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Phosphoramidate derivatives of acyclovir: Synthesis and antiviral activity in HIV-1 and HSV-1 models in vitro

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ABSTRACT

The antiviral activity against HIV and HSV and the chemical stability of ACV phosphoramidate derivatives were studied. The phosphoramidates of ACV demonstrated moderate activity. The best compound appeared to be 9-(2-hydroxymethyl)guanine phosphoromonomorpholidate (**7**), which inhibited virus replication in pseudo-HIV-1 particles by 50% at 50 μM. It also inhibited replication of wild-type HSV-1 (9.7 μM) as well as an acyclovir-resistant strain (25 μM). None of the synthesised compounds showed any cytotoxicity.

1. Introduction

Acyclic analogues of 2′-deoxyguanosine are an important group of medicinal products used for the treatment of human herpes virus (HHV) infections. The most popular drug of the series is 9-[(2-hydroxyethoxy)methyl]guanine (ACV, acyclovir, **1**).[1] Despite the fact that ACV is remarkably active against HSV-1 and HSV-2, it is less active against the varicella-zoster virus (VZV) and even less active against the human cytomegalovirus (HCMV), and it has low oral bioavailability.[2]

The efficacy of ACV may be improved with modifications that would increase its penetration into cells (or its absorption through the stomach) followed by subsequent transformation into the parent compound. A good example of this strategy is the valine ester of ACV, valacyclovir, which reveals higher bioavailability than its parent, ACV.[3]

Infection with HHV strains that are resistant to ACV treatment is an increasing clinical problem in patients with AIDS and other immunosuppressed states. Because of the rapid development of the virus's resistance to these drugs and their high toxicity,[4] many researchers have been prompted to seek new antiviral agents against the HHV family as well as against HIV. Within the background of HIV, other infectious diseases often attack HIV patients, such as the herpes virus, catarrhal diseases and other diseases that tend to decrease of the immunity of the patient. Treatment of these accompanying diseases often causes HIV infection suppression. The antiherpetic drug ACV was previously shown to inhibit HIV in cell cultures that were co-infected with the herpesvirus.[5]

It was discovered that the observed anti-HIV activity of ACV requires phosphorylation of the drug to its monophosphate form by the herpes thymidine kinase during coinfection because HIV does not encode enzymes that recognise ACV as a phosphorylation substrate. Further subsequent phosphorylation into the diphosphate and triphosphate derivatives is promoted by guanosine monophosphate kinase and nucleoside diphosphate kinase, respectively.[6,7] The resulting triphosphate is the derivative that demonstrates biological activity, acting as a chain terminator after ACV-monophosphate is incorporated into the 3′ end of the viral DNA.[5,8] Based on these results, the conclusion was made that anti-HIV activity is revealed only due to ACV-monophosphate and that HHV coinfection is essential for its formation. ACV-MP itself cannot be regarded as an effective anti-HIV drug capable of bypassing the first rate-limiting phosphorylation step because of its instability in biological media and low diffusion through cell membranes. Masking the negatively charged monophosphate with a lipophilic group is a suitable strategy that can overcome all of these limitations. As a result, the phosphoramidate ProTide approach has been developed and successfully implemented to create several nucleosides with antiherpetic and anticancer activities.[9–12] This approach involves masking the charge of a nucleotide's phosphate group with an aryl or amino acid ester substituent. In this

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form, penetration through the cell membranes takes place by passive diffusion. Once inside a cell, phosphoramidate is activated. It initially undergoes enzymatic hydrolysis of the amino acid ester, catalysed by esterases or carboxypeptidases, and thereafter, a spontaneous cyclization occurs along with simultaneous elimination of the aryl group and ring opening of the unstable cycle intermediate in the presence of water. Finally, the phosphoramidase-type enzymes hydrolyse the P–N bond, and the monophosphorylated nucleoside is released.

Recently, the phosphoramidate approach was applied to ACV derivatives, and it was shown that the phosphoramidate derivatives revealed antiviral activity against resistant strains of HSV-1 and HSV-2 as well as against VZV. This activity is attributed to the fact that these compounds do not need to undergo the first phosphorylation step by viral thymidine kinase.[13] It was demonstrated that the ACV derivatives inhibited HIV replication and did not show toxicity.[14] It is noteworthy to mention that because the ACV ProTides exhibit both anti-HIV and antiherpetic activity, they could be considered new double-targeting antivirals. Such drugs can inhibit HIV as well as HSV replication by directly and independently blocking the action of the key enzymes.[15]

The aim of this work is to study the anti-HIV and anti-HSV activities of the synthesised ACV phosphoramidates.

2. Results and discussion

As we previously reported, nucleoside phosphoromonoamidates, unlike phosphorodiamidates, demonstrate in some cases higher antiviral activity than the parent nucleoside.[16] Thus, AZT phosphoromonomorpholidate appeared to be far more active against HIV than AZT phosphorodimorpholidate and AZT itself. Additionally, phosphoromonoamidates, depending on the nature of their amine component, may have higher solubility than the parent nucleoside; therefore, they may be used as depot-forms of the nucleoside monophosphate. Therefore, because ACV phosphoromonoamidate can penetrate through cell membranes and undergo enzymatic reactions, it may also be able to bypass the first phosphorylation step and demonstrate antiviral activity (Fig. 1).

2.1. Synthesis

We previously demonstrated a method for the synthesis of various nucleoside phosphoramidates.[17] However, the ACV phosphoramidates were obtained in extremely poor yields (less than 10%). The main products of this reaction were ACV monophosphate, ACV monophosphate methyl ether and 9-(2-chloroethoxymeth yl)guanine.

Therefore, phosphoramidates of ACV (**3**–**13**) were synthesised as previously described.[18] Thus, ACV (**1**) was suspended in triethylphosphate, cooled to −18 °C, and phosphorus oxychloride was added (Scheme 1, stage a). After 4 h, the newly formed ACV phosphorodichloridate (**2**) was treated with the appropriate amidating agent in dioxane, containing 5% water (Scheme 1, stage b). The phosphorodiamidates were synthesised using the same scheme with an amine in non-aqueous dioxane and without *N*,*N*-diisopropylethylamine as the amidating agent (Scheme 1, stage c).

