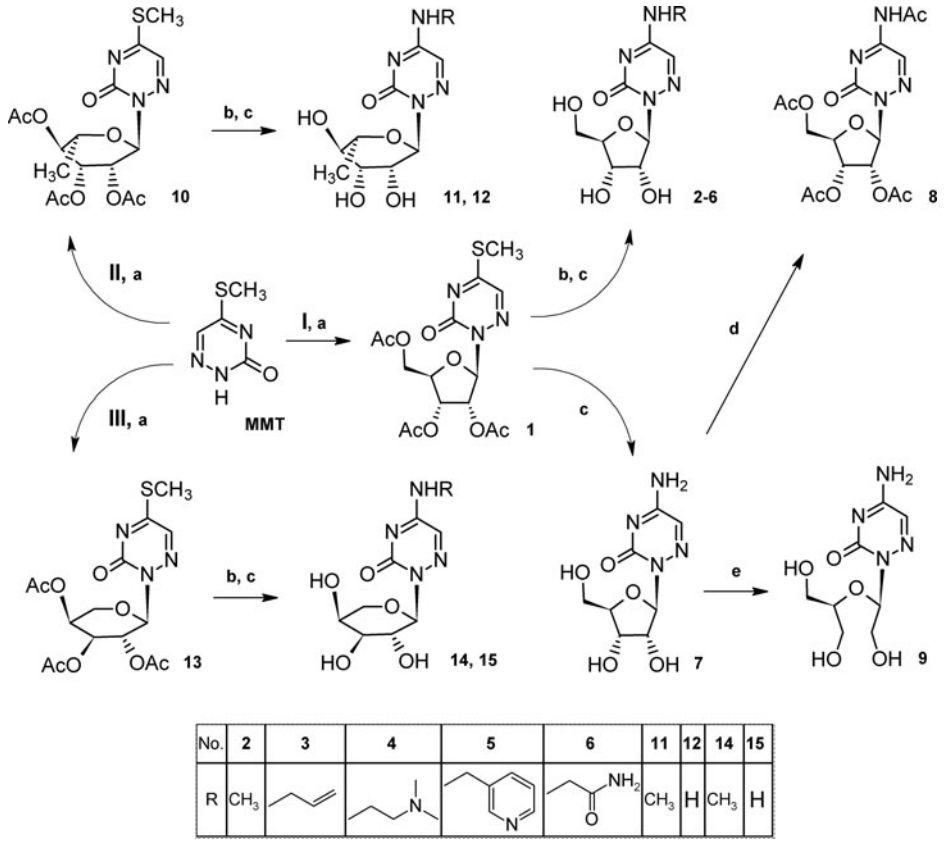
Chemical strategy for the synthesis of 6-azaCyd analogues was based on the bioisosteric substitution of individual atoms, functional groups, and molecular fragments. Efficient methods that have been developed allowed us to obtain compounds in sufficiently high yields.

The synthesis of 6-azaCyd glycoside analogues was performed by a simplified one-pot version of the "silyl condensation" method.[10,14–16] This one-pot procedure involves the formation of silyl ether of heterocyclic aglycone with excess trimethylchlorosilane (TMSCl) and hexamethyldisilasane (HDMS) and its interaction with protected carbohydrate activated by tin(IV) chloride (Schemes 1, 2).[17] As expected, β-anomers of nucleosides were formed in all cases according to NMR data ($J_{1'-2'}$ coupling constants in the range 3.6–4.0 Hz). Glycosylation position was confirmed by a bathochromic shift of UV absorbance maxima of nucleosides in acidic medium (0.1 M HCl), which is a characteristic for *N2*-glycosides of 5-amino-1,2,4-triazune.[15] The

resulting acylated *N2*-glycosides of 5-metylmercapto-1,2,4-triazin-3(2H)-one (MMT) (compounds **1**, **10**, **13)** and 3,5-dithio-(2,3,4,5)-tetrahydro-1,2,4-triazine (DTT) *N2*-riboside (**16**) then underwent amination and/or ammonolyzis for obtaining the corresponding *N*-glycosides of 1,2,4-triazine base (**2–7**, **12**, **15**, **17**) and compounds with modifications at both nucleoside fragments (**11**, **14**). The glycosidic bond of *N2*-triazinyl nucleosides (in contrast to that in *N4*-derivatives) is known to be stable to alkaline hydrolysis.[11] Deblocking of protecting groups in triacetyl glycosides **1**, **10**, **13** by treatment with aqueous ammonia/ethanol (4:1, v/v, RT) resulted in simultaneous substitution of 5-methylmercapto group for amino group and formation of corresponding *N*-glycosides of 6-azacytosine (Scheme 1). This general procedure previously reported by our laboratory was easily applied for 6-azaCyd analogues.[11] Also, the selective amination of triazinebase and ammonolyzis acetoxy-groups ribofuranoside (**16)** into the target 6-azaCyd thio-analogue (**17**) was achieved in fairly good yield by action of methanolic ammonia in a sealed vessel at room temperature (Scheme 2). The treatment of acetylated nucleosides **1**, **10**, **13** with an excess (1.5–2.0 eq.) of appropriate alkylamine in absolute ethanol afforded amino-derivatives. Next, the hydrolysis of acetyl groups of carbohydrate fragment with ammonium hydroxide gave glycosides **2–6**, **11**, **14**, respectively. The *N,O*-tetraacetyl derivative **8** was prepared according to the procedure given in literature.[18]

