Carbocyclic Dinucleoside Polyphosphonates: Interaction with HIV Reverse Transcriptase and Antiviral Activity†

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Carbocyclic α,γ-bis(nucleoside)-5,5′-triphosphonates and α,δ-bis(nucleoside)-5,5′-tetraphosphonates (Ap4A and Gp4G) analogues were shown to be a new type of terminating substrate of HIV reverse transcriptase. They effectively inhibited the DNA synthesis catalyzed by this enzyme in model cell-free systems, but their antiviral activity both in Rat1 fibroblast cell culture bearing MLV reverse transcriptase and in HIV-infected MT-4 cells was low. When a liposome delivery system was used, the antiviral efficacy of the compounds under study was increased.

Introduction

DNA polymerases represent a large group of enzymes catalyzing DNA biosynthesis. These enzymes occur in cells of all types, including mammalian and bacterial cells and viruses. The importance of the study of these enzymes was dramatically increased during the past decades, when the outburst of various viral diseases required new effective drugs.

Constructive blocks for the DNA extension are 2′-deoxynucleoside 5′-monophosphates, which are generated from 2′-deoxynucleoside 5′-triphosphates (dNTP) in the process of the DNA polymerase catalyzed reaction of DNA synthesis. Numerous studies showed that proper modifications in the dNTP structure allow discrimination of DNA polymerases of different origin, particularly, human and viral ones. For example, modifications in the sugar moiety of thymidine, 2′-deoxycytidine, and 2′-deoxyinosine resulted in the design of drugs widely used currently in anti-AIDS therapy. These compounds inhibit HIV DNA polymerase (reverse transcriptase) but only little affect human enzymes.[1−4] However, the efficacy of these drugs is low enough because of the necessity of their intracellular triphosphorylation.[5,6] The use of phosphorylated forms of antiviral nucleosides could shorten or even exclude these transformations of the antiviral nucleoside-based agents.

It was earlier demonstrated in our laboratory that the functionalization of the γ-phosphate in dNTPs (for example, esterification or its replacement with phosphonate groups) did not prevent the recognition of these compounds by HIV reverse transcriptase and increased the selectivity of incorporation.[7] Moreover, α,γ-bis(nucleoside)-5,5′-triphosphonates, which can be regarded as γ-modified nucleoside 5′-triphosphates, also displayed good substrate properties toward some human and bacterial DNA polymerases and HIV reverse transcriptase.[8] We also showed that some triphosphonates of carbocyclic nucleoside analogues were selective inhibitors of DNA synthesis catalyzed by HIV reverse transcriptase but were only moderately active in antiviral tests.[9]

Chart 1

Ia B=Gua X=O; Ib B=Gua X=CF_2; Ic B=Gua X=CBr_2

IIa B=Ade X=O; IIb B=Ade X=CF_2; IIc B=Ade X=CBr_2

III B= Gua

IV B= Ade

Va B = Gua X = O; Vb B = Gua X = CF_2; Vc B = Gua X = CBr_2

VIa B = Ade X = O; VIb B = Ade X = CF_2; VIc B = Ade X = CBr_2

We present in this work the synthesis of dinucleotide polyphosphate analogues bearing a cyclopentenyl residue as a glycone and phosphonate residues in place of phosphate ones (**I**−**IV**) and the evaluation of their substrate potency toward HIV reverse transcriptase in comparison with that of the corresponding nucleoside triphosphate mimetics (**V**−**VI**) (Chart 1). We also studied the inhibition of pSG1 virus replication in rat Rat1 fibroblasts by compounds **I**−**IV** and evaluated their antiviral properties in MT-4 cells infected with HIV using free or liposome-encapsulated forms of the compounds under study.

† This paper is dedicated to the late Prof. A. A. Krayevsky.

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⊥ RC Virology and Biotechnology "Vector".

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Scheme 1

VII B=Gua
VIII B=Ade

Va-c B=Gua
VIa-c B=Ade

III B=Gua
IV B=Ade

Ia-c B=Gua
IIa-c B=Ade

a X=O; **b** X=CF_2; **c** X=CBr_2

Results

Chemistry. We describe herein the synthesis of new carbocyclic α,γ-bis(nucleoside)-5,5′-triphosphonates and α,δ-bis(nucleoside)-5,5′-tetraphosphonates analogues. Previously, for the preparation of diadenosine tetraphosphate analogues bearing β,γ-methylene or β,γ-dihalomethylene bridges, various procedures were used.[10−14] We performed the achiral synthesis of target dinucleoside polyphosphonates **I** and **II** in either a one-step or a two-step procedure using the corresponding monophosphonates **VII** and **VIII** as key intermediates and 1,1′-carbonyldiimidazole (CDI) as an activating agent (Scheme 1). The formation of the side dimer (the corresponding dinucleoside α,α′-diphosphonate) was minimal when the molar excess of CDI was more than 8. Higher yields of the target dinucleoside tetraphosphonates (**I** and **II**) were obtained when the corresponding monophosphonates (**VII, VIII**), rather than diphosphonic acids, were activated. In the one-step procedure, we varied the molar ratio of starting **VII** or **VIII** to diphosphonic acid, trying to achieve the maximum yield of dinucleoside **I** or **II**. When this ratio was 1:0.5, the overall yield of dinucleoside **Ib** and triphosphonate **Vb** did not exceed 10%, although the target tetraphosphonate prevailed. Eventually, in the case of dinucleoside **Ib,** we used a 1.5-fold excess of the diphosphonic acid over the corresponding monophosphonate **VII**. This allowed the isolation of 31% of **Ib** and 11% of **Vb**.

For the preparation of triphosphonate **Vb** in a 72% yield, this ratio was taken as 1:3. It should be mentioned that dibromomethylene derivatives **Ic** and **IIc** were only obtained in the two-step synthesis, that is, with the isolation of the corresponding triphosphonates **Vc** and **VIc** followed by their coupling with CDI-activated monophosphonate **VII** or **VIII**.

In the case of carbocyclic adenosine analogues, the coupling reaction was performed with DMF as a solvent, whereas for guanine derivatives, we used a 1:2 mixture of DMF/hexamethylphosphoramide (HMPA). This allowed an increase in the solubility of both the starting compounds and the products and resulted in an increase in product yields.