2.2. Chemical stability

We studied the chemical stability of the obtained compounds (Table 1).

As shown in Table 1, all of the synthesised compounds hydrolyse rapidly in acidic conditions (pH 2) to ACV monophosphate (**14**). Because the phosphorodiamidates (**3**, **5**, **6** and **12**) are slightly more stable to acid hydrolysis, we did not observe their stepwise hydrolysis to the corresponding phosphoromonoamidates. This result is likely explained by the considerably lower rate of hydrolysis of the first phosphamide bond in the phosphorodiamidates compared to the rate of hydrolysis of the phosphamide bond in the phosphoromonoamidates.

All of the synthesised phosphoromonoamidates (**4**, **7–11** and **13**) were stable at pH 7.4. It should be noted that the phosphorodiamidates (**3**, **5** and **12**), except for ACV phosphorodimorpholidate (**6**), appeared to be unstable and hydrolysed slowly to ACV (**1**) at pH 7.4 (ion-exchange and reversed-phase chromatography conditions). These results agree with the previously reported data,[19] where it is stated that guanosine phosphorodiamidates hydrolyses to guanosine in mildly basic conditions.

In strongly basic conditions (pH 12), the obtained phosphorodiamidates were less stable and hydrolysed to ACV.

It is interesting to note that hydrolysis of the phosphoromonoamidates begins at pH 12, and the formation of ACV (**1**) and ACV monophosphate (**14**) can be observed.

2.3. Antiviral studies

2.3.1. HIV-assay

Modified nucleosides and nucleotides are widely used for the therapy of many viral diseases, including HIV-1 infection.[8,20]

Their mechanism of action involves transformation of these compounds in cells into the corresponding nucleoside triphosphates, which serve as terminating substrates for viral DNA- and RNA-polymerases. Incorporation of nucleotides into the nascent viral DNA/RNA chain inhibits replication and suppresses development of the infection.

We studied the anti-HIV activity of the target phosphoromonoamidates against pseudo-HIV-1 particles in the Jurkat cell line (human T-cell acute lymphoblastic leukaemia) with the G-protein from the vesicular stomatitis virus on its surface.[21]

We considered that our phosphoromonoamidates could be latent forms of ACV monophosphate (**14**). It is known that ACV can demonstrate anti-HIV activity only in herpetic coinfections.[5] However, ProTide derivatives of ACV have demonstrated anti-HIV activity in cell systems in which mixed infection is expected. The

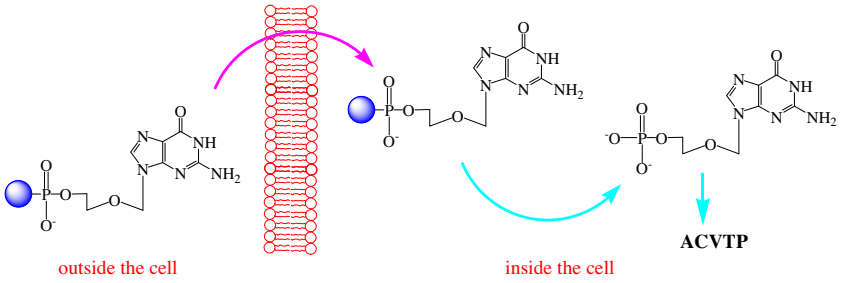
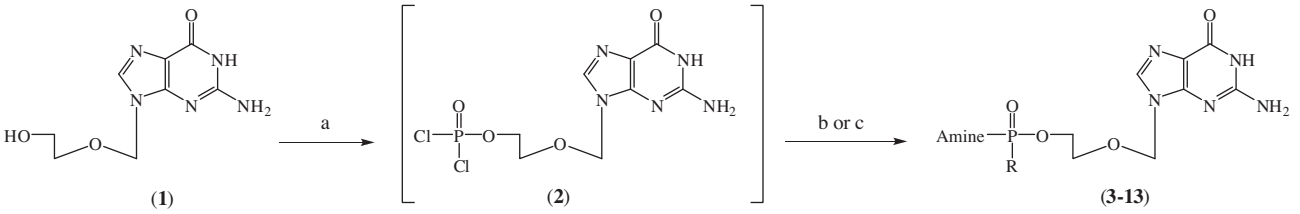
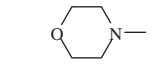
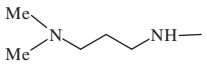
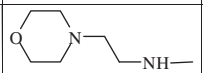
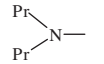
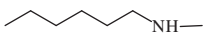
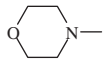
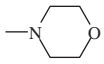
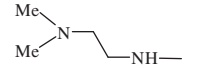


Figure 1. The proposed mechanism of action of the phosphoramidates of acyclovir.



Compound	Amine	R	Compound	Amine	R	Compound	Amine	R
(**3**)			(**7**)		$-O^-NH_4^+$	(**11**)		$-O^-NH_4^+$
(**4**)		$-O^-NH_4^+$	(**8**)		$-O^-NH_4^+$	(**12**)		
(**5**)	H_2N-	$-NH_2$	(**9**)		$-O^-NH_4^+$	(**13**)		$-O^-NH_4^+$
(**6**)			(**10**)		$-O^-NH_4^+$			

Scheme 1. Reagents and conditions: (a) $POCl_3$, triethylphosphate, −18 °C, 4 h; (b) amine/*N*,*N*-diisopropylethylamine/1% aq dioxane, +4 °C, 2 h; (c) amine/dioxane,+4 °C, 4 h.

Table 1
Chemical hydrolysis of obtained phosphoramidates.