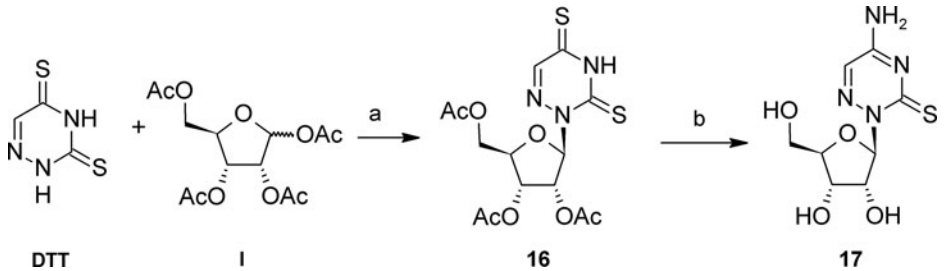
Acyclic 6-azaCyd and 5-methyl-6-azaCyd 2',3'- *seco*-nucleosides (**9**, **9a**) were obtained via the periodate oxidation of carbohydrate residue of corresponding ribofuranosides ($NaIO_4$, 1.1 eq.) followed by the reduction of thus generated aldehyde groups by sodium borohydride as reported previously.[18,19]

The structures of the obtained compounds were confirmed by spectral data (^{1}H NMR, UV, and MS) and their comparison with the spectra of 6-azacytidine and its derivatives with known structure.[9,11] Thus, the position of anomeric proton of ^{exo}N-monosubstituted β-D-ribofuranosyl derivatives **2–6** (δ 5.94–5.96 ppm with a coupling constant value of 3.6–4.0 Hz) and their λ_{max} (266–272 nm) is in agreement with reported data.[9] Chemical shifts of anomeric protons and $J_{1'-2'}$ coupling constants of rhamno- and arabinopyranosyl derivatives **11–15** are close to those of previously reported glucopyranosyl analogues.[11] Note that the NMR spectra of free nucleosides **12**, **15**, as well as *seco*-nucleoside **9**, show the doublet splitting of *exo*-NH_2 group signal characteristic for 6-azacytidine (Δ_δ 0.11–0.12 ppm), which is most pronounced in compounds **9a** [19] and **17** (Δ_δ 0.55 and 0.39 ppm, respectively). The non-equivalence of amino group protons results from its restricted rotation and the asymmetry of 1,2,4-triazinyl heterocycle electronic structure, which is enhanced by the introduction of substituents at C-3 and/or C-6 positions of the ring. It is assumed that one of the NH_2-group protons is involved in intramolecular hydrogen binding to the nitrogen atom *N*-4.[20] This interpretation is consistent with quantum-chemical



SCHEME 1 Synthesis of novel compounds **2–6**, **11**, **12**, **14**, **15**. Reagents and conditions: I – peracetyl-D-ribofuranose, II – peracetyl-L-arabinopyranose, III – peracetyl-L-rhamnopyranose. (a) Peracetyl-sugar, TMSCl, HMDS, $SnCl_4$, CH_3CN, RT, 4–5 hours; (b) amine/ethanol, RT, 0.5 hour; (c) aq. NH_3/ethanol, 4:1 v/v, RT, 16–18 hours; (d) Ac_2O/Py;[18] (e) $NaIO_4$, then $NaBH_4$.[19]

characteristics of 6-azacytidine. This nucleoside exhibits a high dipole moment and can actively participate in the formation of hydrogen bonds and dipole–dipole and π-stacking interactions.[21,22] Formation of similar H-bonds and other interaction can be suggested for its glycoside analogues as well.



SCHEME 2 Synthesis of compounds **16**, **17**. Reagents and conditions: (a) TMSCl, HMDS, $SnCl_4$, CH_3CN, RT, 6 hours; (b) methanolic ammonia, sealed flask, RT, 24 hours.

TABLE 1 The effect of 6-azacytidine derivatives and analogues on the reproduction of AdV5 adenovirus *in vitro*

Compound	Name (short name)	Cell line	Serotype	EC_{50} (μM)*	CC_{50} (μM)*	SI
2	*N4*-methyl-6-azacytidine	Hep-2	Ad-5	0.4	1210	>3000
3	*N4*-allyl-6-azacytidine	Hep-2	Ad-5	56	1700	30
4	*N4*-(dimethylaminoethyl)-6-azacytidine	Hep-2	Ad-5	25.4	920	36
5	*N4*-(pyridine-3-ylmethyl)-6-azacytidine	Hep-2	Ad-5	24.0	560	23
6	*N4*-(carbamoylmethyl)-6-azacytidine	Hep-2	Ad-5	26.0	3320	125
7	6-azacytidine (6-azaCyd)	Hep-2	Ad-5	2.0	760	380
8	*N,O*-tetraacetyl-6-azaCyd	HeLa	Ad-2	0.3	380	>1200
9	6-azaCyd (*seco*)	Hep-2	Ad-2	>100	—	—
9a	5-metyl-6-azaCyd (*seco*)	Hep-2	Ad-2	30	500	16
11	2-α-L-arabinopyranosyl-N^4–methyl-6-azacytosine	Hep-2	Ad-5	31	1000	33
12	2-α-L-arabinopyranosyl-6-azacytosine	Hep-2	Ad-2	>100	> 400	4
14	2-α-L-rhamnopyranosyl-N^4–methyl-6-azacytosine	Hep-2	Ad-5	>100	—	—
15	2-α-L-rhamnopyranosyl-6-azacytosine	Hep-2	Ad-5	>100	—	—
17	2-thio-6-azaCyd	HeLa	Ad-2	0.8	24.0	30.0
18	Ribavirin	Hep-2	Ad-5	33	2000	60
19	Cidofovir	Hep-2	Ad-5	10.7	3560	334

*The average from three independent experiments, standard deviation below 15%.