Figure 1. Dose dependence of guanosine derivatives incorporation into the 3′-end of 14-mer primer of complex **A** under catalysis by HIV reverse transcriptase: **Va** (lanes 5−8) at 0.001, 0.0025, 0.005, 0.01 μM, respectively; **Vb** (lanes 9−12) at 0.01, 0.05, 0.1, 1 μM, respectively; **Ia** (lanes 13−16) at 0.001, 0.0025, 0.0.005, 0.01 μM, respectively; **Ib** (lanes 17−20) at 0.02, 0.05, 0.2, 2 μM, respectively; **III** (lanes 21−24) at 0.02, 0.05, 0.2, 2 μM, respectively; lanes 1−4 show dGTP incorporation at 0.001, 0.0025, 0.005, 0.01 μM, respectively. Left lane corresponds to the position of 14-mer primer.

According to the ^{1}H NMR spectra data, the structures of the targets **I** and **II** were close to symmetrical, which was testified by the presence of one set of signals for each proton of the base, cyclopentene, and the oligophosphate chain. In the proton−phosphorus decoupled ^{31}P NMR patterns of dinucleosides **I** and **II**, two groups of signals were observed, the first of which (a multiplet at 10.20−10.40 ppm) was assigned to α- and α′-P atoms, whereas the other one was characteristic of β- and β′-phosphorus atoms. It can be mentioned that in the case of dinucleoside analogue **Ib** there was fine coupling for each signal within each group, which may imply the distortion of symmetry of the molecule. In contrast, the pattern of parent triphosphonate **Vb** showed the presence of a clear doublet at 10.3 ppm (α-P), a doublet of triplets at 3.8 ppm (β-P), and a doublet of doublets of triplets at −4.7 ppm (γ-P). NMR spectral data, as well as mass spectral data, for the other synthesized compounds were also consistent with the proposed structures.

Substrate Properties of the Compounds under Study toward HIV Reverse Transcriptase. Compounds **I**−**VI** were evaluated as (i) substrates for HIV reverse transcriptase in a one-step elongation reaction of primers in primer−template complexes, (ii) inhibitors of DNA synthesis catalyzed by HIV reverse transcriptase using activated DNA as a primer−template, (iii) terminating substrates in the DNA sequencing assay. The dose-dependent incorporation of guanosine derivatives into the 3′-end of primers (complex **A**) is shown in Figure 1. As can be seen, all the compounds were substrates of HIV reverse transcriptase and could elongate the primers by one nucleotide although with different efficacy. The comparison of lanes 13−16 and 5−8 with lanes 1−4 shows that substrate efficacies of **Ia** and **Va** were close to that of the natural substrate dGTP (lanes 1−4). The introduction of difluoromethylenephosphonate residues **Ib** and **Vb** reduced the activity by at least 1 order of magnitude if compared with the corresponding pyrophosphonyl phosphonates **Ia** and **Va**, respectively. The introduction of dibromomethylenephosphonate fragments **Ic** and **Vc** dramatically affected the activity. The loss of activity for both compounds was several orders of magnitude, so the compounds became essentially inactive (data not shown). The efficacy of diguanosine triphosphonate (**III**) incorporation into the DNA chain (lanes 22−24) was lower than that of tetraphosphonate **Ia** (lanes 13−16). A similar pattern

Table 1. Substrate Concentrations at Which the DNA Synthesis Is Inhibited by 50%[a]

Structure	Compound	IC_{50}, μM
X–P(O)(OH)–O–P(O)(OH)–CH_2–O–(B)]$_2$	**Ia** B=Gua X=O	0.7
	Ib B=Gua X=CF_2	15
	Ib B=Gua X=CBr_2	>250
	IIa B=Ade X=O	2
	IIb B=Ade X=CF_2	20
	IIc B=Ade X=CBr_2	>200
P–O–P(O)(OH)–CH_2–O–(B)]$_2$	**III** B= Gua	8
	IV B= Ade	10
HO–P(O)(OH)–X–P(O)(OH)–O–P(O)(OH)–CH_2–O–(B)	**Va** B=Gua X=O	0.3
	Vb B=Gua X=CF_2	25
	Vc B=Gua X=CBr_2	>200
	VIa B=Ade X=O	0.2
	VIb B=Ade X=CF_2	25
	VIc B=Ade X=CBr_2	>1000

[a] The rate of incorporation of [α-^{32}P]dGTP or [α-^{32}P]dATP into DNA was linear with respect to time. The results were averaged from at least two independent experiments.

was found for adenosine derivatives when complex **B** was used as primer–template (data not shown).

Table 1 shows the concentrations of the compounds under study at which the initial rate of DNA synthesis catalyzed by HIV reverse transcriptase was decreased by 50%. Evidently, the efficacy of inhibition by the compounds under study depended on modifications and corresponded to the efficacy of their incorporation into the DNA chain (Figure 1). The introduction of a difluoromethylenephosphonate group in either guanosine (**Ib** and **Vb**) or adenosine (**IIb** and **VIb**) derivatives sharply increased the concentrations of the compounds necessary for the 50% inhibition of DNA synthesis.

Chain-termination properties of the compounds were assayed for HIV reverse transcriptase in the presence of all four dNTPs following Sanger's procedure.[15] Figure 2 presents the dose-dependent pattern of the DNA synthesis termination by the guanosine derivatives. The synthesis was performed in the presence of M13mp10 phage DNA annealed with 5′-[^{32}P]-labeled 14-mer primer (complex **B**). DNA reaction products were analyzed using 8% denaturing polyacrylamide gel electrophoresis. The primer extension patterns in the presence of **Va** (lanes 7–9), **Vb** (10–12), **Ia** (13–15), and **Ib** (lanes 16–18) were similar to the one observed for the reaction with ddGTP (lanes 4–6). These results imply that the compounds under study were incorporated into the growing DNA chain in place of a guanosine residue in a dose-dependent manner. The subsequent chase containing 50 μM of four dNTPs failed to extend the terminated primer. Thus, the ability of HIV reverse transcriptase to incorporate the guanosine oligophosphonate derivatives into the growing DNA fragments was demonstrated, the compound concentrations necessary for the termination of the elongation reaction being varied with their structure.

Phosphorylation Assay. We studied phosphorylation of **VII** and **VIII** by GMP and AMP kinases, respectively. Figure 3 shows that these enzymes phosphorylated neither of the compounds in vitro, although the sensitivity of the method used by us allowed the detection of phosphorylation products in a 10^4-fold lesser amount than that of phosphorylation products of natural AMP and GMP. In addition, phosphonate **VIII** demonstrated weak inhibitory properties toward AMP kinase; the initial rate of AMP phosphorylation was decreased by 10% at a ratio of **VIII** to AMP of 10:1. In the case of compound **VII** the inhibitory effect was not found.