Compound	Hydrolysis time of the ½ amount of the compound (product of hydrolysis)		
	pH 2	pH 7.4	pH 12
(**3**)	10–15 min, (**14**) [a]	18–24 h, (**1**)	3–4 h, (**1**)
(**4**)	<5 min, (**14**)	>120 h, (**1**)	96–120 h, (**1**)+(**14**) [b]
(**5**)	5–10 min, (**14**) [a]	4–6 h, (**1**)	<1 h, (**1**)
(**6**)	30–40 min, (**14**)[a]	>120 h, (**1**)	18–24 h, (**1**)
(**7**)	<5 min, (**14**)	>120 h, (**1**)	>120 h, (**1**)+(**14**)
(**8**)	<5 min, (**14**)	>120 h, (**1**)	48–72 h, (**1**)+(**14**)[b]
(**9**)	<5 min, (**14**)	>120 h, (**1**)	96–120 h, (**1**)+(**14**) [b]
(**10**)	<5 min, (**14**)	>120 h, (**1**)	48–72 h, (**1**)+(**14**) [b]
(**11**)	<5 min, (**14**)	>120 h, (**1**)	48–72 h, (**1**)+(**14**)[b]
(**12**)	5 min, (**14**) [a]	5–6 h, (**1**)	1–2 h, (**1**)
(**13**)	<5 min, (**14**)	>120 h, (**1**)	48–72 h, (**1**)+(**14**)[b]

(**14**) is ACV monophosphate.
[a] During hydrolysis, the formation of the corresponding monoamidates was not observed.[19]
[b] The main product is ACV (**1**) (greater than 80%).

controlled in vitro safe-screening system we used[22] for the anti-HIV compounds did not contain the herpes viruses. This system utilised the pseudo-HIV-1 particles containing HIV reverse transcriptase and recombinant genomic RNA with the marker gene. As in the case of natural HIV, the viral proteins direct the synthesis of a provirus DNA copy of the recombinant RNA genome and its integration into the host cell's genome.

It was shown that the compounds displayed moderate antiviral activity. Most of the analysed compounds in 20 μM concentrations (Fig. 2) suppressed virus growth by 20–30%. It is noteworthy that phosphoromonomorpholidate (**7**) at the same concentration inhibited virus growth by 50% (Fig. 2A). Increasing the concentrations of the compounds up to 100 μM did not significantly affect virus growth suppression (Fig. 2B). It is also important to note that this inhibition demonstrated dose-dependent character. Therefore, by taking into account the inactivity of ACV in this model, we can conclude that our compounds are latent forms of ACV monophosphate (**14**).

Previously, the antiviral activity of AZT against pseudo-HIV-1 particles was studied, and it was shown that AZT inhibited the infection of eukaryotic cells by pseudo viral particles, though at higher concentrations than by infectious HIV-1. The EC_{50} value was 0.09–0.11 μM,[21] compared to the reference-accepted EC_{50} of 0.003–0.004 μM.[20] The reason for this discrepancy may be caused by the different intracellular contents of the nucleosides and nucleotide kinases[22] and by differences in the rates of specific transporters' expression.[23]

We did not evaluate the phosphorodiamidates because we assumed that these compounds could not be latent forms of ACV monophosphate, (**14**) according to our data on chemical stability (Table 1).

2.3.2. HSV-assay

A considerable drawback of ACV[2] is the emergence of acyclovir-resistant HSV strains because of its widespread use in antiviral therapy. In 95% of the cases, resistance of these strains to ACV is caused by the occurrence of mutations in the viral thymidine kinase (TK) gene. In other rare cases, it is associated with mutations in the viral DNA polymerase gene.[24] Therefore, strain resistance is usually explained by an increase in substrate specificity of viral thymidine kinase that ceases to phosphorylate ACV to ACV monophosphate. One approach to overcome this limitation is to synthesise derivatives of ACV that are in a latent form of ACV monophosphate.

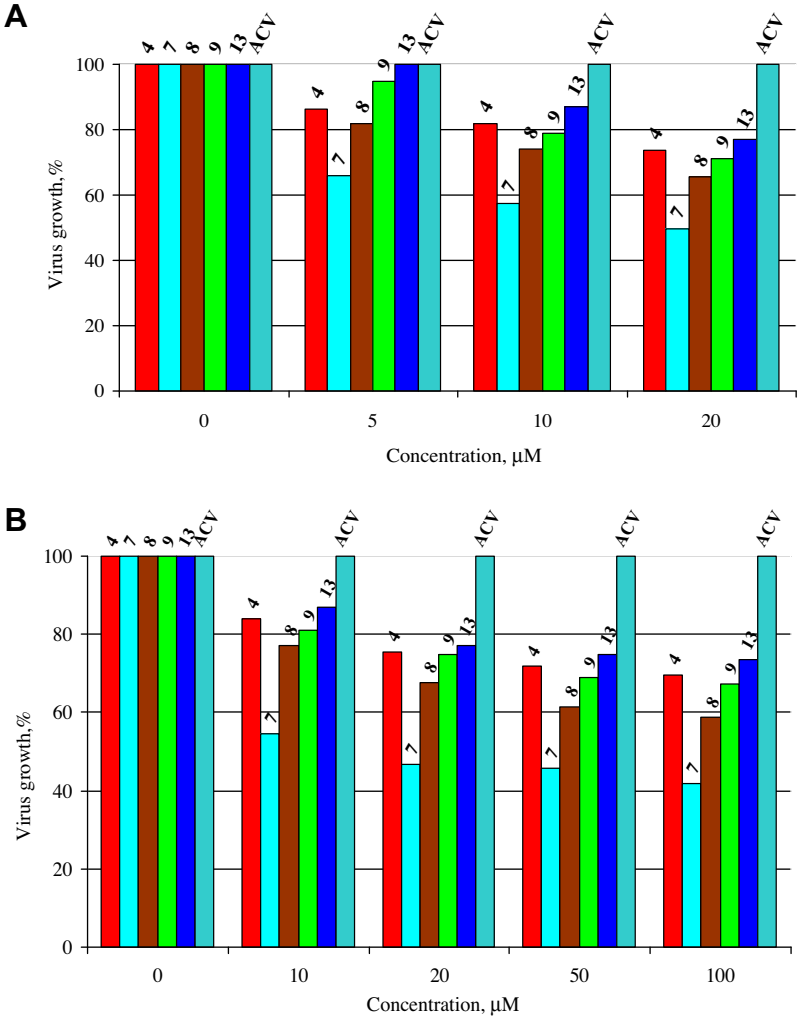


Figure 2. Anti-HIV-1 activity of acyclovir phosphoromonoamidates.