ANTIVIRAL ACTIVITY

Anti-adenoviral activity of 6-azaCyd derivatives was evaluated using the cytomorphological test developed previously by us that allows to precisely calculate the number of infected cells by the presence of AdV-induced DNA-containing intranuclear inclusion bodies. The correctness of this method was confirmed by the fluorescent antibodies method (MFA) according to the presence of major structural protein hexon in nucleus. Both methods demonstrated correlation between anti-adenoviral activity index values for 6-azaCyd, 6-azauridine, and ribavirin.[12,14,23] This direct quantitative virus-specific method allows correct determination of anti-adenoviral effect of tested compounds and has several advantages over the indirect assays, such as cytopathic effect (CPE), plaque formation, or viability of infected cells (MTT test) in the primary screening of compounds.

As can be seen in Table 1, 6-azaCyd (**7**) is an efficient and selective inhibitor of AdV with EC_{50} of 2.0 μM and a selectivity index (SI) of 380.

It should be noted that ribavirin (**18**) and cidofovir (**19**) reference compounds studied under the same experimental conditions appeared to be much weaker AdV inhibitors (EC_{50} 33 and 10.7 μM, respectively) than 6-azaCyd.

The modified 6-azaCyd derivatives **2–6**, **17** containing various substituents at C-3 and C-5 positions of 1,2,4-triazinyl aglycone demonstrated significant structure-dependent anti-adenoviral activity. They inhibited AdV reproduction with inhibitory concentration ranging from 0.4 to 56 μM and low cytotoxicity. Compounds **4**, **5**, and **6** showed decrease in antiviral effect (EC_{50} 24–26 μM) in comparison with the parent compound, but these values are close to those obtained for ribavirin. The lowest cytotoxicity was demonstrated by compound **6**. The most efficient compounds in this series of 6-azaCyd derivatives were *N4*-methyl-6-azaCyd (**2**) and 2-thio-6-azaCyd (**17**), with EC_{50} values being 0.4 μM and 0.8 μM, respectively. However, these compounds demonstrated very different cytotoxicity levels that have an effect on selectivity index. The most active compound **2** has high SI value (>3000), whereas it was low for compound **17** (SI $\sim$ 30). As shown in Table 1, compound **8** with substituents located at both moieties of nucleoside molecule also appeared to be an efficient AdV inhibitor (EC_{50} 0.3 μM, SI value = 1200).

Seco-nucleoside **9** with unsubstituted aglycones, α-L-arabinopyranosyl-6-azaCyt (**12**) and α-L-rhamnopyranosyl-6-azaCyt (**15**), as well as other glycosides containing carbohydrate fragments other than ribofuranose, have lost their ability to inhibit AdV replication (EC_{50} > 100 μM). Presence of a methyl group at C-5 or C-6 position of aglycone of *seco*-nucleoside **9a** and α-L-arabinopyranoside **11**, respectively, enhanced their anti-adenoviral activity (EC_{50} $\sim$ 30 μM) but they showed no increase in antiviral effect in comparison with the parent compound. However, the same modification of α-L-rhamnopyranoside **14** was inefficient (EC_{50} > 100 μM).

Previous studies on anti-adenoviral activity of various 6-azaCyd analogues, including 2′-deoxy- and 2′,3′-dideoxynucleosides,[9] did not reveal structural elements enhancing the antiviral effect. In this work, we studied a wide range of compounds. *N*-glycopyranosides of 6-azacytosine and *seco*-nucleoside **9** were inactive against AdV even at high concentrations (>100 μM), which may be due to their structural features, especially the conformation state of carbohydrate fragment in these compounds resulting in the loss of their affinity to viral targets. Similar results concerning the decrease or loss of activity after the substitution of ribofuranose residue for other sugars were obtained with ribavirin[24] and 6-azacytidine glycoside analogues.[11] At the same time, significant increase in anti-adenoviral activity observed for compounds **2**, **9a**, and **11** in comparison with **7**, **9**, and **12** was probably caused by the presence of CH_3-group at C-5 or C-6 position of triazinyl aglycone. It is known that the introduction of a lipophilic methyl group into the compound structure may enhance its interaction with

appropriate target area. In addition, electron-donor properties of methyl group can affect molecule ionization that is frequently reflected in biological characteristics of compounds as well. Thus, the presence of *N4*-methyl group in compound **2** led to almost five-fold increase of its anti-adenoviral activity as compared with the parent 6-azaCyd. However, in the case of α-L-rhamnopyranoside **14**, the presence of CH_3 groups at both *N4*-positions of aglycone did not lead to expected result. It can be supposed that the C-5′ methyl group of a carbohydrate moiety found in compounds **14** and **15** may significantly restrict the conformational possibilities of nucleosides and their interaction with the target.[25] Elongation of alkyl chain of *N4*-substituent (compounds **3**, **4**, and **6**) or the introduction of pyridyl group (**5**) resulted in the weakening of biological effect (Table 1). Thus, methyl substituent is an important pharmacophoric element for enhancing the anti-adenoviral activity for **9a** and **11** glycoside analogues and *N4*-methyl derivative **2**.

Compound **17** is one of the best AdV inhibitors, but at the same time, it has the highest cytotoxicity. This effect may be possibly associated with the easiness of oxidation of C-3 thiol group in its imine tautomer of triazinyl ring with a formation of disulfide bond with biopolymers.

Compound **8** acylated at amino and hydroxyl groups (depot form of 6-azaCyd) exhibited high anti-adenoviral activity, perhaps due to the increased lipophilicity.

The inhibitory effect of 6-azaCyd was analyzed in our previous studies also at the level of viral proteins synthesis. Electrophoretic analysis of ^{14}C-labeled viral proteins synthesized in 48 hours after infecting and cell treatment with 6-azaCyd showed the absence of some early and late proteins being synthesized prior and after DNA replication, respectively. These proteins required for DNA replication include the DNA-binding protein (DBP, 72 kDa), the precursor of terminal protein (pTP, 75 kDa), which may form durable heterodimer with AdV-polymerase and serves as a primer for AdV DNA replication and AdV DNA polymerase, and 140 kDa protein. Blocking of the synthesis of major structural protein (hexon) was also demonstrated by the fluorescent antibodies technique.[8,23] It should be noted that the effect of 6-azaCyd on the synthesis of viral proteins is comparable with that of ribavirin reported by us previously,[14]

The 6-azaCyd inhibition of the formation of intranuclear DNA-containing inclusion bodies and the synthesis of early virus-induced and structural viral proteins allow us to suppose that 6-azaCyd is able to completely block the expression of adenoviral genome.

