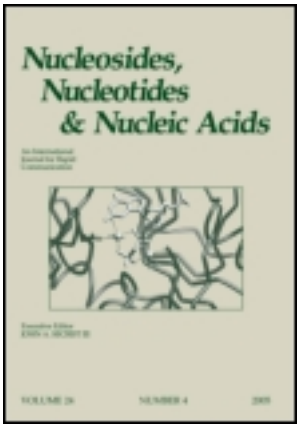

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AZT 5′-Cholinephosphate as an Anti-HIV Agent: The Study of Biochemical Properties and Metabolic Transformations Using Its ^{32}P-Labelled Counterpart

Dmitry V. Yanvarev [a], Elena A. Shirokova [a], Maria V. Astapova [b] & Yury S. Skoblov [b]

[a] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russian Federation

[b] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation

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AZT 5′-CHOLINEPHOSPHATE AS AN ANTI-HIV AGENT: THE STUDY OF BIOCHEMICAL PROPERTIES AND METABOLIC TRANSFORMATIONS USING ITS ^{32}P-LABELLED COUNTERPART

Dmitry V. Yanvarev and Elena A. Shirokova □ *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russian Federation*

Maria V. Astapova and Yury S. Skoblov □ *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation*

□ *Biochemical and metabolic transformations of 3′-azido-3′-deoxythymidine 5′-choline phosphate (**1**) were studied using its ^{32}P-labelled counterpart for the evaluation of possible reasons for its enhanced anti-HIV activity. An effective synthesis of ^{32}P-labelled **1** with a specific activity > 1,000 Ci/mmol was developed by esterification of ^{32}P-phosphoric acid with choline in the presence of BrCN followed by the coupling of the resulting choline phosphate with 3′-azido-3′-deoxythymidine (AZT). Chemical and enzymatic stabilities of **1** as well as the dynamics of penetration through HL-60 cell membranes were studied at the concentrations comparable to its antiviral concentrations. The products of intracellular transformations of the studied nucleotide were identified.*

Keywords 3′-Azido-3′-deoxythymidine 5′-[^{32}P]-choline phosphate; [^{32}P]orthophosphoric acid; Phosphorylation; Synthesis of [^{32}P]-labeled nucleotides

INTRODUCTION

Synthetic nucleoside analogues are widely used in medical practice for therapy of viral infections. For many of them, the mechanism of action involves intracellular phosphorylation into the corresponding triphosphates that are incorporated into the growing chain of viral DNA and terminate the DNA synthesis.[1] This mechanism implies that the efficacy of inhibition of viral reproduction is affected by at least several factors, in particular, the ability of the compound to pass through the cell membrane, the efficacy of

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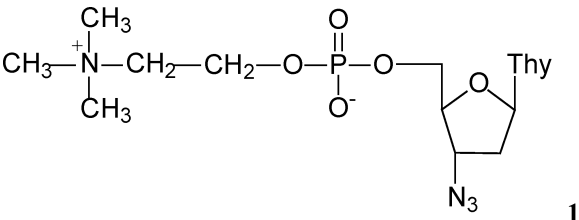
Address correspondence to Dmitry V. Yanvarev, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov st., Moscow, 119991, Russian Federation. E-mail: vsupport@bk.ru

the triphosphorylation cascade, and the ability of the resulting nucleoside triphosphate to be recognized by viral DNA polymerases.

One of the trends in the design of novel highly effective antiviral agents is further modifications of the known synthetic analogues affording lower toxicity, better penetration into cells, and/or prolonged action. The nucleoside modified in this way is transformed in vivo (cell or organism) into the parent nucleoside and affects the virus according to the known mechanism. 3′-Azido-3′-deoxythymidine 5′-*H*-phosphonate (AZT 5′-*H*-phosphonate, phosphazide, Nicavir) can serve a successful example of this approach. This drug has been used in anti-HIV therapy since 1999 as a less toxic AZT "substituent" and its in vivo precursor.[2]

Earlier we showed that some modified AZT derivatives inhibited the HIV replication in cell cultures more effectively than the parental nucleoside.[3–5] One of these compounds was AZT 5′-choline phosphate **1**: its antiviral activity measured as IC_{50} (the concentration, at which 50% of the virus replication is inhibited) was 0.6 nM versus 17 nM of AZT, whereas their toxicities were identical.[6] The latter is not surprising because, as a component of many natural compounds, a choline phosphate group must not contribute to the compound toxicity. Obviously, the anti-HIV activity of this compound is due to its ability to be intracellularly converted into AZT 5′-triphosphate, which terminates the synthesis of viral DNA. According to the chemical structure, compound **1** can donate either/both AZT 5′-monophosphate or/and AZT. For the understanding of the mechanism, we synthesized AZT 5′-choline phosphate labelled with phosphorus-32 (**1**) with a high specific activity (>1000 Ci/mmol) and studied some of its biochemical and metabolic properties in cell cultures. The use of the radioactive phosphorus enabled the study of both intracellular transformations of the phosphate group and alterations in the penetration of the tested compound through cell membranes at micromolar concentrations and those close to the concentrations of 90% inhibition of HIV-1 replication (IC_{90}). It also allowed us to compare the dynamics of penetration into cells of the studied AZT choline phosphate **1** with that of parental AZT.