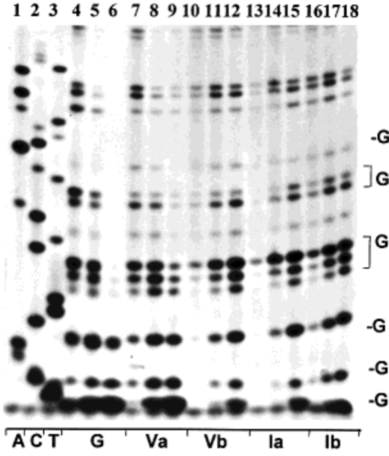

Figure 2. Radioautograph of the chain-terminating sequencing reaction with guanosine derivatives using HIV reverse transcriptase and M13mp10 phage DNA annealed with [5′-^{32}P]-14-mer primer (complex **B**). The letters on the right side indicate the position of guanosine residues in the DNA chain after the primer. Lanes 1–3 show the DNA sequence with 1 μM ddATP, 1 μM ddCTP, and 2 μM ddTTP, respectively, lanes 4–6 with 0.2, 1, and 5 μM of ddGTP, lanes 7–9 with 0.2, 1, and 5 μM of **Va**, lanes 10–12 with 0.2, 1, and 5 μM of **Vb**, lanes 13–15 with 0.2, 1, and 5 μM of **Ia**, and lanes 16–18 with 0.2, 1, and 5 μM of **Ib**.

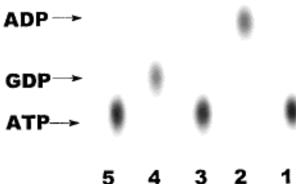

Figure 3. Phosphorylation reaction of **VII** (lane 5) and **VIII** (lane 3) by GMP and AMP kinases, respectively: (lane 1) [γ-^{32}P]ATP position; (lane 2) [β-^{32}P]ADP after phosphorylation of AMP by [γ-^{32}P]ATP with AMP-kinase; (lane 3) phosphorylation of **VIII** by AMP-kinase; (lane 4) [β-^{32}P]GDP after phosphorylation of GMP by [γ-^{32}P]ATP with GMP-kinase; (lane 5) phosphorylation reaction of **VII** by GMP kinase.

Enzymatic Stability. The enzymatic stability of guanosine derivatives **Ia,b** was evaluated in human blood serum as a model of a biologically active medium containing various types of hydrolyzing enzymes.

As is seen in Figure 4, compound **Ib** was hydrolyzed to give two products, monophosphonate **VII** and triphosphonate **Vb**, both products being accumulated at the same rate for the first 3 h. The amount of monophosphonate **VII** was constantly increased, and after 22 h, it was virtually a single product detected in the reaction mixture. The accumulation profile of triphosphonate **Vb** was more intricate. For the first 3 h, its accumulation

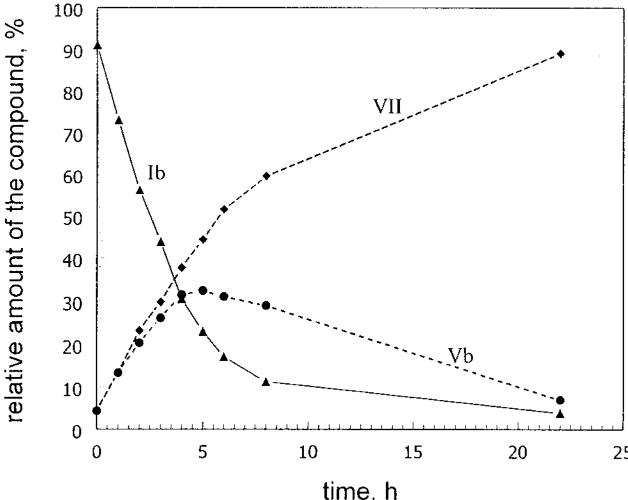

Figure 4. Time dependence of tetraphosphonate **Ib** hydrolysis in human blood serum. The total amount of the compounds loaded on the column was taken as 100%. The results are averaged from three independent experiments.

Table 2. Stability of Dinucleoside Tetraphosphosphonates **Ia**,**b**

compound	retention time,[a] min	half-life at 37 °C,[b] h: human blood serum	half-life at 37 °C,[b] h: phosphate buffer, pH 7.5
Ia	20.6 ± 0.1	3.0 ± 0.3	12 ± 0.5
Ib	20.0 ± 0.1	3.0 ± 0.3	15 ± 0.5
Vb	16.5 ± 0.1	7.0 ± 0.5	>24
VII	17.0 ± 0.1		
dGTP	12.3 ± 0.1	0.30 ± 0.03	>24

[a] HPLC system was used for the analysis. [b] Each value was averaged from at least three independent experiments. Authentic **Vb**, **VII**, and dGTP were taken as controls.

rate was comparable with that of monophosphonate. During the next 2−3 h, the amount of **Vb** remained essentially the same and then slowly decreased, which can be accounted for by its own hydrolysis.[9] Hydrolysis of compound **Ia** proceeded in a similar fashion. On the basis of HPLC analysis, hydrolysis rate curves were plotted and half-lives were determined (Table 2). Compounds **Ia** and **Ib** demonstrated close enzymatic resistance, which was 1 order of magnitude higher than that of natural dGTP. The corresponding difluoromethylene triphosphosphonate **Vb** was twice as stable under the conditions used. It is interesting to note that the introduction of a β,β'-difluoromethylene fragment in place of the pyrophosphate residue did not affect the stability of the tested compounds in blood serum.

Antiviral Properties. Two systems were used for the evaluation of antiviral activity of compounds **I**−**IV**: (a) Rat1 fibroblast cell culture infected by recombinant retrovirus pSG1, containing Moloni murine leukemia reverse transcriptase; (b) MT-4 cells infected with HIV-1.