We evaluated the antiherpetic activity of compounds (**7**) and (**9**). It was shown that these compounds in non-toxic concentrations suppress virus-induced cytopathic effects in cell cultures infected with HSV-1/L_2, that is, their antiviral effect is pronounced as demonstrated by the SI values. It is important to note that the compounds retained their antiviral activity even in the HSV-1/L_2/R model strain, which is strongly resistant to ACV. The corresponding data are given in Table 2. The data presented show that the antiviral activity of compounds (**7**) and (**9**) is retained for HSV-1/L_2/R at concentrations 2.5 times higher than those needed for HSV-1/L_2. For both viral models, the EC_{50} values of compounds (**7**) and (**9**) were comparable with the ACV H-phosphonate EC_{50} value.[25] Like ACV H-phosphonate, derivatives (**7**) and (**9**) showed less activity than ACV against the parent strain of HSV-1 but appeared to be significantly more active in the TK^--strain of HSV-1/L_2/R.

In addition, we evaluated the antiherpetic activity of phosphorodimorpholidate (**6**). We predicted that it would at least show anti-HSV activity against the wild strain of HSV due to hydrolysis to ACV. However, phosphorodimorpholidate (**6**) appeared to be inactive in this viral model at the concentration range studied (Table 2).

All the investigated compounds showed low toxicity in Vero E6 cell cultures and Jurkat cells. Their cytotoxic effects were not observed even at the maximum concentration studied (2000 μM).

Table 2
Cytotoxic properties and antiherpetic activity of a series of ACV derivatives in Vero E6 cell culture.

Compound	HSV-1/L_2		HSV-1/L_2/R		CC_{50}, μM
	EC_{50}, μM	SI	EC_{50}, μM	SI	
(**6**)	>500	NA	>500	NA	>2000
(**7**)	9.7	>206	25	>80	>2000
(**9**)	15	>133	37	>54	>2000
ACV	1.7	>1176	530	>3.8	>2000
ACV H-phosphonate[a]	20	>100	41	>49	>2000

SI–selectivity index, CC_{50}/EC_{50}; NA–Not Applicable.
[a] Data from.[25]

3. Conclusions

In summary, we synthesised several phosphoramidates of ACV (**3**–**13**). We studied the chemical stability of all the compounds obtained. It was shown that all of the ACV phosphoramidates (**3**–**13**) are rapidly hydrolysed to ACV monophosphate (**14**) at pH 2. It was also shown that ACV phosphorodiamidates (**3**, **5**, **6** and **12**) are unstable at neutral and basic pH conditions. The ACV phosphoromonoamidates (**4**, **7**–**11** and **13**) are stable at neutral pH and are slowly hydrolysed at pH 12, leading to a mixture of products (**1**) and (**14**).

The anti-HIV and anti-HSV activities of the obtained compounds were studied. All of the studied compounds (except phosphorodiamidate (**6**)) showed moderate HIV-1 and HSV-1 activity against

normal and resistant strains of HSV. The best results were obtained with phosphoromonomorpholidate (**7**). All of the studied compounds were non-toxic.

Therefore, ACV phosphoromonoamidates derivatives could be latent forms of ACV monophosphate (**14**) and represent new, promising antiviral drugs with a broad spectrum of action.

4. Experimental section

4.1. General methods

The morpholine, 2-morpholinethylamine, 3-dimethylamino-1-propylamine, hexylamine, *N*,*N*-diisopropylethylamine and phosphorus oxychloride were purchased from Fluka (Switzerland). The *N*,*N*-dimethylethylenediamine and dipropylamine were obtained from Aldrich (Germany). The pyrrolidine and 30% aqueous ammonia were supplied from Acros (Belgium). The solvents were purified by standard procedures.

ACV (GlaxoWellcom, Great Britain) was used as the reference drug.

The reaction process was checked using TLC on precoated Kieselel $60F_{254}$ plates (Merck, Germany) in the following systems, (A) 4:1 chloroform:methanol, (B) 60:35:5 chloroform:methanol:25% aqueous ammonia and (C) 4:1 dioxane:25% aqueous ammonia.

Column chromatography was performed on ion-exchange DEAE-Toyopearl 650 M resin (Tosoh Corporation, Japan) and on reversed-phase silica gel, LiChroprep RP-18 (40–63 μm) (Merck, Germany).

UV spectra were registered on a Shimadzu UV-2401PC spectrophotometer (Shimadzu Corporation, Japan) in water, in the range of 200–300 nm.

NMR spectra (δ, ppm; *J*, Hz) were registered on an AMX III-400 spectrometer (Bruker, USA) with a working frequency of 400 MHz for ^{1}H NMR (sodium 3-trimethylsilyl-1-propanesulfonate was the internal standard for D_2O), 162 MHz for ^{31}P NMR (85% H_3PO_4 was the external standard) and 101 MHz for ^{13}C NMR at 27 °C. All ^{31}P-NMR and ^{13}C-NMR were proton-decoupled.

Mass spectra were registered on a MALDI Ultraflex spectrometer (Bruker Daltonics, Germany).