Herein we report the synthesis of labelled AZT 5′-choline phosphate **1** and present the results of the study of its penetration into HL-60 cells, the composition of metabolites and potencies of some enzymes to degrade the studied nucleotide.



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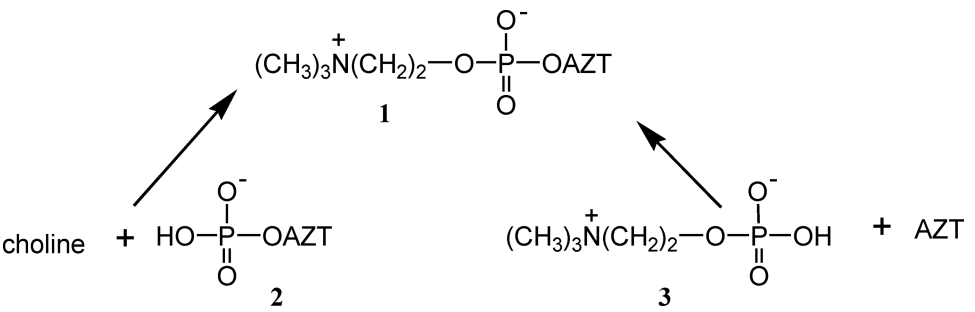
RESULTS

Chemistry

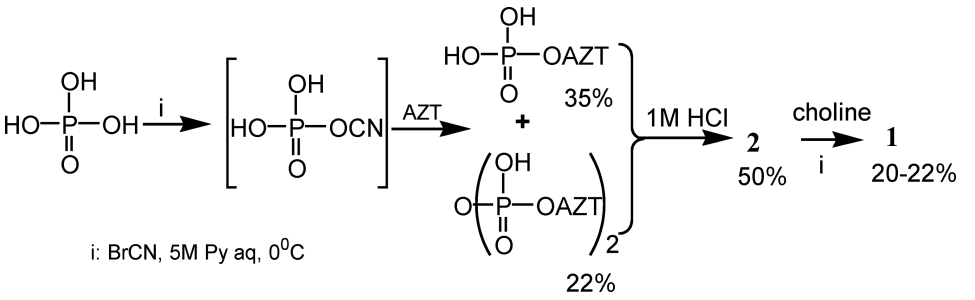
Phosphorylation of an alcoholic hydroxy group with phosphorus oxychloride is the most common chemical way to obtain the corresponding phosphates (including nucleoside phosphates).[7] The method is reproducible and allows high yields irrespective of the nucleoside nature. However, this approach is inapplicable for radiochemical synthesis of compounds with the high specific activity, since the specific activity of $^{32}POCl_3$ as a radioactive starting compound is markedly lower than that of ^{32}P-orthophosphoric acid. In addition, ^{32}P-orthophosphoric acid is substantially cheaper and more stable than $^{32}POCl_3$. With all this in mind, we used carrier-free ^{32}P-orthophosphoric acid for the synthesis of phosphorus-labeled compound **1**.

Two approaches can be used for the preparation of the target phosphate **1** (Scheme 1). The first approach includes phosphorylation of a nucleoside followed by the coupling of the resulting nucleotide with choline. The second method includes esterification of ^{32}P-phosphoric acid with choline followed by the coupling of the resulting choline phosphate with the nucleoside.



SCHEME 1 Approaches to the synthesis of target phosphate **1**.

The scheme resulting in AZT monophosphate **2** at the first stage seemed to be more convenient, as the intermediate nucleotide is easy to detect and isolate by HPLC. Chemically, a phosphorus-32-labelled nucleotide with the specific activity of at least 1000 Ci/mmol can be obtained by interaction of the corresponding nucleoside and $H_3{}^{32}PO_4$ in the presence of the coupling agent. The nucleoside and the coupling agent are used in this reaction in large excess (10-fold or more) relative to phosphoric acid. We performed the synthesis of nucleotide **2** in the presence of BrCN (Scheme 2), which proved to be more effective for AZT 5′-phosphorylation than commonly used coupling agents (DCC, TPS-Cl, and trichloroacetonitrile).[8] The reaction proceeded fast ($\leq$1 minute) even at 0°C, and the further incubation only increased the amount of side products, particularly, symmetrical

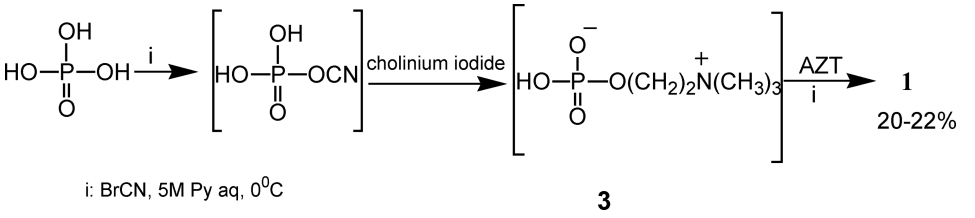


SCHEME 2 Synthesis of phosphate **1** via AZT-5′-phosphate.

bis-AZT 5′,5″-diphoshate. However, acid hydrolysis of the latter allowed an essential increase in the yield of the target **2**: after the 30 minutes incubation of the reaction mixture with 1M HCl at room temperature, the yield of monophosphate **2** achieved 50% relative to starting $H_3{}^{32}PO_4$.