As can be seen in Figure 5, the activity of dinucleoside derivative **Ib** in Rat1 fibroblast cell culture infected by pSG1 retrovirus was 3 orders of magnitude lower than that of AZT, although about 1 order of magnitude higher than the activity of the corresponding triphosphonate **Vb** and monophosphonate **VII**. For the other dinucleotide analogues, the inhibitory concentrations at which the virus replication was inhibited by 50% essentially

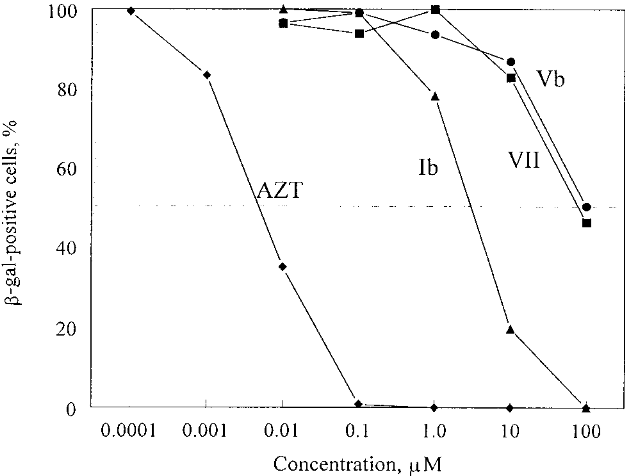

Figure 5. Dose dependence of inhibition of recombinant retrovirus pSG1 in Rat1 fibroblast cell culture by **Ib.** The data are averaged from two independent experiments.

Table 3. Anti-HIV Effect of the Compounds under Study in MT-4 Cell Culture

compd	CD_{50},[a] μM	ID_{50} [b] for free forms, μM	ID_{50} [b] for liposome-entrapped forms,[c] μM
Ia	>12	>12	0.5
Ib	84	60	
IIa	131	2	1.2
IIc	>110	5.4	
III	56	9.0	
IV	146	6.6	
Va	20.5	7.2	<1
Vb	144	>200	
VIa	212	>10	<1
VIc	>160	80	
VII	153	138	
VIII	>320	201	
AZT	140	0.004	
D4T	314	0.24	

[a] Compound concentration required to cause a 50% inhibition of cell proliferation. [b] Compound concentration required to cause a 50% inhibition of HIV reproduction. [c] Incorporation of the compounds into liposomes was 40−50%. Each value was averaged from two independent experiments.

did not differ from the corresponding triphosphonates and were about 7−10 μM.

The data on antiviral activity of dinucleoside analogues **I**−**IV** in MT-4 cells infected with HIV are presented in Table 3. In this system the differences in ID_{50} among the tested compounds were rather significant. The most active compounds were diadenosine tetraphosphonates **IIa** and **IIc**. Triphosphonate **Vb** and monophosphonate **VII** did not display any anti-HIV activity, which is consistent with the results for the fibroblast cell culture. In contrast, the activity of dinucleoside **Ib** was much lower if compared with the data for Rat1 cells. It can also be mentioned that there is no correlation between the antiviral effect and the inhibition of HIV reverse transcriptase in cell-free systems.

Intracellular Uptake. To estimate the effect of the penetration of the compounds under investigation on HIV inhibition into MT-4 cells, we used phosphatidylcholine-composed liposomes as a drug delivery system. Table 3 shows the anti-HIV efficacy of free and liposome-encapsulated compounds. The obtained data demonstrated that the compounds encapsulated into lipo-

somes inhibited virus reproduction more effectively than free compounds. In particular, the use of a liposome delivery system allowed at least a 10-fold increase in the activity of compounds **Ia**, **Va**, and **VIa**.

Discussion

In the course of the study of nucleotides modified at the triphosphate residue as substrates and/or inhibitors of HIV reverse transcriptase, our attention was diverted to carbocyclic dinucleoside polyphosphonates. Carbocyclic nucleosides are of special interest because of their metabolic stability in vivo and antiviral activity found for some analogues.[16] The presence of a carbocyclic ring makes them resistant to nucleoside phosphorylases and hydrolases, which smoothly cleave nucleosides. At the same time the structural similarity to the natural nucleosides allows these analogues to behave as substrates or inhibitors of some enzymes.[17−19]

Recently we reported the synthesis and some properties of nucleoside triphosphate mimetics bearing a cyclopentenyl residue as a glycone and a triphosphonate chain in place of a triphosphate.[9] It was also shown in our laboratory that bis(2′-deoxynucleoside)-5′,5′-tetraphosphates and bis(2′-deoxynucleoside)-5′,5′-triphosphates were good substrates for several human DNA polymerases and HIV reverse transcriptase.[8] On the basis of carbocyclic dinucleoside analogues bearing a polyphosphonate chain, we hoped to get new tools for the investigation of HIV reverse transcriptase. Moreover, proper modifications of the polyphosphate chain or its replacement by a phosphonate residue might open new fields for the design of a new type of potential anti-HIV agent, stable in blood serum and hydrophobic enough for penetration through cell walls.

In this report, we showed that carbocyclic diguanosine and diadenosine tetra- and triphosphonates **Ia**, **IIa**, **III**, and **IV** were good substrates for HIV reverse transcriptase, triphosphonates **III** and **IV** being less potent than tetraphosphonates **Ia** and **IIa** (Figure 1 and Table 1). This is consistent with our previous data on higher efficacy of natural dinucleoside α,δ-tetraphosphates as substrates for HIV reverse transcriptase than dinucleoside α,γ-triphosphates.[8] The introduction of a CF_2 group into the polyphosponate chain caused a 10-fold decrease in the activity of the compounds obtained. It is worth mentioning that according to the TLC and HPLC data, the synthesized dinucleoside tri- and tetraphosphonates displayed higher lipophilicity of the products if compared with the corresponding triphosphates (for example, the difference in the retention time of **Ib** and **Vb** exceeded 3 min). We hoped that higher lipophilicity as well as higher stability in blood serum could allow these compounds to be good inhibitors of HIV reproduction in cell cultures. Unfortunately, the compounds synthesized were moderately active in cell cultures. We believed, however, that the inhibitory effect was specific and associated with the action of dinucleoside oligophosphonates rather than with their degradation products. We demonstrated that potential decomposition products (**VII** or **VIII**) could not be phosphorylated by monophosphate kinases and did not affect virus reproduction. We assumed that one of the reasons for their weak activity might be poor penetration through cell membranes. Indeed, when a liposome system was used for delivering compounds **Ia** and **IIa** into cells, we observed an increased antiviral effect, although this increase was not as pronounced as we expected. We cannot also explain the lack of correlation between the inhibitory effects of the tested compounds in cell and cell-free systems. All this implies that compounds of this type may suffer various transformations in cells prior to their incorporation by reverse transcriptase.