4.2. Synthesis

4.2.1. Standard procedure A: Synthesis of phosphoromonoamidates

Phosphorus oxychloride (1.50 mol/equiv) was added to a suspension of ACV (1.00 mol/equiv) in triethylphosphate (1 ml) pre-cooled to −18 °C. The mixture was kept at −18 °C for 4 h. Then, it was treated with the amidating agent (amine (3.00 mol/equiv) and *N*,*N*-diisopropylethylamine (6.00 mol/equiv) in 1% aqueous dioxane (1 ml)). The reaction was maintained at +4 °C for 2 h, neutralised by a saturated $NaHCO_3$ solution (10 ml) pre-cooled to +4 °C, and extracted with ether (10 ml). The aqueous extract was applied onto a DEAE-Toyopearl column (20 × 175 mm) and eluted with a linear gradient of NH_4HCO_3 (0 → 0.25 M, 600 ml). The target fraction was concentrated by evaporation under vacuum; the residue was diluted with water, re-evaporated (2 × 30 ml) and additionally chromatographed on a RP-18 column (15 × 140 mm) and eluted with a linear gradient of acetonitrile (0 → 30%, 400 ml) in 0.05 M NH_4HCO_3. The fraction containing the target product was freeze-dried from water.

4.2.2. Standard procedure B: Synthesis of phosphorodiamidates

Phosphorus oxychloride (1.50 mol/equiv) was added to a suspension of ACV (1.00 mol/equiv) in triethylphosphate (1 ml) pre-cooled to −18 °C. The mixture was kept at −18 °C for 4 h then treated with the amine (10.00 mol/equiv) in dioxane (1 ml). It was then kept at +4 °C for an additional 2 h and partitioned between ether (10 ml) and water (20 ml). The aqueous extract was chromatographed on a DEAE-Toyopearl column (20 × 175 mm), eluted with water and additionally chromatographed on a RP-18 column (15 × 140 mm) and eluted with a linear gradient of acetonitrile (0 → 30%, 400 ml) in 0.05 M NH_4HCO_3. The fraction containing the target product was freeze-dried from aqueous acetonitrile.

4.2.3. 9-(2-Hydroxyethoxymethyl)guanine, phosphorodipyrrolidate (3)

Product (**3**) was prepared according to standard procedure B from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and pyrrolidine (156 mg, 2.2 mmol). According to the NMR data, product (**3**) contained ACV admixture (**1**) in the quantity of 25%. UV (H_2O): λ_{max} 249 nm (9800). ^{1}H-NMR (D_2O, 400 MHz): δ 7.76 (s, 1H, H-8), 5.48 (s, 2H, H-1′), 3.99–4.04 (m, 2H, H-3′), 3.75–3.78 (m, 2H, H-2′), 2.86–3.12 (m, 8H, CH_2NCH_2), 1.90–2.05 (m, 8H, $CH_2CH_2CH_2CH_2$). ^{31}P-NMR (D_2O, 162 MHz): δ 19.95.

4.2.4. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromonopyrrolidate (4)

Product (**4**) was prepared according to standard procedure A from ACV (60.0 mg, 0.266 mmol), phosphorus oxychloride (61.3 mg, 0.4 mmol) and pyrrolidine (57 mg, 0.8 mmol). Purification and freeze-drying yielded 48 mg (48%) of (**4**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (9700). ^{1}H-NMR (D_2O, 400 MHz): δ 7.91 (s, 1H, H-8), 5.49 (s, 2H, H-1′), 3.84–3.89 (m, 2H, H-3′), 3.70–3.73 (m, 2H, H-2′), 2.84–2.89 (m, 4H, CH_2NCH_2), 1.62–1.66 (m, 4H, $CH_2CH_2CH_2CH_2$). ^{31}P-NMR (D_2O, 162 MHz): δ 9.06. ^{13}C-NMR (D_2O, 101 MHz): δ 160.93 (C-4), 156.11 (C-2), 154.13 (C-6), 142.14 (C-8), 119.03 (C-5), 75.04 (C-1′), 69.27 (d, *J* = 7.67 Hz, C-3′), 66.18 (d, *J* = 5.01 Hz, C-2′), 56.74 (d, *J* = 3.61 Hz, CH_2NCH_2), 24.41 (d, *J* = 2.89 Hz, $CH_2CH_2CH_2CH_2$). HRMS (ESI^-) calcd for $C_{12}H_{15}N_5O_5P$ $(M\text{-}NH_4)^-$ 357,1077 $(M\text{-}NH_4)^-$; found 357,1044.

4.2.5. 9-(2-Hydroxyethoxymethyl)guanine, phosphorodiamidate (5)

Phosphorus oxychloride (51.0 mg, 0.33 mmol) was added to a suspension of ACV (50.0 mg, 0.22 mmol) in triethylphosphate (1 ml) pre-cooled to −18 °C. The mixture was maintained at −18 °C for 4 h with the following treatment with 30% aqueous ammonia (150 μl, 2.2 mmol), and then it was kept at +4 °C for another 2 h and partitioned between ether (10 ml) and water (20 ml). The aqueous extract was chromatographed on a DEAE-Toyopearl column (20 × 175 mm) and eluted with a linear gradient of a NH_4HCO_3 water solution (0 → 0.25 M, 600 ml). The fraction collected in the concentration range of 0.03–0.06 M NH_4HCO_3 and was concentrated by evaporation under vacuum. The residue was diluted with water, re-evaporated (2 × 30 ml) and additionally chromatographed on a RP-18 column (15 × 140 mm) and eluted with a linear gradient of acetonitrile (0 → 40%, 400 ml) in 0.05 M NH_4HCO_3. According to the NMR data, product (**5**) contained 50% of the ACV admixture (**1**). UV (H_2O): λ_{max} 249 nm (9900). ^{1}H-NMR (D_2O, 400 MHz): δ 7.88 (s, 1H, H-8), 5.48 (s, 2H, H-1′), 3.99–4.03 (m, 2H, H-3′), 3.75–3.77 (m, 2H, H-2′). ^{31}P-NMR (D_2O, 162 MHz): δ 21.55.