Monophosphate **2** obtained at the first stage (Scheme 2) must be isolated and purified prior to the next step, since the choline hydroxy group is considerably less reactive than that of AZT. After reversed-phase HPLC, nucleotide **2** was esterified with choline under the conditions described for the first step to give 25–30% of target **1**. However, the total yield of product **1** was only 12–15%.

We used the other route in the hope to increase the product yield using choline phosphate **3** as an intermediate compound (Scheme 3).



SCHEME 3 Synthesis of phosphate **1** via choline phosphate.

Variations of the reaction conditions (e.g., the reagent ratio, temperature, or reaction time) only insignificantly influenced on the yield of the esterification product, which ranged from 25% to 30%. The esterification with the nucleoside was performed without isolating ^{32}P-choline phosphate **3**. The coupling of the latter with AZT was very effective and gave 90% of diester **1**. Although the total yield did not exceed 25%, it was significantly higher than that of compound **1** obtained according to Scheme 2. The specific activity of the product was 1000 Ci/mmol and the radioactive purity exceeded 95%. This allowed us to study metabolic transformations of compound **1**.

For the identification of the synthesized **1–3** by NMR and UV spectroscopies, we synthesized these compounds using the indicator amount of

radioactivity (specific activity of 0.1 Ci/mmol) and isolated them under the conditions described in the Experimental section.

Cellular Metabolic Studies

The dynamics of penetration of compound **1** into cells was studied in HL-60 cell culture. The dynamics of permeation of the radioactivity into cells is shown in Figure 1.

As is seen in Figure 1, the level of radioactivity was constantly growing during the 24-hour period and the curve lacked the saturation area. For a better understanding of this fact, we studied the spectrum of metabolites containing the ^{32}P isotope in the water-soluble fraction of the cellular lysate. We showed by HPLC that at any time point within the 0- to 24-hour time period only 3–6% of the total radioactivity found in the cell was present in cytoplasm as unchanged diester **1**, whereas the rest portion of radioactive products consisted of ester **3** and orthophosphate in a 4:1 ratio.

Obviously, following such effective intracellular destruction of diester **1** its concentration gradient inside and outside the cell must remain persistent within the experimental time period. The linearity of the curve in Figure 1 confirms the postulate. As diester **1** is hydrolyzed in the cultural medium by only 3–4% after 24 hours, the radioactive phosphorus isotope is likely to pass into cell as unchanged nucleotide **1**.

We established the intracellular distribution of metabolites containing the ^{32}P isotope by successive washing off the cells (after the 24-hour incubation), cryolysis, extraction of the water-insoluble fraction with a 1:1 chloroform-methanol mixture similarly to the procedure for the lipid isolation,[4] and two-dimensional TLC identification of the products. The results are shown in the diagram (Figure 2a). The water-soluble fraction contained 66% of the total intracellular radioactivity, 20% was found in the lipid one, and 14% of radiolabelled products was detected in the fraction, which was soluble neither in water nor in the chloroform-methanol mixture

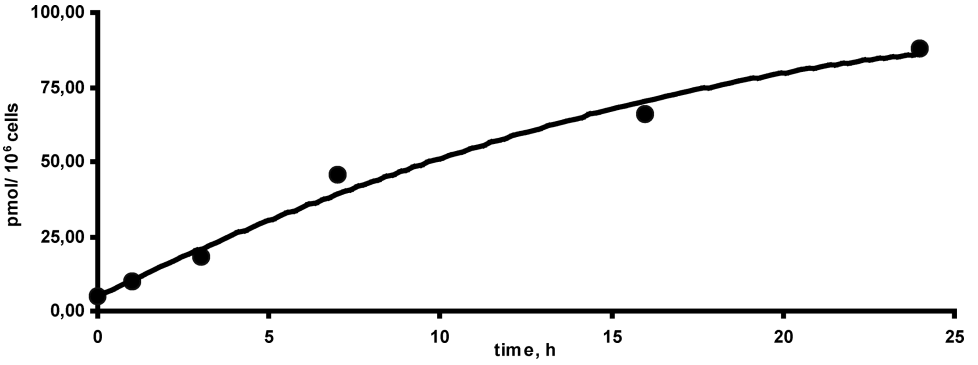


FIGURE 1 Dynamics of penetration of diester **1** into HL-60 cells.