CONCLUSIONS

A series of new 6-azaCyd analogues and derivatives were synthesized and their antiviral activity against AdV reproduction in cell cultures was

evaluated. The results of the present study showed the increase of antiadenoviral effect of **2**, **8**, and **17** in comparison with 6-azaCyd and reference compounds (ribavirin and cydofovir). Two compounds were found to exhibit the activity exceeding five to seven times that of 6-azaCyd, namely **2** (*N4*-methyl-6-azaCyd) and **8** (*N,O*-tetraacetyl-6-azacytidine). Anti-adenoviral action of compounds **2**, **9a**, and **11** is determined by the presence of CH_3 group, which may be a key pharmacophore in antiviral activity of the studied modified nucleosides. High antiviral effect of *N4*-methyl-6-azacytidine against AdV (EC_{50} 0.4 μM, SI > 3000) suggests its further investigation as a potential treatment for AdV infection.

EXPERIMENTAL

Chemistry

All reagents and solvents for synthesis were from UkrOrgSyntez (Ukraine), Fluka (Switzerland), and Reachim (Russia); they were purified and dried by standard methods. Reference compounds 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin, Virazole) and 1-(S)-[3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine (Cydofovir, Vistide) were obtained from Gilead Sciences (USA).

Thin-layer chromatography (TLC) was performed on Silica gel $60F_{254}$ plates (Merck, Germany) in the solvent system chloroform/methanol (9:1 or 14:1, v/v). The glycosides of 1,2,4-triazines were visualized on TLC plates using a specific color reaction with Dische's reagent. Column chromatography was carried out on silica gel (63–200 mesh, Merck) in the gradient of methanol in chloroform. The absorption spectra were recorded with a Shimadzu UV-3100 spectrophotometer (Japan). ^{1}H NMR spectra were recorded on a Varian Mercury 400 spectrometer (400 MHz) in DMSO-d_6 with tetramethylsilane as an internal standard; chemical shifts are given in ppm. Chromato-mass spectra were obtained with an Agilent 1100 LC/MSD SL instrument (Germany) in a positive mode. Melting points of new compounds were determined using a Boetius PNMK 05 apparatus (Nagema, Germany).

General procedure for the preparation of nucleoside intermediates 1, 10, 13. To a solution of 4-methylmercapto-1,2,4-triazin-3(2H)-on**e** (MMT base, 0.429 g, 3 mmol) and corresponding peracylated sugar (3.3 mmol) in absolute acetonitrile (25 mL) was added hexamethyldisilazane (HMDS, 0.52 mL, 2.4 mmol), trimethylchlorosilane (TMSCl, 0.6 mL, 4.8 mmol), and $SnCl_4$ (0.72 mL, 6.0 mmol), and the mixture was stirred under anhydrous conditions for 4–6 hours at room temperature. The reaction was monitored by TLC. The solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography in a gradient

of MeOH (0–5%) in $CHCl_3$. The acetylated nucleosides were obtained as chromatographically homogeneous oils (yields 75–82%).

***N2*-(2′,3′,5′-tri-*O*-acetyl)-β-D-ribofuranosyl-5-methylmercapto-1,2,4-triazin-3(2H)-one** (**1**). Colorless foam (0.98 g, 82%). UV ($CHCl_3$): λ_{max} 232, 302 nm; λ_{min} 257 nm. ^{1}H NMR: δ 7.97 (s, 1H, C5H), 6.11 (d, 1H, 1′-H, J = 3.2 Hz), 5.38 (dd, 1H, 2′-H), 5.21 (dd, 1H, 3′-H), 4.30 (m, 2H, 4′-H, 5′-H), 4.07 (m, 1H, 5′-H), 2.56 (s, 3H, SCH3), 2.08–2.02 (m, 9H, 3Ac). LC–MS: m/z 402.3 $[M+H]^+$.

General procedure for the synthesis of *N4*-derivatives of 6-azacytidine (2-6). To a suspension of 1.0 mmol intermediate **1** in 8 mL of ethanol was added an appropriate amino component (1.5–2 mmol), and the reaction mixture was stirred at room temperature for several hours (TLC control). The reaction mixture was concentrated to dryness under reduced pressure to afford clear oil. Deacylation of glycoside derivatives was carried out with a mixture of 25% aqueous ammonia and ethanol (4:1, v/v) for 16 hours at 18°C. The solvent was removed *in vacuo* and the residue was crystallized from ethanol.

2-(β-D-ribofuranosyl)-5-methylamino-1,2,4-triazin-3(2H)-one (2). Yield from **1**: 0.19 g (73%), mp: 219–221°C. UV (H_2O): λ_{max} 272 nm (lg ε 3.96). ^{1}H NMR: δ 8.45 (d, 1H, NH, J = 4.2 Hz), 7.46 (s, 1H, C5H), 5.94 (d, 1H, 1′-H, J = 3.6 Hz), 4.97 (d, 1H, 2′-OH, J = 4.2 Hz), 4.75 (d, 1H, 3′-OH, J = 5.6 Hz), 4.43 (t, 1H, 5′-OH, J = 5.6; 6.0 Hz), 4.18 (dd, 1H, 2′-H), 3.98 (dd, 1H, 3′-H), 3.76 (t, lH, 4′-H), 3.49 (m, 1H, 5′-H_β), 3.38 (m, 1H, 5′- H_α), 2.84 (d, 3H, CH_3, J = 4,8 Hz). LC–MS: m/z 259.2 $[M+H]^+$.