4.2.6. 9-(2-Hydroxyethoxymethyl)guanine, phosphorodimorpholidate (6)

Product (**6**) was prepared according to standard procedure B from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and morpholine (192 mg, 2.2 mmol). Purification and freeze-drying gave 49 mg (50%) of (**6**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (10000). ^{1}H-NMR (D_2O,

400 MHz): δ 7.78 (s, 1H, H-8), 5.37 (s, 2H, H-1′), 3.86–3.88 (m, 2H, H-3′), 3.71–3.73 (m, 2H, H-2′), 3.43–3.45 (m, 8H, CH_2OCH_2), 2.77–2.79 (m, 8H, CH_2NCH_2). ^{31}P-NMR (D_2O, 162 MHz): δ 15.92. ^{13}C-NMR (D_2O, 101 MHz): δ 159.67 (C-4), 154.64 (C-2), 152.08 (C-6), 138.66 (C-8), 117.44 (C-5), 73.04 (C-1′), 70.60 (d, *J* = 8.04 Hz, C-3′), 66.44 (d, *J* = 4.92 Hz, C-2′), 64.39 (d, *J* = 4.01 Hz, CH_2OCH_2), 45.21 (d, *J* = 3.86 Hz, CH_2NCH_2). MS (ESI^+) *m*/*z* 443,1711 (M).

4.2.7. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromonomorpholidate (7)

Product (**7**) was prepared according to standard procedure A from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and morpholine (57.5 mg, 0.66 mmol). Purification and freeze-drying gave 40 mg (45%) of (**7**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (10200). ^{1}H-NMR (D_2O, 400 MHz): δ 7.90 (s, 1H, H-8), 5.47 (s, 2H, H-1′), 3.83–3.87 (m, 2H, H-3′), 3.70–3.73 (m, 2H, H-2′), 3.52 (t, 4H, *J* = 4.40 Hz, CH_2OCH_2), 2.84 (dt, 4H, *J* = 4.40 Hz, *J* = 4.80 Hz, CH_2NCH_2). ^{31}P-NMR (D_2O, 162 MHz): δ 8.18. ^{13}C-NMR (D_2O, 101 MHz): δ 160.21 (C-4), 155.07 (C-2), 152.94 (C-6), 140.28 (C-8), 118.01 (C-5), 72.81 (C-1′), 70.05 (d, *J* = 8.21 Hz, C-3′), 65.84 (d, *J* = 5.08 Hz, C-2′), 63.51 (d, *J* = 4.11 Hz, CH_2OCH_2), 46.51 (d, *J* = 3.79 Hz, CH_2NCH_2). MS(ESI^+) *m*/*z* 374,1119 ($M+H^+$), 396,0944 ($M+Na^+$), 412,0685 ($M+K^+$); HRMS (ESI^-) calcd for $C_{12}H_{18}N_6O_6P$ $(M\text{-}NH_4)^-$:373,1026 $(M\text{-}NH_4)^-$; found 373,0085.

4.2.8. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromono-N-morpholinoethylamidate (8)

Product (**8**) was prepared according to standard procedure A from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and 2-morpholinoethylamine (86.0 mg, 0.66 mmol). Purification and freeze-drying gave 11.5 mg (12%) of (**8**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (9800). ^{1}H-NMR (D_2O, 400 MHz): δ 7.79 (s, 1H, H-8), 5.34 (s, 2H, H-1′), 3.86–3.89 (m, 2H, H-3′), 3.81 (br s, 4H, CH_2OCH_2), 3.73–3.75 (m, 2H, H-2′), 2.92–2.98 (m, 6H, $CH_2CH_2NH + CH_2NCH_2$), 2.81–2.87 (m, 2H, CH_2CH_2NH). ^{31}P-NMR (D_2O, 162 MHz): δ 9.26. ^{13}C-NMR (D_2O, 101 MHz): δ 159.08 (C-4), 154.39 (C-2), 153.38 (C-6), 140.99 (C-8), 118.14 (C-5), 74.81 (C-1′), 69.34 (d, *J* = 5.56 Hz, C-3′), 66.71 (d, *J* = 7.64 Hz, C-2′), 66.37 (2s, CH_2OCH_2), 53.61 (d, *J* = 2.58 Hz, CH_2CH_2NH), 53.07 (2s, CH_2NCH_2), 43.11 (d, *J* = 4.04 Hz, CH_2CH_2NH). HRMS (ESI^-) calcd for $C_{14}H_{20}N_6O_6P$ $(M\text{-}NH_4)^-$ 416,1448 $(M\text{-}NH_4)^-$; found 416,1419.

4.2.9. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromono-dipropylamidate (9)

Product (**9**) was prepared according to standard procedure A from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and dipropylamine (67 mg, 0.66 mmol). Purification and freeze-drying yielded 36.5 mg (41%) of (**9**) as a white lyophilisate. UV (H_2O): λ_{max} 249 nm (10000). ^{1}H-NMR (D_2O, 400 MHz): δ 7.91 (s, 1H, H-8), 5.48 (s, 2H, H-1′), 3.77–3.81 (m, 2H, H-3′), 3.71–3.73 (m, 2H, H-2′), 2.63–2.70 (m, 4H, $CH_2CH_2CH_3$), 1.26–1.36 (m, 4H, $CH_2CH_2CH_3$), 0.71 (t, 6H, *J* = 7.48 Hz, $CH_2CH_2CH_3$). ^{31}P-NMR (D_2O, 162 MHz): δ 10.76. ^{13}C-NMR (D_2O, 101 MHz): δ 161.67 (C-4), 156.80 (C-2), 154.47 (C-6), 142.78 (C-8), 118.92 (C-5), 75.70 (C-1′), 71.88 (d, *J* = 7.68 Hz, C-3′), 65.89 (d, *J* = 5.45 Hz, C-2′), 50.73 (d, *J* = 3.43 Hz, $CH_2CH_2CH_3$), 24.24 (d, *J* = 2.22 Hz, $CH_2CH_2CH_3$), 13.45 ($CH_2CH_2CH_3$). MS (ESI+) *m*/*z* 388,1624 $(M\text{-}NH_3)$, 410,1443 $(M\text{-}NH_4$+Na), 426,1183 $(M\text{-}NH_4$+K); HRMS (ESI^-) calcd for $C_{14}H_{24}N_6O_5P$ $(M\text{-}NH_4)^-$: 387,1546 $(M\text{-}NH_4)^-$; found 387,1508.