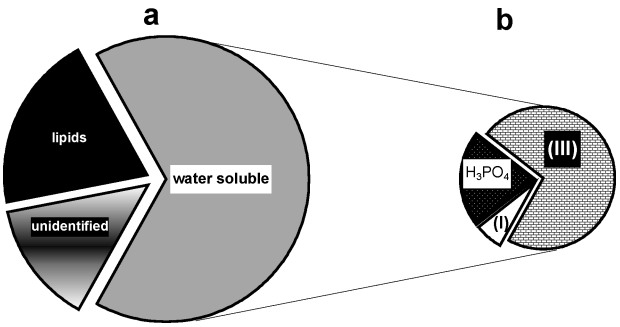


FIGURE 2 (a) Intracellular distribution of the radioactive label after the incubation of HL-60 cells with compound **1**. (b) The ^{32}P distribution in the water-soluble fraction of the cellular lyzate.

("unidentified fraction"). The product distribution of the water-soluble fraction is shown in Figure 2b. As is seen, the portions of choline phosphate **3**, H_3PO_4, and diester **1** totaled to 73%, 21%, and 6%, respectively.

The product composition in the lipid fraction isolated as described in Folch et al.[9] was studied by two-dimensional TLC.[10] The major radioactive metabolic products were phosphatidyl choline and phosphatidyl ethanolamine (Figure 3). The content of phosphatidic acid was considerably lower.

We also compared the dynamics of concurrent penetration into the cells of the tested diester **1** and the parent AZT. To this end, we used [6-^{3}H]-AZT[2] and detected the products based on the ^{32}P and ^3H levels. The results of this experiment are shown in Figure 4.

As is seen in Figure 4, the intracellular amount of [6-^{3}H]-AZT was higher than that of ^{32}P-labelled product(s) for the first 8 hours. Since diester **1** was quantitatively metabolized to AZT in cells, one can claim that the AZT amount resulted from diester **1** constitutes 94–97% of the intracellular ^{32}P

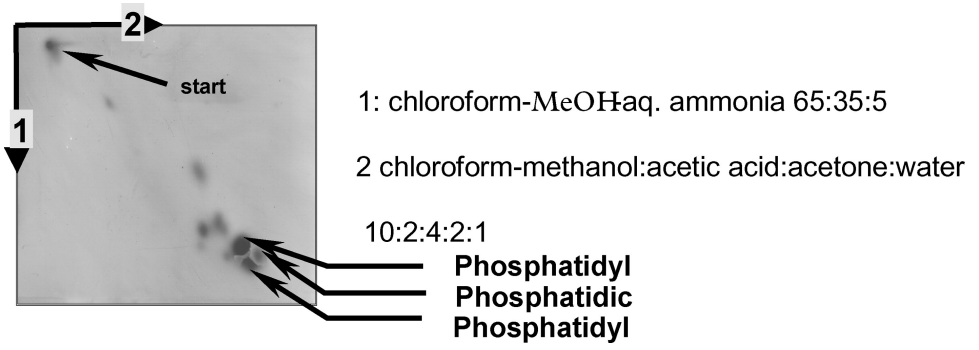


FIGURE 3 The TLC pattern of ^{32}P-labelled metabolites in the lipid fraction. Identification of the products was performed by comparison of their mobilitites with those of the corresponding unlabelled authentic reference compounds.

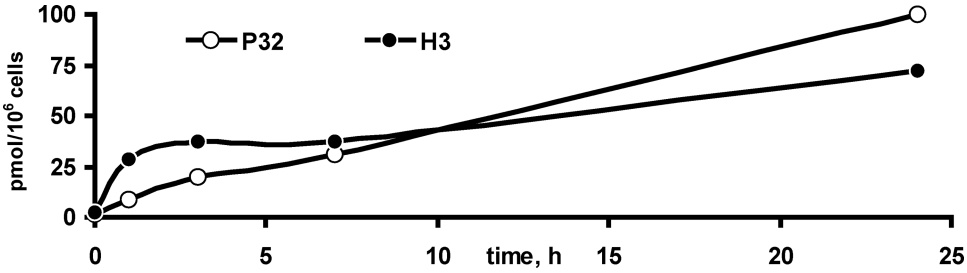


FIGURE 4 Dynamics of the cellular uptake of diester [^{32}P]-**1** and [6-^{3}H]-AZT. Both compounds were added simultaneously, each at the concentration of 4 μM. The presented data are mean values of two independent experiments made in two parallels.

amount. Hence, during the first 8 hours the intracellular AZT concentration from [6-^{3}H]-AZT was higher than that formed from the degraded **1**. Then the rate of [6-^{3}H]-AZT penetration markedly fell, whereas the AZT from diester **1** was accumulated, and the concentration difference between these two products achieved approximately 30% after 24 hours. However, it is noteworthy that the behavior of the penetration curve of compound **1** negligibly changed in the presence of the equivalent amount of AZT after the 6- to 7-hour incubation.

We also evaluated how the presence of a tenfold excess of AZT affected the cellular uptake of compound **1** (Figure 5). As is seen in Figure 5, AZT obviously influenced upon the penetration of **1** into the cells, but this influence was insignificant and cannot be explained by routine concurrent interactions.