2-(β-D-ribofuranosyl)-5-allylamino-1,2,4-triazin-3(2H)-one (3). Yield from **1**: 0.169 g (56%), mp: 147–149°C. UV (H_2O): λ_{max} 273 nm (lg ε 3.84). ^{1}H NMR: δ 8.55 (t, 1H, NH), 7.52 (s, 1H, C5H), 7.33 (s, 1H, HC =), 5.95 (d, 1H, 1′-H, J = 4.0 Hz), 5.14–5.27 (dd, 2H, CH_2), 4.95 (d, 1H, 2′-OH, J = 5.6 Hz), 4.72 (d, 1H, 3′-OH, J = 6.0 Hz), 4.39 (t, 1H, 5′-OH, J = 5.6; 6.0 Hz), 4.20 (dd, 1H, 2′-H), 3.95–4.02 (m, 3H, CH_2-N, 3′-H), 3.78 (t, 1H, 4′-H), 3.52 (m, 1H, 5′-H_α), 3.40 (m, 1H, 5′- H_β). LC–MS: m/z 285.0 $[M+H]^+$.

2-(β-D-ribofuranosyl)-5-(2-dimethylaminoethyl)-amino-1,2,4-triazin-3 (2H)-one (4). Yield from **1**: 0.22 g (70%), mp: 240-242°C. UV (H_2O): λ_{max} 267.5 nm (lg ε 3.95). ^{1}H NMR: δ 8.36 (br.s, 1H, NH), 7.55 (s, 1H, C5H), 2.21 (s, 6H, CH_3), 5.94 (d, 1H, 1′-H, J = 3.6 Hz), 4.97 (d, 1H, 2′-OH, J = 4.2 Hz), 4.76 (d, 1H, 3′-OH, J = 7.0 Hz), 4.44 (t, 1H, 5′-OH, J = 5.6; 6.0 Hz), 4.18 (dd, 1H, 2′-H), 3.98 (dd, 1H, 3′-H), 3.76 (dd, 1H, 4′-H), 3.51 (m, 1H, 5′-H_α), 3.38–3.97 (m, 3H, CH_2, 5′-H_β), 2.43 (t, 2H, CH_2). LC–MS: m/z 316.2 $[M+H]^+$.

2-(β-D-ribofuranosyl)-5-(pyridin-3-ylmethyl)-amino-1,2,4-triazin-3(2H)-one (5). Yield from **1**: 0.20 g (62%), mp: 117–120°C. UV (H_2O): λ_{max} 267 nm (lg ε 3.95). ^{1}H NMR: δ 8.95 (br.s, 1H, NH), 8.54 (s, 1H, Py), 8.44 (d, 1H, Py), 7.74 (dd, 1H, Py), 7.54 (s, 1H, C5H), 6.61 (t, 1H, Py), 5.94 (d, 1H, 1′-H, J = 4.0 Hz), 4.97 (d, 1H, 2′-OH), 4.74 (d, 1H, 3′-OH), 4.55 (d, 2H,

CH_2, J = 7.6 Hz), 4.44 (t, 1H, 5′-OH), 4.18 (dd, 1H, 2′-H), 3.98 (dd, 1H, 3′-H), 3.76 (dd, 1 H, 4′-H), 3.51 (m, 1H, 5′-H_α), 3.43 (m, 1H, 5′-H_β), 1.07 (s, 1H, Py). LC–MS: m/z 336.0 $[M+H]^+$.

2-(β-D-ribofuranosyl)-5-(carbamoylmethyl)-amino-1,2,4-triazin-3(2H)-one (6). Yield from **1**: 0.181 g (60%), mp: 110–112°C. UV (H_2O): λ_{max} 271 nm (lg ε 3.96). ^1H NMR: δ 8.95 (br.s, 1H, NH), 7.68 (s, 1H, C5H), 7.55 (s, 1H, NH_2), 7.17 (s, 1H, NH_2), 5.98 (d, 1H, 1′-H, J = 3.9 Hz), 5.20 (d, 1H, 2′-OH, J = 5.4 Hz), 4.96 (d, 1H, 3′-OH, J = 6.0 Hz), 4.67 (t, 1H, 5′-OH, J = 5.7; 6.0 Hz), 4.22 (dd, 1H, 2′-H, J = 4.0; 5.6 Hz), 3.97 (dd, 1H, 3'-H, J = 6.0; 6.0 Hz), 3.89 (d, 2H, CH_2, J = 5.2 Hz), 3.76 (dd, 1H, 4′-H, J = 5.6; 6.0 Hz), 3.52–3.38 (m, 2H, 5′- H_α, 5′-H_β). LC–MS: m/z 302.0 $[M+H]^+$.

2-(β-D-ribofuranosyl)-5-amino-1,2,4-triazin-3(2H)-one (6-azaCyd, 7). Yield from **1**: 0.226 g (93%), mp: 223–225°C. UV (H_2O): λ_{max} 264 nm (lg ε 3.89), UV (0,1 M HCl): λ_{max} 285 (lg ε 3.96). ^1H NMR: δ 7.91, 7.77 (2s, 2H, NH_2), 7.45 (s, 1H, C5H), 5.94 (d, 1H, 1′-H, J = 4.0 Hz), 4.95 (d, 1H, 2′-OH, J = 5.2 Hz), 4.72 (d, 1H, 3′-OH, J = 6.0 Hz), 4.12 (t, 1H, 5′-OH, J = 5.2; 6.0 Hz), 4.20 (dd, 1H, 2′-H, J = 4.0; 5.6 Hz), 3.98 (dd, 1H, 3'-H, J = 6.0; 6.0 Hz), 3.77 (dd, 1H, 4′-H, J = 5.6; 4.4 Hz), 3.52 (m, 1H, 5′- H_α), 3.40 (m, 1H, 5′-H_β). LC–MS: m/z 245.1 $[M+H]^+$.