4.2.10. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromono-N,N-dimethylaminoethylamidate (10)

Product (**10**) was prepared according to standard procedure A from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and dimethylaminoethylamine (58 mg, 0.66 mg). Purification and freeze-drying gave 8.8 mg (10%) of (**10**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (9700). ^{1}H-NMR (D_2O, 400 MHz): δ 7.79 (s, 1H, H-8), 5.34 (s, 2H, H-1′), 3.92–3.96 (m, 2H, H-3′), 3.68–3.72 (m, 2H, H-2′), 3.22 (t, 2H, *J* = 6.08 Hz, $(CH_3)_2NCH_2CH_2NH$), 2.80 (t, 2H, *J* = 6.08 Hz, $(CH_3)_2NCH_2CH_2NH$), 2.68 (s, 6H, CH_3N). ^{31}P-NMR (D_2O, 162 MHz): 10.86. ^{13}C-NMR (D_2O, 101 MHz): δ 160.94 (C-4), 157.11 (C-2), 153.18 (C-6), 143.10 (C-8), 119.01 (C-5), 74.91 (C-1′), 69.35 (d, *J* = 5.27 Hz, C-3′), 65.42 (d, *J* = 6.99 Hz, C-2′), 58.56 (d, *J* = 4.21 Hz, $(CH_3)_2$ NCH_2CH_2), 45.72 ($(CH_3)N$), 42.25 (d, *J* = 2.51 Hz, $(CH_3)_2NCH_2CH_2$). HRMS (ESI^-) calcd for $C_{12}H_{18}N_6O_5P$ $(M\text{-}NH_4)^-$ 374,1342 $(M\text{-}NH_4)^-$; found 374,1322.

4.2.11. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromono-3-dimethylamino-1-propylamidate (11)

Product (**11**) was prepared according to standard procedure A from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and 3-dimethylamino-1-propylamine (67.5 mg, 0.66 mmol). Purification and freeze-drying yielded 9.8 mg (11%) of (**11**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (9600). ^{1}H-NMR (D_2O, 400 MHz): δ 7.79 (s, 1H, H-8), 5.34 (s, 2H, H-1′), 3.92–3.96 (m, 2H, H-3′), 3.68–3.72 (m, 2H, H-2′), 3.65-3.69 (m, 2H, $CH_2CH_2CH_2NH$), 3.00 (t, 2H, *J* = 6.78 Hz, $(CH_3)_2NCH_2CH_2CH_2$), 2.73 (s, 6H, $(CH_3)_2N$), 1.71–1.79 (m, 2H, $CH_2CH_2CH_2NH$). ^{31}P-NMR (D_2O, 162 MHz): δ 10.55. ^{13}C-NMR (D_2O, 101 MHz): δ 162.01 (C-4), 156.83 (C-2), 154.74 (C-6), 142.83 (C-8), 120.01 (C-5), 75.02 (C-1′), 71.49 (d, *J* = 4.76 Hz, C-3′), 65.89 (d, *J* = 8.03 Hz, C-2′), 54.61 (d, *J* = 4.12 Hz, $(CH_3)_2NCH_2CH_2CH_2$), 45.17 ($(CH_3)_2N$), 43.24 (d, *J* = 2.57 Hz, $(CH_3)_2NCH_2CH_2CH_2$), 26.97 (d, *J* = 2.29 Hz, $(CH_3)_2NCH_2$ CH_2CH_2). HRMS (ESI-) calcd for $C_{13}H_{20}N_6O_5P$ $(M\text{-}NH_4)^-$ 388,1499 $(M\text{-}NH_4)^-$; found 388,1465.

4.2.12. 9-(2-Hydroxyethoxymethyl)guanine, phosphorodihexylamidate (12)

Product (**12**) was prepared according to standard procedure B from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and hexylamine (223 mg, 2.2 mmol). According to NMR data product (**12**) contained ACV admixture (**1**) in the quantity of 30%. UV (H_2O): λ_{max} 250 nm (9600). ^{1}H-NMR (D_2O, 400 MHz): δ 7.93 (s, 1H, H-8), 5.56 (s, 2H, H-1′), 3.87–3.91 (m, 2H, H-3′), 3.72–3.76 (m, 2H, H-2′), 2.46–2.53 (m, 4H, CH_2NH), 1.07–1.24 (m, 16H, $CH_3(CH_2)_4CH_2NH$), 0.78 (t, 3H, *J* = 7.32, CH_3). ^{31}P-NMR (D_2O, 162 MHz): δ 16.05.

4.2.13. 9-(2-Hydroxyethoxymethyl)guanine, phosphoromonohexylamidate (13)