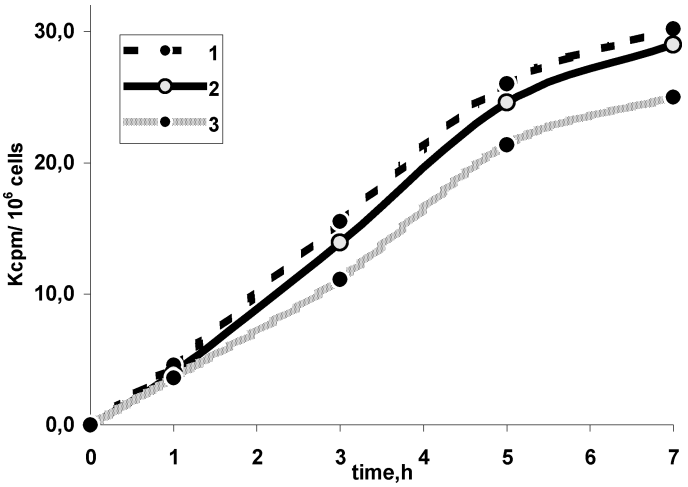


FIGURE 5 Dynamics of penetration of diester [^{32}P]-**1** (*1*) in the absence of AZT; (*2*) the presence of 1 μM AZT and (*3*) 10 μM AZT. Concentration of diester **1** in the cultural medium was 1 μM; specific activity of 4 Ci/mmol. The data are mean values of three independent experiments.

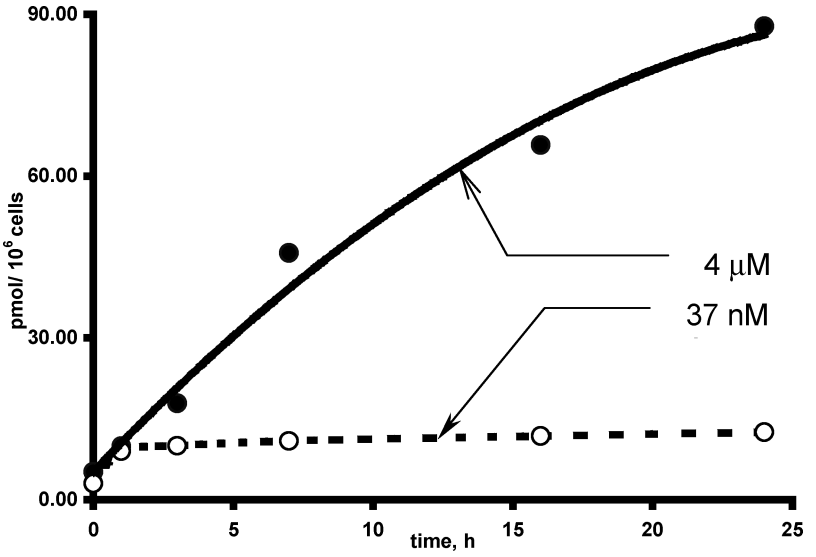


FIGURE 6 Dynamic curves of diester [^{32}P]-**1** at the concentrations of 37 nM and 4 μM. Specific activity of compound **1** was 4.2 Ci/mmol. The presented data are mean values of two independent experiments made in two parallels.

The cellular uptake was also studied at the 37 nM concentration of diester **1**, which is close to its IC_{90} value (18 nM[6]) (Figure 6). Unlike the curve corresponding to the concentration of 4 μM, the curve for the 37 nM concentration has a saturated area after 1–2 hours of incubation. Thus, a 100-fold increase in the intercellular concentration of nucleotide **1** entails only a 7-fold increase in its intracellular concentration.

Enzymatic Stability

Table 1 demonstrates the data on the chemical and enzymatic stability of phosphate **1**. The half-life ($T_{1/2}$) of nucleotide **1** in the phosphate buffer at 37°C in the pH range of 5–8 exceeded 24 hours, whereas in 90% normal human serum it amounted to 6.3 hours. The only hydrolysis product was AZT in both cases. Taking into account that the hydrolysis rate of nucleotide **2** was one order of magnitude higher under these experimental conditions if compared with diester **1** (data not shown), it is not surprising that monophosphate **2** was not found in the reaction mixture.

The behavior of the enzymes contained in the HL-60 cellular lyzate was studied as described in Skoblov et al.[2] After centrifugation at 14000 *g* in 100 mM PBS, the supernatant was found to hydrolyze compound **1** nearly 5 times slower than the suspension of the pellet after centrifugation. The product spectra were identical in both cases.

TABLE 1 Chemical and enzymatic stability of nucleotide **1**

	Product content[a] at $T_{1/2}$, %		
	AZT	(2)	Half-life
Phosphate buffer (pH 5–8)	90	10	>24 h
Snake venom preparation	50	50	1 h
Snake venom phosphodiesterase	3	97	5 min
Snake venom 5′-nucleotidase	98,5	1,5	24 h
Phospholipase C	99	1	18 h
Cellular membrane preparation	93	7	32 min
Water-soluble fraction of the cellular lyzate	99	1	2.5 h
Human blood serum	99	1	6.3 h

[a]The sum of AZT and (2) is taken as 100%.