2-β-D-(2', 3', 5'- tri-*O*-acetyl)-*N5*-acetylamino-1,2,4-triazin-3(2H)-one (8). Compound **8** was obtained as reported previously.[18] Yield 94%, mp: 147–150°C (EtOH). UV (H_2O): λ_{max} 265 nm (lg ε 4.06). ^1H NMR: δ 11.50 (s, 1H, NH), 8.72 (s, 1H, C5H), 6.21 (d, 1H, 1′-H, J = 3.2 Hz), 5.58 (t, 1H, 2′-H, J = 3.5 Hz), 5.40 (t, 1H, 3′-H, J = 5.6 Hz), 4.32 (m, 2H, 4'-H, 5'-H), 4.06 (dd, 1H, 5'-H, J = 4.4 Hz), 2.18 (s, 3H, Ac), 2.08 (2s, 6H, 2Ac), 2.02 (s, 3H, Ac).

5-Amino-2-[2′-hydroxy-1′-(2″-hydroxy-1″-hydroxymethyl-ethoxy)-ethyl]-1,2,4-triazin-3(2H)-one (9) was synthesized as a white solid by the procedure described in our paper.[19] Yield 92%, mp: 143–146°C. UV (H_2O): λ_{max} 263.5 nm (lg ε 3.79).

2-(α-L-arabinopyranosyl)-5-methylamino-1,2,4-triazin-3(2H)-one (11). Yield from **10**: 0.162 g (63%), mp: 225–228°C. UV (H_2O): λ_{max} 275.5 nm (lg ε 3.94). ^1H NMR: δ 8.49 (d, 1H, NH, J = 4.6), 7.55 (s, 1H, C5-H), 5.92 (d, 1H, 1′-H, J = 3.6 Hz), 5.42 (d, 1H, 2′-OH), 5.25 (d, 1H, 3′-OH), 4.72 (t, 1H, 4′-OH), 4.41 (dd, 1H, 2′-H), 3.83 (s, 2H, 3′-H, 4′-H), 3.53 (m, 1H, 5′-H_α), 3.40 (m, 1H, 5′-H_β), 2.80 (d, 3H, CH_3, J = 4.6 Hz). LC–MS: m/z 259.2 $[M+H]^+$.

2-(α-L-arabinopyranosyl)-5-amino-1,2,4-triazin-3(2H)-one (12). Yield from **10**: 0.157 g (61%), mp: 240–242°C. UV (H_2O): λ_{max} 264 nm (lg ε 3.82), UV (0,1 M HCl): λ_{max} 270 nm (lg ε 3.94). ^1H NMR: δ 7.88, 7.76 (2s, 2H, NH_2), 7.53 (s, 1H, C5-H), 5.91 (d, 1H, 1′-H, J = 5.6 Hz), 5.27 (d, 1H, 2′-OH, J = 6.4 Hz), 5.13 (d, 1H, 3′-OH, J = 6.0 Hz), 4.56 (t, 1H, 4′-OH), 4.40 (dd, 1H, 2′-H), 3.83 (m, 2H, 3′-H, 4′-H), 3.55 (m, 1H, 5′-H_α), 3.41 (m, 1H, 5′-H_β). LC–MS: m/z 245.0 $[M+H]^+$.

2-(α-L-rhamnopyranosyl)-5-methylamino-1,2,4-triazin-3(2H)-one (14). Yield from **13**: 0.176 g (56%), mp: 245–248°C. UV (H_2O): λ_{max} 274 nm (lg ε 3.95). ^{1}H NMR: δ 8.58 (d, 1H, NH, J = 4.4 Hz), 7.44 (s, 1H, C5-H), 5.93 (d, 1H, 1′-H, J = 4.0 Hz), 4.672 (d, 1H, 2′-OH), 4.66 (d, 1H, 3′-OH), 4.45 (s, 1H, 4′-OH), 3.99 (d, 1H, 2′-H, J = 3.6 Hz), 3.93 (br s, 1H, 3′-H), 3.64 (t, 1H, 4′-H, J = 6.4; 6.8 Hz), 3.31 (d, 1H, 5′-H), 2.51 (d, 3H, CH_3, J = 4,4 Hz), 1.183 (d, 3H, CH_3, J = 6,4 Hz). LC–MS: m/z 273.0 $[M+H]^+$.

2-(α-L-rhamnopyranosyl)-5-amino-1,2,4-triazin-3(2H)-one (15). Yield from **13**: 0.168 g (65%), mp: 262–264°C. UV (H_2O): λ_{max} 271.3 nm (lg ε 3.65), UV (0,1 M HCl): λ_{max} 279.7 (lg ε 4.01). ^{1}H NMR: δ 7.79, 7.68 (2s, 2H, NH_2), 7.43 (s, 1H, C5-H), 5.93 (d, 1H, 1′-H, J = 3.6 Hz), 4.66 (s, 1H, 2′-OH), 4.61 (s, 1H, 3′-OH), 4.45 (s, 1H, 4′-OH), 3.90–4.00 (dd, 2H, 3′-H; 2′-H), 3.65 (t, 1H, 4′-H, J = 6.4; 7.0 Hz), 3.30 (m, 1H, 5′-H), 1.18 (d, 3H, CH_3, J = 7.0 Hz). LC–MS: m/z 259.2 $[M+H]^+$.