Product (**13**) was prepared according to standard procedure A from ACV (50.0 mg, 0.22 mmol), phosphorus oxychloride (51.0 mg, 0.33 mmol) and hexylamine (67.0 mg, 0.66 mmol). Purification and freeze-drying yielded 15 mg (17%) of (**13**) as a white lyophilisate. UV (H_2O): λ_{max} 250 nm (9700). ^{1}H-NMR (D_2O, 400 MHz): δ 7.92 (s, 1H, H-8), 5.49 (s, 2H, H-1′), 3.84–3.89 (m, 2H, H-3′), 3.71–3.74 (m, 2H, H-2′), 2.56–2.62 (m, 2H, CH_2NH), 1.22–1.29 (m, 4H, $CH_3(CH_2)_2CH_2CH_2CH_2NH$), 1.07–1.13 (m, 4H, $CH_3CH_2CH_2(CH_2)_3NH$), 0.77 (t, 3H, *J* = 7.32 Hz, CH_3). ^{31}P-NMR (D_2O, 162 MHz): δ 8.56. ^{13}C-NMR (D_2O, 101 MHz): δ 164.08 (C-4), 156.37 (C-2), 155.01 (C-6), 141.14 (C-8), 119.92 (C-5), 74.78 (C-1′), 69.88 (d, *J* = 5.11 Hz, C-3′), 65.03 (d, *J* = 8.04 Hz, C-2′), 45.17 (d, *J* = 3.33 Hz, $CH_2(CH_2)_4CH_3$), 31.75 ($(CH_2)_3CH_2CH_2CH_3$), 28.82 (d, *J* = 2.2 Hz, $CH_2CH_2(CH_2)_3CH_3$), 25.79 (d, *J* = 2.5 Hz, $(CH_2)_2CH_2(CH_2)_2CH_3$), 22.37 ($(CH_2)_4CH_2CH_3$), 14.14 (CH_3). HRMS (ESI^-) calcd for $C_{14}H_{24}N_6O_5P$ $(M\text{-}NH_4)^-$: 387,1546 $(M\text{-}NH_4)^-$; found 387,1578.

4.3. Chemical stability

Hydrolysis of the compounds (**3-13**) was performed in acidic (pH 2, KCl and HCl, 25 ml of 0.2 M KCl and 6.5 ml of 0.2 M HCl

diluted to 100 ml), neutral (pH 7.4, phosphate buffer, 25 ml of 0.2 M KH_2PO_4 and 19.5 ml of 0.2 M KOH diluted to 100 ml) and basic (pH 12, KCl and KOH, 25 ml of 0.2 KCl and 6 ml of 0.2 KOH diluted to 100 ml) conditions at 25 °C and with 0.02 mM of the studied compounds. The samples were taken (10 μl) at regular intervals and were analysed by TLC in a system of dioxane:ammonia (8:2). Substance containing zones were cut out and washed with aqueous dioxane. The amount of product was estimated by their UV-absorption at 250 nm.

The experiments were performed three times for each phosphoramidate. The averaged data are presented in Table 1; the experimental error did not exceed 20%.

4.4. Antiviral investigation

4.4.1. HIV assay

HEK293 (human embryonic kidney) cells were seeded in Petri dishes with a diameter of 100 mm in the amount of 3.5–4.0 × 10^6 cells per dish, 12–14 h prior to the transfection onset. They were used as packaging cells in which the assembly of recombinant lentiviral pseudo-HIV-1 particles occurred.

DNA of the lentiviral vector containing the marker gene of the green fluorescent protein and the plasmids directing the synthesis of the proteins that are required for formation of the pseudo-HIV-1 particles were introduced into the HEK293 cells via calcium phosphate transfection. The infectious pseudo-HIV-1 particles were collected 24 h following transfection in 12 h intervals.[26]

The virus was titrated onto HEK293 cells seeded in 24-well plates 24 h prior to infection. The level of cell fluorescence was measured 48 h post infection using an Epics 4XL Beckman Coulter flow cytofluorimeter (USA). The titre of the virus (T) was determined using the formula,

$$T = \frac{N * P}{V}$$

where N - the number of the cells in each well, P - the percentage of the induced cells in population, V - the volume of the virus-containing supernatant.

The samples with virus titre from 5 × 10^5 to 5 × 10^6 were used in this study.

To determine anti-HIV-1 activity, the aqueous solutions of the compounds were added to the cells, and 2–8 h later, these cells were infected with pseudo-HIV-1 particles. The relative infection levels were measured 48 h post infection by flow cytometry using an Epics 4XL Beckman Coulter Cytometer (USA).

The cytotoxicity of the compounds was determined by measuring the uninfected Jurkat cells stained by trypan blue (Invitrogen Corporation, USA). Briefly, the cells were grown in the presence of the compounds for 48 h, resuspended in fresh medium containing 0.4% trypan blue and incubated for an additional 5 min. The number of viable (unstained) and non-viable (stained) cells was estimated using a Neubauer chamber. The per cent of viable cells in the population was determined as a ratio of the unstained cells to the total number of cells.

4.4.2. HSV-1 assay

A Vero E6 monolayer culture was grown in Eagle medium supplemented with 7% foetal calf serum, L-glutamine, penicillin and streptomycin.

The HSV-1/L2 strain was obtained from the State collection of viruses from the Ivanovsky Institute of virology of the Ministry of Health and Social Development of the Russian Federation. The viruses were propagated at 37 °C in Vero E6 cell cultures in a growth medium containing a 1:1 mixture of the Eagle and 199 media.

The HSV-1/L_2/R mutant ACV-resistant strain was obtained by serial passages in Vero E6 cells in the presence of increasing concentrations of ACV, according to previously reported methods.[27,28] In the first passage, the multiplicity of infection was 1 PFU/cell; in further passages, the multiplicity decreased to 0.1 PFU/cell. The resulting viral strain was used to obtain the viral clone resistant to ACV and was characterised as TK^-. Before use, each clone was plaque-purified three times on Vero E6 cells according to a standard protocol.[29]

The antiviral activity was measured by the ability of each compound to inhibit the development of the virus-induced cytopathogenic effect[30] using 96-well plastic plates (Linbro, Flow Lab, UK), as previously described.[25,28] The Vero E6 monolayer cultures were infected with the virus (0.1 plague-forming unit per cell) and grown at 37 °C in the presence of 5% CO_2. The antiviral activity was determined 48 h post infection when 95–100% of the cytopathogenic effect was observed in the untreated control cells, and the EC_{50} values were determined (the concentration in which the virus-induced cytopathogenic effect was reduced by 50% compared with the control infected culture).

The CC_{50} values (the concentration that causes death to 50% of the uninfected cells after 72 h of incubation) were assessed by staining the Vero E6 cells with trypan blue in 96-well plastic plates.[31]

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