We also attempted to determine the enzymes capable of degrading nucleotide **1**. The total enzymatic preparation of snake venom smoothly hydrolyzed compound **1** to give a mixture of choline, AZT and **2** with $T_{1/2}$ of about 60 minutes. By this moment AZT and monophosphate **2** were accumulated in the reaction mixture in a 1:1 ratio. Since monophosphate **2** was digested by snake venom nucleotidase, the hydrolysis products after the 4-hour incubation were found as a mixture of AZT, choline, and orthophosphate.

Snake venom phosphodiesterase very effectively hydrolyzed diester **1** to give choline and nucleotide **2** (with the half-life of 3–5 minutes).

Diester **1** was a poor substrate for 5′-nucleotidase. Its degradation proceeded 1000 times slower if compared with that of nucleotide **2** and only 2- to 3-fold exceeded the rate of chemical hydrolysis. This enzymatic activity was likely due to the residual activity of phosphodiesterase contained in the preparation as a 0.1% admixture.

Snake venom phospholipase C digested diester **1** nearly 50 times slower than it degraded phosphatidyl choline and gave choline phosphate **3** and AZT as the reaction products (data not given).

DISCUSSION

The enzymatic approach reported by Johnson and Walseth[11] is the most effective method for the preparation of nucleotides labeled with radioactive phosphorus isotopes and is successfully used for their production. However, the method has serious limitations due to enzyme specificity and cannot be employed for the preparation of modified nucleotides including AZT derivatives. The standard chemical synthesis of ^{32}P-labelled nucleotides consists of phosphorylation of the excess of the nucleoside with ^{32}P-orthophosphoric acid in the presence of a coupling agent. With AZT as an example, we recently compared the efficacy of coupling agents

commonly used in phosphorylation reactions, such as DCC, water-soluble carbodiimide, trichloroacetonitrile, and TPSCl with the efficacy of BrCN[8] known as the coupling agent in oligonucleotide ligation.[12] We showed that the use of BrCN was beneficial for the synthesis of ^{32}P-AZTMP **2** if compared not only with carbodiimides and trichloroacetonitrile, but also with TPSCl. The yield of phosphate **2** in the presence of BrCN was comparable with that in the presence of TPSCl, but the product isolation was much more difficult in the latter case. Taking into account this experience, we used BrCN for the synthesis of diester **2**.

Chemical phosphorylation of choline with ^{32}P-orthophosphoric acid was considerably less effective than that of AZT. The use of a multifold excess of choline was inapplicable because of low solubility of choline and its salts in organic solvents, particularly, at the reaction temperatures (0°C). Therefore, we synthesized diester **1** using Scheme 2.

The dynamics of the cellular uptake of compound **1** was studied in HL-60 cell culture, which was previously used by us for the metabolic studies of AZT 5′-H-phosphonate.[2] The curves presenting the penetration of diester **1** at the concentration of 4 μM differ from those for AZT (Figures 2 and 4): for diester **1**, the value of intracellular radioactivity was rising linearly with the time of the experiment, whereas for AZT, it was rapidly growing during the first 3 hours, and then the penetration rate evidently reduced (Figure 4). At any time point after 8 hours the intracellular level of ^{3}H-AZT was lower than that after the incubation with diester **1**. At the same time the interdependence of **1** and AZT on the penetration was insignificant (Figure 5). This effect may be accounted for by the existence of various routes of penetration for **1** and AZT and/or by competitive passing of other nucleotides, for example, thymidine, contained in the cultural medium at higher concentrations than diester **1**.

An absolutely different dynamic curve was obtained when the concentration of nucleotide **1** was decreased by approximately 100 times (37 nM). The saturation area was rapidly achieved, and the level of intracellular radioactivity was only 7 times lower, whereas the extracellular concentration declined by 100 times. This contrast to the dynamics of penetration of the parent AZT allows a suggestion that compound **1** may have some other channels to cross the cytoplasmic membrane. However, it should be noted that at the concentration of 37 nM the contribution of extracellular hydrolysis of diester **1** was higher and achieved 30% after 24 hours. In addition, the result also may be partially affected by nonspecific adsorption of radioactive metabolites on the cellular membrane.

The data of enzymatic hydrolysis showed that snake venom phosphodiesterase effectively hydrolyzed compound **1** to give choline and phosphate **2**. Like 5′-nucleotidase, spleen phosphodiesterase could not hydrolyze diester **1**, whereas phospholipase C digested it to give AZT and choline phosphate **3**. The comparison of these data with the dynamics and the composition of

hydrolysis products in HL-60 cells makes it possible to assume that diester **1** is likely to be affected by phosphodiesterase I and then by phosphatase (or 5′-nucleotidase) in blood serum, whereas in HL-60 cells it is primarily a target for phospholipase C bound to the cell membrane.

The analysis of the composition of phosphorus-32-labelled metabolic products in the HL-60 cellular lyzate showed that compound **1** was degraded to AZT, which was then phosphorylated to the corresponding phosphate derivatives. Thus, diester **1** is an AZT rather than AZT 5′-phosphate prodrug. A higher anti-HIV activity of compound **1** if compared with AZT may be due to a better cellular uptake rather than specific metabolic transformations. This statement correlates well with the earlier obtained data on the metabolism of AZT *H*-phosphonate.[2] The latter phosphonate also was rapidly hydrolyzed to AZT in HL-60 cells, but its less effective penetration into cells (one-third of that of AZT) was in the author's opinion the reason for a decreased antiviral activity.