Experimental Section

General Methods. Chemicals and solvents were from Aldrich. Anhydrous solvents were used without further drying. Phosphonates **VII** and **VIII** were synthesized as described before.[19,20] The synthesized compounds were prepared as ammonium salts. UV spectra were recorded on a Shimadzu UV-1201 spectrophotometer in water at pH 7.0. ^{1}H NMR spectra were registered on a Bruker AMX III-400 spectrometer (400 MHz) with tetramethylsilane as an internal standard. ^{31}P NMR spectra (162 MHz) were registered with P−H decoupling, taking 85% H_3PO_4 as an external standard and D_2O as a solvent. ^{19}F NMR (367 MHz) spectra were recorded in D_2O. Mass spectra were registered on a COMPACT MALDI-4 (Kratos Analytical) spectrometer. Column chromatography was performed on DEAE Toyopearl (HCO_3^+) (Toyosoda, Japan) and LiChroprep RP-18 (25−40 μm). TLC was carried out on Kieselgel 60 F254 plates (Merck) in iPrOH/water/25% aqueous ammonia 7:2:1 or dioxane/water/25% aqueous ammonia 6:4:1. Human blood serum was a kind gift from Dr. V. Chernikov (Institute of Gene Biology, RAMS). HIV-1 reverse transcriptase was obtained from Amersham Life Science (lot no. 21A). T4 polynucleotide kinase was from Sigma (lot 12H-0442). Single-stranded M13mp10 phage DNA was isolated from the cell culture media of the recipient *E. coli* K12XL1 strain according to the reported procedure.[21] Oligonucleotides were a kind gift of Dr. S. Surzhikov (Engelhardt Institute of Molecular Biology, Moscow, Russia). [γ-^{32}P]ATP (specific activity 6000 Ci/mmol), [γ-^{32}P]dATP (specific activity 3000 Ci/mmol), and [α-^{32}P]dGTP (specific activity 3000 Ci/mmol) were obtained from IPPE (Obninsk, Russia).

General Procedure for the Activation of (±)-Phosphonate (VII or VIII). To a solution of the bis(tributylammonium) salt of phosphonate **VII** or **VIII** (0.1 mmol) in DMF (1 mL) and HMPA (2 mL), CDI (130 mg, 0.8 mmol) was added, and the mixture was stirred at 25 °C for 3 h. Methanol (65 μL, 2 mmol) was added and after 0.3 h of stirring, methanol was removed in a vacuum. The resulting solution was stored in an argon atmosphere at room temperature and used in the subsequent reactions as a stock solution.

***P*,*P*′-Bis{[4-(guanine-9-yl)cyclopent-2-enyl]oxymethylphosphonyl}diphosphate, Ia, and 9-[4-(Diphosphoryloxyphosphonylmethoxy)cyclopent-2-enyl]guanine, Va.** A 0.5 M solution of bis(tributylammonium) pyrophosphate in DMF (0.2 mmol, 400 μL) was added under argon with stirring to the stock solution of activated phosphonate **VII** (0.1 mmol) prepared as described above, and the reaction mixture was stirred for 6 h at room temperature. The reaction solution was loaded on a DEAE-Toyopearl column and eluted in a linear gradient of NH_4HCO_3 (0−0.4 M). Triphosphonate **Va** was eluted at 0.3 M NH_4HCO_3, and dinucleotide **Ia** was eluted at 0.35 M NH_4HCO_3. The products were repurified by reverse-phase chromatography on a LiChroprep RP-18 column (water). The target fractions were lyophilized to give 24% (19 mg) of **Ia** and 57% (28 mg) of **Va.**

Compound Ia. UV: λ_{max} 252.2 nm (ϵ 18 000). ^{1}H NMR (D_2O, δ, ppm): 1.90 (1H, dt, J = 14.3 and 4.0 Hz, 5′β-H), 2.99 (1H, dt, J = 14.3 and 7.2 Hz, 5′α-H), 3.69 (2H, d, J = 9.0 Hz, OCH_2P), 5.08 (1H, m, 4′-H), 5.32 (1H, m, 1′-H), 6.15 (1H, m, 2′-H), 6.40 (1H, m, 3′-H), 7.83 (1H, s, 8-H). ^{31}P NMR (δ, ppm): 8.8 (d, J = 22 Hz, P_α), −22.7 (d, P_β). MS (m/e): 796.1 [M^+].

Compound Va. UV: λ_{max} 252.5 nm (ϵ 10 300). ^{1}H NMR (δ, ppm): 1.90 (1H, dt, J = 14.3 and 4.0 Hz, 5′β-H), 2.99 (1H, dt, J = 14.3 and 7.2 Hz, 5′α-H), 3.69 (2H, d, J = 9.0 Hz, OCH_2H), 5.08 (1H, m, 4′-H), 5.32 (1H, m, 1′-H), 6.15 (1H, m, 2′-H), 6.40

(1H, m, 3′-H), 7.83 (1H, s, 8-H). ^{31}P NMR (δ, ppm): 7.8 (d, J = 27 Hz, P_α), −7.8 (d, J = 20 Hz, P_γ), −22.8 (dd, J = 27 and 20 Hz, P_β). MS (m/e): 487.3 [M$^+$].

***P,P′*-Bis{[4-(guanine-9-yl)cyclopent-2-enyl]oxymethylphosphonyl}difluorodiphosphonate, Ib, and 9-[4-(Difluoromethyldiphosphonyl)oxyphosphonylmethoxy)cyclopent-2-enyl]guanine, Vb. Ib** and **Vb** were prepared in a similar fashion from activated phosphonate **VII** (0.1 mmol) and 0.5 M tributylammmonium difluoromethylenediphosphonate (0.15 mmol, 300 μL) in a yield of 31% (13 mg) and 11% (6 mg), respectively.

Compound Ib. UV: λ_{max} 252.2 nm (ϵ 18 000). ^{1}H NMR (δ, ppm): 1.90 (1H, dt, J = 14.3 and 4.0 Hz, 5′β-H), 2.99 (1H, dt, J = 14.3 and 7.2 Hz, 5′α-H), 3.69 (2H, d, J = 9.0 Hz, OCH_2H), 5.08 (1H, m, 4′-H), 5.32 (1H, m, 1′-H), 6.15 (1H, m, 2′-H), 6.40 (1H, m, 3′-H), 7.83 (0.5 H, s, 8-H), 7.85 (0.5 H, s, 8-H). ^{31}P NMR (δ, ppm): 10.4 (d, $J_{P\alpha,P\beta}$ = 17 Hz, P_α), −5.7 (dt, $J_{P\beta,F}$ = 84 Hz, P_β). ^{19}F NMR (δ, ppm): −42.6 (t, $J_{F,P}$ = 84 Hz). MS (m/e): 830.3 [M$^+$].