2-(β-D-Ribofuranosyl)-5-amino-1,2,4-triazin-3(2H)-thione (2S-6-azaCyd, 2-thio-6-azacytidine, 17). The acetylated 2-β-D-ribofuranosyl-1,2,4-triazin-3,5(2H,4H)-dithione was prepared by the reaction of 2,4-dithio-6-azauracil[25] (0.145 g, 1 mmol) with tetraacetylribofuranose (0.35 g, 1 mmol) using the conventional methodology. Purification by silica gel flash chromatography (chloroform–methanol, 95:5 v/v) gave 0.31 g (77%) of the product **16** as a red-brown solid. It was dissolved in methanolic ammonia (15 mL), and gaseous ammonia was passed through this solution for 2 hours at room temperature. The mixture was kept in a sealed flask for 24 hours. The solvent was removed *in vacuo* and the residue was crystallized from ethanol. Yield 0.17 g (73%), mp: 235-239°C (lit. 235–238°C[26]). UV (EtOH): λ_{max} 242 nm (lg ε 4.09); 282 nm (lg ε 3.83). ^{1}H NMR: δ 8.53, 8.14 (2s, 2H, NH_2), 7.74 (s, 1H, C5H), 7.04 (d, 1H, 1′-H, J = 3.6 Hz), 5.07 (d, 1H, 2′-OH, J = 4.8 Hz), 4.76 (d, 1H, 3′-OH, J = 6.4 Hz), 4.43 (t, 1H, 5′-OH, J = 4.8; 6.0 Hz), 4.12 (dd, 1H, 2′-H, J = 4.0; 4,8 Hz), 4.00 (dd, 1H, 3′-H, J = 6.0; 5.6 Hz), 3.80 (dd, 1H, 4′-H, J = 6.0; 6.0 Hz), 3.54 (m, 1H, 5′-H), 3.45 (m, 1H, 5′-H). LC–MS: m/z 261.0 $[M+H]^+$.

BIOLOGY

Cells and Viruses

Reference strains of AdV (types 2 and 5) obtained from the Institute of Microbiology, Semmelweis University Medical School (Budapest, Hungary) and monolayer culture Hep-2 cells were used in the study. Cells were grown in a medium containing equal volumes of medium 199 and Eagle medium, supplemented with 10% of heat-inactivated fetal bovine serum (Sigma), 100 U/mL of penicillin and 100 mg/mL of streptomycin. Infected cells were incubated in maintenance medium (Eagle medium without serum).

Antiviral and Cytotoxicity Assays

Anti-AdV activity was determined by a method developed in our laboratory, and is based on the calculation of infected cells with DNA-containing intranuclear inclusion bodies induced by the virus.[14] In preliminary experiments, we have found the virus infection dose that in 48 hours post-infection induced intranuclear inclusion bodies in 70–80% of infected cells. Cells were grown in tubes with strips of cover glasses. In 48 hours, cells were infected with AdV and after 1 hour of the adsorption of virus at room temperature, cells were washed with Hank's solution and pre-incubated in maintenance medium containing test compounds at varying concentrations. The infected cells, which were not treated with the assayed compounds, were used as a positive control of virus infection. After 48 hours of incubation at 37°C, the cells were fixed with 96% ethanol, washed with Hank's solution, stained with 0.01% acridine orange solution, and the number of infected cells with DNA-containing inclusion bodies was counted using a luminescent microscope LM-2 (LOMO, Russia). On each of the three slides, 500 cells were counted and the percentage of infected cells was determined. The 50% effective concentration (EC_{50}) was determined as the compound concentration decreasing the percentage of infected cells with intranuclear inclusion bodies by 50% in comparison with the virus control.

The cytotoxicity of the compounds was determined in Hep-2 cells by a standard MTT test, a colorimetric assay for cell viability.[27] Hep-2 cells growing in 96-well plates were treated by serial dilutions of tested compounds for four days. The compound concentration that reduced the absorbance of mock-infected cells at 570 nm by 50% of that of the control was defined as 50% cytotoxic concentration (CC_{50}). Selectivity index was determined as a ratio of minimal cytotoxic concentration (CC_{50}) to minimal effective concentration (EC_{50}). Three independent experiments were performed to determine the CC_{50} and EC_{50} values for each compound presented in the Table.

ACKNOWLEDGMENT

The authors are grateful to Dr. Igor Dubey for valuable discussions and support.

REFERENCES

1. Waye, M.M.Y.; Sing, C.W. Anti-viral drugs for human adenoviruses. *Pharmaceuticals* **2010**, 3, 3343–3354.
2. De Clercq, E. A 40-year journey in search of selective antiviral chemotherapy. *Annu. Rev. Pharmacol. Toxicol.* **2011**, 51, 1–24.
3. Lenaerts, L.; De Clercq, E.; Naesens, L. Clinical features and treatment of adenovirus infections. *Rev. Med. Virol.* **2008**, 357–374.