EXPERIMENTAL

[^{32}P]-Orthophosphoric acid with a specific activity of 8000 Ci/mmol (296 PBq/mol) was obtained from the Institute of Reactor Materials (Zarechnyi, Russia); [6-^{3} H]-AZT was prepared as described in Sidorov et al.;[14] the solvents and reagents were dried directly before use. Cholinium iodide was from Acros; 98% H_3PO_4 was from Fluka, AZT was a kind gift of AZT Association (Russia). Snake venom (*Crotalus attrox*), snake venom phosphodiesterase (*Crotalus attrox*) and 5′-nucleotidase (*Crotalus attrox*) were from Fluka. Phospholipase C (*Clostridium perfringens*) was from Serva (Heidelberg, Germany).

HPLC was performed on a Gilson chromatograph (France) supplied with a UV detector type 315 and a flow-rate Beckman 170 radioactive detector. Reversed-phase HPLC was performed on a Nucleosil 100 C-18 (5 μm) column (4 × 150 mm) eluting with buffer A: 50 mM TEAB; buffer B: 75% ethanol; gradient: 0–5 minute, 0% B; 5–7 minute, 0–12% B; 7–20 minute, 12–20% B; 20–30 minute, 20–50% B; the flow rate was 0.5 ml/minute. Ion-exchange HPLC was performed on a Nucleosil-NH_2 column (4 × 50 mm) eluting with buffer C: water; buffer D: 500 mM NaH_2PO_4: 0–40 minute, 0–30% D; 40–50 minute, 100% D; 50–60 minute, 100–0% D; the flow rate was 0.4 ml/minute. TLC was performed on Kieselgel 60 F_{254} plates (Merck) developing in dioxane-aq. ammonia-*i*-propanol-water 3:3:3:1.

NMR spectra were registered on an AMX III-400 spectrometer (Bruker) with the working frequency of 400 MHz for ^{1}H and Me_4Si as an internal standard; 162 MHz for ^{31}P NMR (with phosphorus-proton interaction decoupling, 85% H_3PO_4 as an external standard); and 100 MHz for ^{13}C NMR. UV absorption spectra were registered on a Specord M-40

spectrophotometer in water. Radioactivity was measured on a LS-counter SL-4000 Intertechnique (France) by the Cherenkov method. The products were detected by autoradiography and analyzed on an InstantImager (Packard Instrument Company, USA). Quantitative TLC analysis of reaction mixtures was performed using the Imager.

3′-Azido-2′,3′-dideoxythymidine 5′-choline Phosphate (1)

Orthophosphoric acid (10 nmol, 10 μl of 1 mM solution in water) and absolute acetonitrile (40 μl) were added to a solution of 10 mCi [^{32}P]H_3PO_4 in 0.5 mM HCl, and the solvent was evaporated. The residue was dissolved in 5 M aqueous pyridine (20 μl), and cholinium iodide (1 μmol, 231 μg) was added. The reaction mixture was kept for 10 minutes at 0°C, a solution (6 μl) of 50 mM BrCN (0.3 μmol) in acetonitrile was added, and after 20 minutes at 0°C the solvent was evaporated. The residue was dissolved in 5 M aqueous pyridine (20 μl), and AZT (1 μmol, 267 μg) and a solution (6 μl) of 50 mM BrCN (0.3 μmol) in acetonitrile were added. After 10 minutes, 30% aq. ammonia (10 μl) was added, the solvent was evaporated, and the residue was coevaporated with water (50 μl). The residue was dissolved in 50 mM TEAB (50 μl) and applied on a reversed-phase HPLC column eluted in the system given above. The yield of the product was 18–20%, the radiochemical purity 99%, specific activity 1,000 Ci/mmol. R_f: 0.6; UV: λ_{max} 266.3 nm (8440) (pH 7.0, water), $\lambda_{max}/\lambda_{minute}$ 3.3; retention time: 18.5 minute. ^{1}H-NMR: 7.63d (J 1 Hz, 1H, H-6); 6.20t (J 6.5 Hz, 1H, H-1′); 4.43 dt (J 6.5 and 11 Hz, 1H, H-3′); 4.25 m (2 H, $\underline{CH_2}$ O choline); 4.12–4.04 m (3H, 2H-5′+H-4′); 3.61 m (2 H, $\underline{CH_2}$ N choline); 3.16 s (3H, Me_3 choline) 2.47 t (J 6.5 Hz, 2H, 2 H-2′); 1.86 s (3H, Me-Thy). ^{31}P-NMR: −0.16 s. ^{13} C-NMR: 166.8, 151.9 (C-2 and C-4); 137.7 (C-6); 111.9 (C-5); 85.4 (C-1′); 83.1 d (J 8.5, C-4′); 66.3 ts (J_{NC} 3.9, $\underline{CH_2}$ N choline); 65.4 d (J 5.2, C-5′); 60.5 (C-3′); 59.8 d (J 4.9, $\underline{CH_2}$O choline); 54.3 ts (J_{NC} 3.7, Me_3 choline); 36.5 (C-2′); 12.0 (Me-Thy).