Compound Vb. UV: λ_{max} 252.5 nm (ϵ 10 300). ^{1}H NMR (δ, ppm): 1.90 (1H, dt, J = 14.3 and 4.0 Hz, 5′β-H), 2.99 (1H, dt, J = 14.3 and 7.2 Hz, 5′α-H), 3.69 (2H, dt, J = 9.0 Hz, OCH_2H), 5.08 (1H, m, 4′-H), 5.32 (1H, m, 1′-H), 6.15 (1H, m, 2′-H), 6.40 (1H, m, 3′-H), 7.83 (1H, s, 8-H). ^{31}P NMR (δ, ppm): 10.3 (d, $J_{P\alpha,P\beta}$ = 33 Hz, P_α), 3.8 (dt, $J_{P\gamma,P\beta}$ = 59 Hz, $J_{P\gamma,F}$ = 82 Hz, P_γ), −4.7 (ddt, $J_{P\beta,F}$ = 88 Hz, P_β). ^{19}F NMR (δ, ppm): −42.6 (dd, $J_{F,P\alpha}$ = 82 Hz, $J_{F,P\beta}$ = 88 Hz). MS (m/e): 519.9 [M$^+$].

***P,P′*-Bis{[4-(guanine-9-yl)cyclopent-2-enyl]oxymethylphosphonyl}dibromodiphosphonate, Ic. Ic** was prepared from triphosphonate **Vc** (0.05 mmol) and activated phosphonate **VII** (0.1 mmol) similarly to the preparation of **Ia** in a yield of 35%. UV: λ_{max} 251.2 nm (ϵ 18 000). ^{1}H NMR (δ, ppm): 1.90 (1H, dt, J = 14.0 and 4.0 Hz, 5′β-H), 3.04 (1H, m, 5′α-H), 3.96 (2H, d, J = 9.4 Hz, OCH_2H), 4.85 (1H, m, 4′-H), 5.25 (1H, m, 1′-H), 6.10 (1H, m, 2′-H), 6.40 (1H, m, 3′-H), 7.85 (1H, s, 8-H). ^{31}P NMR (δ, ppm): 9.7 (d, J = 26 Hz, P_α), −0.8 (d, P_β). MS (m/e): 952.6 [M$^+$].

9-[4-(Difluoromethyldiphosphonyl)oxyphosphonylmethoxy)cyclopent-2-enyl]guanine, Vc. Vc was synthesized from a solution of 0.5 M tributylammmonium dibromomethylenediphosphonate in DMF (0.3 mmol, 600 μL) and activated phosphonate **VII** (0.1 mmol) as described above in a yield of 72%. UV: λ_{max} 251.5 nm (ϵ 10 300). ^{1}H NMR (δ, ppm): 1.94 (1H, dt, J = 14.4 and 4.0 Hz, 5′β-H), 2.99 (1H, dt, J = 14.4 and 7.2 Hz, 5′α-H), 3.96 (2H, d, J = 9.4 Hz, OCH_2H), 4.87 (1H, m, 4′-H), 5.35 (1H, m, 1′-H), 6.13 (1H, m, 2′-H), 6.42 (1H, m, 3′-H), 7.85 (1H, s, 8-H). ^{31}P NMR (δ, ppm): 9.7 (d, $J_{P\alpha,P\beta}$ = 34 Hz, P_α), 8.4 (d, $J_{P\gamma,P\beta}$ = 14 Hz, P_γ), 2.5 (dd, P_β). MS (m/e): 643.2 [M$^+$].

Adenosine analogues **IIa**−**c** and **VIa**−**c** were prepared in a similar fashion.

Compound IIa. Yield 16%. UV: λ_{max} 261.3 nm (ϵ 27 000). ^{1}H NMR (δ, ppm): 1.97 (1H, m, J = 15.0, 8.0, and 4.0 Hz, 5′β-H), 3.02 (1H, m, 5′α-H), 3.72 (2H, d, J = 9.0 Hz, OCH_2H), 4.95 (1H, m, 4′-H), 5.50 (1H, m, 1′-H), 6.23 (1H, m, 2′-H), 6.47 (1H, m, 3′-H), 8.14 (1H, s, 8-H), 8.18 (1H, s, 2-H). ^{31}P NMR (δ, ppm): 9.5 (d, J = 22 Hz, P_α), −22.7 (d, P_β). MS (m/e): 764.1 [M$^+$].

Compound VIa. Yield 23%. UV: λ_{max} 261.5 nm (ϵ 15 000). ^{1}H NMR (δ, ppm): 1.97 (1H, m, J = 15.0, 8.0, and 4.0 Hz, 5′β-H), 3.02 (1H, m, 5′α-H), 3.72 (2H, d, J = 9.0 Hz, OCH_2H), 4.95 (1H, m, 4′-H), 5.50 (1H, m, 1′-H), 6.23 (1H, m, 2′-H), 6.47 (1H, m, 3′-H), 8.14 (1H, s, 8-H), 8.18 (1H, s, 2-H). ^{31}P NMR (δ, ppm): 9.9 (d, J = 26 Hz, P_α), −9.6 (d, J = 20 Hz, P_γ), −22.7 (dd, P_β). MS (m/e): 471.2 [M$^+$].

Compound IIb. Yield 27%. UV: λ_{max} 261.4 nm (ϵ 27 000). ^{1}H NMR (δ, ppm): 1.97 (1H, m, J = 15.0, 8.0, and 4.0 Hz, 5′β-H), 3.02 (1H, m, 5′α-H), 3.72 (2H, d, J = 9.0 Hz, OCH_2H), 4.95 (1H, m, 4′-H), 5.50 (1H, m, 1′-H), 6.23 (1H, m, 2′-H), 6.47 (1H, m, 3′-H), 8.14 (1H, s, 8-H), 8.18 (1H, s, 2-H). ^{31}P NMR (δ, ppm): 9.7 (d, J = 21 Hz, P_α), −6.1 (dt, $J_{P\beta,F}$ = 84 Hz, P_β). ^{19}F NMR (367 MHz, D_2O, δ, ppm): −42.6 (t, $J_{F,P}$ = 84 Hz). MS (m/e): 798.1 [M$^+$].