4. Kinchington, P.R.; Romanowski, E.G.; Gordon, Y.J. Prospects for adenovirus antivirals. *J Antimicrob. Chemother.* **2005**, 55, 424–429.
5. Sorm, F.; Smrt, J.; Chernetsky, V. 6-Azacytidine – A new antimetabolite. *Experientia* **1961**, 17, 64.
6. Handschumacher, R.E., Skoda, J., Sorm, F. Metabolic and biochemical effects of 6-azacytidine in mice with Ehrlich ascites carcinoma. *Coll. Czech. Chem. Commun.* **1963**, 28, 2983–2990.
7. Petrusha, N.A. Some toxicopharmacological properties of 6-azacytidine. *Farmakologiya i Toksikologiya (Russ)* **1987**, 50, 75–76.
8. Alexeeva, I.; Dyachenko, N.; Nosach, L.; Zhovnovataya, V.; Rybalko, S.; Lozitskaya, R.; Fedchuk, A.; Lozitsky, V.; Gridina, T.; Shalamay, A.; Palchikovskaja, L.; Povnitsa, O. 6-Azacytidine – Compound with wide spectrum of antiviral activity. *Nucleosides Nucleotides Nucleic Acids* **2001**, 20, 1147–1152.
9. Alexeeva, I.V.; Palchikovskaya, L.G.; Shalamay, A.S.; Nosach, L.N.; Zhovnovataya, V.L.; Povnitsa, O.Yu.; Dyachenko, N.S. N_4-amino-acid derivatives of 6-azacytidine: Structure–activity relationship. *Acta Biochim. Polon.* **2000**, 47, 95–101.
10. Nosach, L.N.; Dyachenko, N.S.; Butenko, S.I.; Zhovnovataya, V.L.; Timofeeeva; M.Ya., Tarasyshyn, L.A.; Alexeeva, I.V., Chernetsky, V.P. The effect of 6-zacytidine on expression of adenovirus genome, in *New Approaches to Chemotherapy of Virus Infection*, ed. R. A. Kukain, Zinatne, Riga, Russia, **1991**, pp. 87–94.
11. Alexeeva, I.V.; Palchikovskaya, L.I.; Usenko, L.S.; Nosach, L.N.; Zhovnovataya, V.L.; Dyachenko, N.S. The glycosylic analogues of 6-azacytidine: Synthesis and antiviral activity. *Biopolym. Cell* **2004**, 20, 435–439.
12. Zarubaev, V.V.; Sukhinin, V.P.; Nosach, L.N.; Dyachenko, N.S.; Povnitsa, O.Y.; Zhovnovataya, V.L.; Alexeeva, I.V.; Palchikovskaya, L.I. Effect of 6-azacytidine on the course of experimental adenoviral infection in newborn Syrian hamsters. *Antivir. Chem. Chemother.* **2007**, 19, 44–51.
13. Zarubaev, V.V.; Garshinina, A.V.; Kalinina, N.A.; Shtro, A.A.; Belyaevskaya, S.V.; Slita, A.V.; Nebolsin, V.E.; Kiselev, O.I. Activity of ingavirin (2-[1H-imidazol-4-yl)ethylamino]-5-oxo-hexanoic acid) against human respiratory viruses *in vivo* experiments. *Pharmaceuticals* **2011**, 4, 1518–1534.
14. Nosach, L.N. Anti-adenoviral assay, based on the quantitative detection of infected cells containing virus-induced intranuclear inclusion bodies. *Antivir. Res.* **2011**, 90, A61.
15. Alexeeva, I.V.; Palchykovskaya, L.I.; Shalamay, A.S.; Ognyanik, S.S.; Morgart, N.V.; Petrusha, N.A. The synthesis and biological activity of N1-substituted 6-azacytosines. *Khim. Pharm. Zhurn.* **1994**, 4, 16–20.
16. Vorbruggen, H.; Bennua, B. Nucleoside synthesis. XXV. A new simplified nucleoside synthesis. *Chem. Ber.* **1981**, 114, 1279–1286.
17. Niedballa, U.; Vorbruggen, H. A general synthesis of *N*-glycosides 6. On the mechanism of the stannic chloride catalyzed silyl Hilbert–Johnson reaction. *J. Org. Chem.* **1976**, 41, 2084–2086.
18. Chernetsky, V.P.; Alexeeva, I.V. Synthesis of 6-azacytidine and its derivatives. *Khimiya Geterotsykl. Soed* **1967**, 6, 1109–1113.
19. Ognyanik, S.S.; Shalamay, A.S.; Alexeeva, I.V.; Lysenko, N.A. Synthesis of nonglycosidic analogues of 6-azacytidine and 6-azauridine. *Ukr. Khim. Zh.* **1995**, 61, 52–55.
20. Samijlenko, S.P.; Alexeeva, I.V.; Palchykivska, L.H.; Kondratyuk, I.V.; Stepanyugin, A.V.; Shalamay, A.S.; Hovorun, D.M. Structural peculiarities of 6-azacytosine and its derivatives imply intramolecular H-bonds. *J. Mol. Struct.* **1999**, 484, 31–38.
21. Platonov, M.O.; Sudakov, O.O.; Palchykovska, L.G.; Alexeeva, I.V.; Boyko, Yu. V.; Hovorun, D.M. Nonempirical quantum-chemical conformational analysis of 6-azacytidine, modified nucleoside with a wide spectrum of biological activities. *Dopovidi NAN Ukrainy* **2004**, 3, 163–169.
22. Iermak, Ie. L.; Kruglova, O.B.; Palchykovska, L.H.; Alexeeva, I.V. Spectrophotometrical study of mechanisms of cytidine analogues and ethidium bromide binding with DNA. *Biopolym. Cell* **2007**, 23, 529–537.
23. Nosach, L.N.; Dyachenko, N.S.; Zhovnovataya, V.L. Antiadenoviral activity of substance and medical form of the ribamidyl in cell culture. *Mikrobiol. Zh.* **2009**, 71, 60–67.
24. Sidwell, R.W. In vitro and in vivo inhibition of DNA viruses by ribavirin, in *Clinical Applications of Ribavirin*, eds. R.A. Smith, V. Knight, J.A.D. Smith, Academic Press, New York, NY, 1984, pp. 19–32.

25. Mikhailov, S.N.; Meshkov, S.V.; Kuznetsov, D.A.; Lysov, Yu. P.; Gorelik, E. Sh.-B.; Fomitcheva, M.V.; Beigelman, L.N.; Padyukova, N. Sh. Conformational peculiarities of 5′-C-methylnucleosides. *Bioorg. Chem.* **1989**, 15, 969–975.
26. Fu, Y.L.; Parthasarathy, R.; Bobek, M. Synthesis of 2-thio-6-azacytidine and 2,2′-anhydro-1-(2-thio-β-D-arabinofuranosyl)-6-azacytosine. *J. Carbohydrates Nucleosides Nucleotides* **1978**, 5, 79–87.
27. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J. Immunol. Meth.* **1983**, 65, 55–64.