Enzymatic Tests

Unlabelled diester **1** was applied onto a Dowex 50 (H^+) column, eluted with 10% ammonia, the solvent was removed in vacuum, and the residue was dissolved in water up to the concentration of 114 μM and used in the further experiments as a stock solution.

Hydrolysis with the cellular lyzate. The suspension of HL-60 cell culture ($12{\cdot}10^6$ cells) in 5 mM Tris-HCl (500 μl, pH 7.5) was subjected to four freezing-melting cycles using liquid nitrogen and centrifuged for 5 minute at 14,000 g. A solution of 55 mM $MgCl_2$ in 500 mM Tris-HCl (5 μl, pH 7.5) was added to the clear lysate (50 μl), the mixture was heated thermostatically at 37°C for 5 minute and 114 μM diester **1** (2 μl, 3.2 Ci/mmol) was

added. Aliquots (2.5 μl) were taken out, applied onto a TLC silica gel plate, eluted in the system given above, and analyzed using the Imager.

Hydrolysis with membrane preparations. The HL-60 cells (12×10^6) cultivated, washed and degraded as described in the section "Cellular experiments." The resulting suspension was centrifuged for 5 minute at 14,000 g, and the supernatant was removed. The membrane fraction was washed with PBS cooled to 5°C (2×5 ml), suspended in 5 mM Tris-HCl buffer (200 μl, pH 7.5), heated thermostatically at 37°C for 5 minutes, and diester **1** (114 μM 4 μl) was added. Aliquots (0.5 μl) were taken in certain intervals, chromatographed by TLC, and analyzed using the Imager.

Hydrolysis with human blood serum. Human blood serum (100 μl) was heated at 37°C for 5 minutes and 114 μM diester **1** with a specific activity of 2.9 Ci/mmol (4 μl) was added. Aliquots (0.5 μl) were taken out and added to methanol (10 μl). The suspension was centrifuged for 2 minutes at 14,000 g, supernatant was concentrated in vacuum to the volume of 4–5 μl, applied onto a TLC silica gel plate, eluted in the system given above, and analyzed using the Imager.

*Hydrolysis with total snake venom preparation (*Crotalus attrox*).* The preparation (0.25 mg) was dissolved in 50 mM Tris-HCl (pH 8.0, 200 μl) containing 10 mM $MgCl_2$. The solution was heated at 37°C for 5 minutes and a solution of 114 μM diester **1** (8 μl, 3.2 Ci/mmol) was added. Aliquots were taken, applied onto a TLC silica gel plate, eluted in the system given above, and analyzed using the Imager.

Hydrolysis with phosphodiesterase. The enzyme (0.5 U according to the di(p-nitrophenyl) phosphate test) was dissolved in 50 mM Tris-HCl (pH 8.0, 50 μl) containing 10 mM $MgCl_2$ and 0.11 M NaCl. The solution was heated at 37°C for 5 minutes and a solution of 114 μM diester **1** (2 μl, 2.8 Ci/mmol) was added. Aliquots were taken, applied onto a TLC silica gel plate, eluted in the system given above, and analyzed using the Imager.

Hydrolysis with 5′-nucleotidase. The enzyme (1 U according to the 5′-AMP-dephosphorylation test) was dissolved in 50 mM Tris-HCl (pH 8.0, 50 μl) containing 10 mM $MgCl_2$ and 0.11 M NaCl. The solution was heated at 37°C for 5 minutes and a solution of 114 μM diester **1** (2 μl, 3.2 Ci/mmol) was added. Aliquots (0.5 μl) were taken, applied onto a TLC silica gel plate, eluted in the system given above, and analyzed using the Imager.

Cellular Experiments

Promyelocytic leukemia cells HL-60 were cultivated in the RPMI–1640 medium containing 2 mM *L*-Gln and 10% calf serum at 37°C in the atmosphere with 5% CO_2 up to the density of 10^6 cells/ml. The radioactive **1** (4–5 Ci/mmol) was added and after 0, 1, 3, 7, and 24 hours two aliquots (3 ml) were taken out in parallel and centrifuged at 180 g for 4 minutes at 4°C. The cells were washed twice 5 ml with PBS cooled to 5°C and

suspended in 5 mM Tris-HCl buffer (200 μl, pH 7.5). The cells were degraded by the fourfold freezing-melting procedure in liquid nitrogen, methanol (400 μl) was added, and the resulting suspension was centrifuged (2 minutes at 14,000 g). The supernatant was concentrated in vacuum at 37°C. The residue was dissolved in TEAB (25 μl) and analyzed by HPLC.

The lipid composition of the membrane fraction was analyzed by silica gel TLC with unlabelled authentic samples of the corresponding lipid components as reference compounds. Phospholipids were detected by the Vaskovsky and Kostevsky method.[13]

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