Compound VIb. Yield 24%. UV: λ_{max} 261.5 nm (ϵ 15 000). ^{1}H NMR (δ, ppm): 1.97 (1H, m, J = 15.0, 8.0, and 4.0 Hz, 5′β-H), 3.02 (1H, m, 5′α-H), 3.72 (2H, d, J = 9.0 Hz, OCH_2H), 4.95 (1H, m, 4′-H), 5.50 (1H, m, 1′-H), 6.23 (1H, m, 2′-H), 6.47 (1H, m, 3′-H), 8.14 (1H, s, 8-H), 8.18 (1H, s, 2-H). ^{31}P NMR (δ, ppm): 9.6 (d, $J_{P\alpha,P\beta}$ = 34 Hz, P_α), 3.6 (dt, $J_{P\gamma,P\beta}$ = 57 Hz, $J_{P\gamma,F}$ = 73 Hz, P_γ), −2.9 (ddt, $J_{P\beta,F}$ = 91 Hz, P_β). ^{19}F NMR (δ, ppm): −42.6 (dd, $J_{F,P\gamma}$ = 73 Hz, $J_{F,P\beta}$ = 91 Hz). MS (m/e): 504.9 [M$^+$].

Compound IIc. Yield 31%. UV: λ_{max} 262.2 nm (ϵ 27 000). ^{1}H NMR (δ, ppm): 1.89 (1H, m, 5′β-H), 3.02 (1H, m, 5′α-H), 3.93 (2H, d, J = 10 Hz, OCH_2H), 4.84 (1H, m, 4′-H), 5.39 (1H, m, 1′-H), 6.08 (1H, m, 2′-H), 6.41 (1H, m, 3′-H), 8.05 (1H, s, 8-H), 8.08 (0.5H, s, 2-H), 8.10 (0.5H, s, 2-H). ^{31}P (δ, ppm): 9.8 (d, J = 26 Hz, P_α), −0.8 (d, P_β). MS (m/e): 919.9 [M$^+$].

Compound VIc. Yield 68%. UV: λ_{max} 262.5 nm (ϵ 27 000). ^{1}H NMR (δ, ppm): 1.98 (1H, dt, J = 14.3 and 4.0 Hz, 5′β-H), 3.09 (1H, dt, J = 14.3 and 7.8 Hz, 5′α-H), 3.96 (2H, d, J = 9.4 Hz, OCH_2H), 4.90 (1H, m, 4′-H), 5.55 (1H, m, 1′-H), 6.21 (1H, m, 2′-H), 6.49 (1H, m, 3′-H), 8.21 (1H, s, 8-H), 8.25 (1H, s, 2-H). ^{31}P NMR (ppm): 9.6 (d, $J_{P\alpha,P\beta}$ = 33 Hz, P_α), 8.6 (d, $J_{P\gamma,P\beta}$ = 14 Hz, P_γ), 2.9 (dd, P_β). MS (m/e): 627.7 [M$^+$].

***P,P′*-Bis{[4-(guanine-9-yl)cyclopent-2-enyl]oxymethylphosphonyl}phosphate, III**. A solution of 1 M tributylammmonium orthophosphate (200 μL) was added to the solution of activated phosphonate **VII** prepared as described above, and the reaction mixture was stirred at room temperature for 6 h. The target **III** was isolated as described for compound **Ia** in a yield of 36% (13 mg). UV: λ_{max} 250.2 nm (ϵ 16 000). ^{1}H NMR (δ, ppm): 1.90 (1H, dt, J = 14.3 and 4.0 Hz, 5′β-H), 2.99 (1H, dt, J = 14.3 and 7.2 Hz, 5′α-H), 3.69 (2H, d, J = 9.0 Hz, OCH_2H), 5.08 (1H, m, 4′-H), 5.32 (1H, m, 1′-H), 6.15 (1H, m, 2′-H), 6.40 (1H, m, 3′-H), 7.83 (0.5 H, s, 8-H), 7.85 (0.5 H, s, 8-H). ^{31}P NMR (δ, ppm): 9.1 (d, J = 27 Hz, P_α), −22.3 (t, P_β). MS (m/e): 716.1 [M$^+$]

Adenosine analogue **IV** was prepared in a similar fashion. Yield 42%. UV: λ_{max} 261.3 nm (ϵ 25000). ^{1}H NMR (δ, ppm): 1.87 (1H, m, 5′β-H), 2.98 (1H, m, 5′α-H), 3.84 (2H, d, J = 9.0 Hz, OCH_2H), 4.65 (1H, m, 4′-H), 5.35 (1H, m, 1′-H), 6.03 (1H, m, 2′-H), 6.37 (1H, m, 3′-H), 8.07 (0.5H, s, 8-H), 8.09 (0.5H, s, 8-H), 8.11 (0.5H, s, 2-H), 8.13 (0.5H, s, 2-H). ^{31}P NMR (δ, ppm): 9.1 (d, J = 24 Hz, P_α), −22.4 (d, P_β). MS (m/e): 684.2 [M$^+$].

Enzymatic Stability. A solution (25 μL) containing 0.5 mM compounds under study in 100% human blood serum was incubated at 37 °C. The aliquots were taken out after certain intervals and frozen in liquid nitrogen. After thawing, the precipitated proteins were removed by centrifugation (6 min at 12 000 rpm) and the products were analyzed by HPLC using a Nucleosil RP-18 column (0.5 μm, 4 mm × 15 mm). Elution was performed in a linear gradient of 70% ethanol (0−25%) in 0.05 M triethylammonium bicarbonate buffer (pH 7.5−8.0) for 25 min. The retention times of the compounds are shown in Table 2.

Reverse Transcriptase Assays. Oligonucleotide primers were labeled at the 5′-terminus using T4 polynucleotide kinase, and template−primer complexes were prepared according to the standard procedure.[22] One-step primer extension reactions (6 μL) contained 1 nM primer−template (complex **A**), 0.3 U of enzyme, various concentrations of the substrate analogues (see figure captions), and the buffer according to the supplier's recommendations. The reactions were performed for 30 min at 37 °C. The reaction products were separated by 14% denaturing polyacrylamide gel electrophoresis, and the gels obtained were radioautographed. Termination properties of the compounds were evaluated according to the standard DNA sequencing assay.[15] Reaction mixtures (6 μL) contained 1 nM primer−template (complex **B**), 1 μM dNTP bearing the same base as the corresponding terminating substrate, 20 μM three other dNTP, and a terminating substarate at various concentrations as shown in the figure captions. The reactions were carried out at 37 °C for 10 min followed by the addition of four dNTP up to a final concentration of 50 μM. After 10 min,

reaction products were analyzed by 8% denaturing polyacrylamide gel electrophoresis, and the gels were radioautographed.

The primer–template complexes used for the study are as follows.

Primer–template complex **A**:

3′-... TTT GCT GCC GGT CAC GG T TGG ...

5′-[^{32}P]-AAA CGA CGG CCA GT ...

Primer–template complex **B**:

3′-... CAT TTT GCT GCC GGT CAC GGT TCG AAC CCG ACG ...

5′-[^{32}P]-GTA AAA CGA CGG CC ...

The ability of the compounds to inhibit DNA synthesis catalyzed by HIV reverse transcriptase was routinely assayed in the reaction mixture (8 μL) containing 2.5 μg of activated calf thymus DNA per sample, 30 μM three natural dNTP, 2 μM [α-^{32}P]dATP for the study of adenosine derivatives or 2 μM [α-^{32}P]dGTP for guanosine derivatives, various concentrations of inhibitors, 2 U of HIV reverse transcriptase, and the buffer according to the supplier's recommendations. After 5 min of incubation at 37 °C, the reaction mixtures were loaded onto Whatman DE-81 disks. The filters were washed with 0.2 M NaCl containing 0.5 mM EDTA and then fixed with ethanol. The incorporation of the radiolabeled substrate into the growing DNA chain was measured by liquid scintillation counting.

AMP and GMP Kinase Assay. AMP and GMP kinases with specific activities of 0.2 and 0.15 U/mg, respectively, were isolated from human placenta as described in ref 23. Both enzymes lacked ATP-degrading activity.

The reaction mixture (20 μL) contained 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM AMP or its counterpart **VIII**, 5 μM ATP, 2 μCi [γ-^{32}P]ATP, and 0.01 U of AMP kinase. After 20 min of incubation at 37 °C, the aliquot was loaded on a PEI cellulose TLC plate, developed in 0.5 M KH_2PO_4 (pH 4), dried, and exposed with X-ray film. The inhibition of AMP kinase was carried out in the reaction mixture (total volume of 20 μL) containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM AMP, 1 mM **VIII**, 20 μM ATP, 2 μCi [γ-^{32}P]ATP, and 0.0001 U of AMP kinase. After incubation at 37 °C for 0, 5, 10, 20, and 40 min, the aliquots were loaded on a PEI cellulose TLC plate, developed as described above, dried, and exposed with X-ray film. The areas of the TCL plate containing labeled [γ-^{32}P]ATP and [β-^{32}P]ADP were cut out, and their radioactivity was measured using a liquid scintillation counter. The initial rate of the enzymatic reaction was calculated under conditions when the conversion did not exceed 25%. The experiments with GMP kinase and compound **VII** were carried out in a similar fashion.

Antiviral Activity. Two systems were used to test antiviral activity of the compounds. (a) One system was Rat1 cells infected with recombinant retrovirus (pSG1) bearing the gene of Moloni murine leukemia reverse transcriptase and encoding β-galactosidase. The recombinant virus construction was described earlier.[24,25] The blue cell colonies expressing β-galactosidase were counted after the 24 h cell incubation with the compounds under study as described in ref 9. The results were expressed as a percentage of β-galactosidase-positive cells in the presence of inhibitors compared with the proportion of β-galactosidase-positive cells in the control. (b) The other system was MT-4 cells infected with HIV-1 strain (GKV-4046). The multiplicity of infection (MOI) was 0.2–0.5 units per cell. MT-4 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 300 mg/mL L-glutamine, 80 μg/mL gentamycin, and 30 μg/mL lincomycin at 37 °C in 5% CO_2 atmosphere. For infecting, the cells at a concentration of 2×10^6/mL and with viability exceeding 90% were used.

Cytotoxicity. MT-4 cells were cultured in the presence of various doses of the tested compounds (0.001–100 μg/mL, three replicates for each dose) on a 96-well cultural plate for 3 days. The concentration and viability of MT-4 cells were measured by the trypan blue-dye exclusion colorimetric assay, and the CD_{50} for each compound was calculated.

Anti-HIV Assay. The infected cells were cultured in the presence of various doses of the tested compounds (0.001–100 μg/mL, three replicates for each dose) on a 96-well cultural plate for 3 days. Anti-HIV activity of the tested compounds was assessed by the measurement of the p24 antigen amount using immunoassay.[26] The cell concentration and viability were estimated using the calorimetric assay as described above.

Liposome Delivery System. Liposomes composed of egg phosphatidylcholine (egg PC; ICN Biomedicals, Irvine, CA) were prepared by the technique of hydration of a thin-lipid film as described in ref 27 with slight modifications. Briefly, the phospholipid was first dissolved in a chloroform/ethanol (1:1 vol/vol) solution in a 50 mL round-bottom flask, and the solvent was then removed on a rotary evaporator with subsequent formation of a thin-lipid film on the flask wall. Multilamellar vesicles (heterogeneous population of 0.8–5.0 μm in diameter) containing tested compounds were prepared by mechanical agitation of the dry thin-lipid film for 30 min at 25 °C under nitrogen atmosphere with each of the tested compounds, which were dissolved in a phosphate-buffered saline (PBS; pH = 7.0) at a 1:124 drug/lipid molar ratio (final concentration of each compound was 1 mM). The resulting multilamellar vesicles were then sequentially extruded through polycarbonate membranes (Nuclepore, Cambridge, Massachusetts, USA) with pore diameters of 1.0, 0.8, 0.4, 0.2, and 0.1 μm using a polypropylene 13 mm holder (Millipore Corp., Bedford, MA) with plastic syringes.

The average diameter of the extruded liposomes was 0.16 $\pm$ 0.04 μm. Vesicle size distribution was evaluated by negative stain electron microscopy.

The amount of the drug encapsulated into liposomes was estimated after centrifugation of liposomes at 100 000*g* for 1 h followed by phospholipid extraction from the pellet with a 2:1 chloroform/methanol mixture. The concentrations of the tested compounds in the water phase were calculated using molar extinction coefficients of the compounds under study (see Experimental Section).

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