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5'-Aminocarbonyl phosphonates as new AZT depot forms: antiviral properties, intracellular transformations and pharmacokinetic parameters

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RUNNING TITLE PAGE

a) AZT 5'-aminocarbonyl phosphonates as new AZT depot forms

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c) 30 text pages

8 tables

8 figures

16 references

247 words in the Abstract

359 words in the Introduction

1464 words in the Discussion

d) AIDS- Acquired ImmunoDeficiency Syndrome; AZT - 3'-azido-3'-deoxythymidine; AZT-MP - 3'-azido-3'-deoxythymidine 5'-monophosphate; HIV – human immunodeficiency virus; DNA - deoxyribonucleic acid; NMR - nuclear magnetic resonance; UV – ultraviolet; TEAB –triethyl ammonium bicarbonate; HPLC - high-pressure liquid chromatography; GSIOC - Gilson Serial Input/Output Channel; C_{max} - maximum plasma concentration; T_{max} - time to maximum plasma concentration; AUC - area under concentration-time curve from 0 to ∞; CL - total clearance; MRT - mean residence time; $T_{1/2}$ –elimination half-life; LD_{50} – mean lethal dose; CD_{50} – cytotoxic dose; ID_{50} – inhibitory dose; SI – selectivity index; SD - standard deviation; Thy - thymine; T - thymidine; DCC – dicyclohexyl carbodiimide; C_c - cumulation coefficient

ABSTRACT

The main disadvantages of 3'-azido-3'-deoxythymidine (Zidovudine®, AZT), the most common anti-HIV drug, are toxicity and a short half-life in the organism. The introduction of an H-phosphonate group into the AZT 5' position resulted in significant improvement of its therapeutical properties and allowed a new anti-HIV drug Nikavir®. In this work we described a new group of AZT derivatives, namely, AZT 5'-aminocarbonylphosphonates. The synthesized compounds displayed antiviral properties in cell cultures infected with HIV-1 and the capacity to release the active nucleoside in animals (rabbits and dogs) in a dose-dependent manner. The compounds were less toxic in MT-4 and HL-60 cell cultures and experimental animals when compared with Zidovudine. Major metabolites found in MT-4 cells after their incubation with AZT 5'-aminocarbonyl phosphonate **1** were AZT and AZT 5'-phosphate (25 and 55%, respectively). Among the tested compounds, phosphonate **1** was the most effective AZT donor and its longest $T_{1/2}$ and T_{max} values in the line phosphonate **1** - Nikavir - Zidovudine imply that compound **1** is an extended depot-form of AZT. Although bioavailability of AZT following oral administration of phosphonate **1** was lower than those of Nikavir and Zidovudine (8% against 14% and 49%), we expect that this reduction would not cause essential fall of antiviral activity but noticeably decrease toxicity due to gradual accumulation of AZT in blood and the absence of sharp difference between C_{max} and C_{min}. Such a combination of properties makes the compounds of this group promising for further studies as extended-release forms of AZT.

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INTRODUCTION

The progress towards the treatment of HIV infections has steadily increased in the past two decades. At the end of the 1980's the life expectancy of HIV-infected patients was only 2 to 5 years, whereas today many patients often survive between 10 to 15 years, however, once the infection progresses to full blown AIDS, the mortality rate is 100%. Currently, more than 20 drugs have been approved for treatment of HIV, these include inhibitors of crucial viral enzymes such as reverse transcriptase, protease, and integrase. Despite significant progress in the design of anti-HIV drugs, many problems remain such as toxicity and side effects, as well as rapid elimination from the body resulting in more frequent dosing. More significantly, the development of drug-resistant strains has increased dramatically. As a result, there is a critical need for more effective and less toxic therapeutics.

In that regard, HIV reverse transcriptase has proven to be an attractive target. HIV RT inhibitors are primarily modified nucleosides that are converted by way of an intracellular cascade of phosphorylations to the corresponding triphosphates. The triphosphates are then incorporated into the growing DNA chain, which results in termination of DNA synthesis. As the efficacy of the triphosphates is low, drug doses must therefore be high, which generally leads to significant toxicity. One solution is to design the corresponding depot form, i.e., a prodrug-like derivative capable of revealing the active compound in the organism at a controlled rate (Stańczak and Ferra, 2006). Many depot forms of anti-HIV drugs have been developed (Beaumont et al., 2003; Calogeropoulou et al., 2003) and some have resulted in new drugs (Kearney et al., 2004; Lyseng-Williamson et al., 2005; De Clercq and Field, 2006). In particular, the depot form of AZT, Nikavir® (AZT *H*-phosphonate, phosphazide, AZT 5'-hydrogenphosphonate), proved to be highly effective and has been approved in Russia for the prevention and treatment of AIDS (Kravchenko, 2004; Skoblov et al., 2004; Kukhanova and Shirokova, 2005).

In this work we describe preliminary biological data for several new AZT depot analogues (phosphonates **1-5,** Fig. 1), including their anti-HIV properties in cell systems, cellular uptake, intracellular transformations (for phosphonate **1** as an example) and pharmacokinetic and toxicological data.

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METHODS

Chemistry

All reagents and solvents were purchased from Acrus (Belgium).

The syntheses of compounds **1-3** and **5** have been described previously (Shirokova et al., 2004; Ias'ko et al., 2006). Compound **4** was obtained from AZT 5'-ethoxycarbonylphosphonate and *n*-butylamine. AZT was a gift from the "AZT Association" (Moscow, Russia).

NMR spectra were obtained on an AMX III-400 spectrometer (Bruker) with the working frequency of 400 MHz for ^{1}H NMR (Me_4Si as an internal standard for organic solvents and sodium 3-trimethylsilyl-1-propanesulfonate for D_2O), 162 MHz for ^{31}P NMR (with phosphorus-proton interaction decoupling, 85% H_3PO_4 as an external standard) and 100.6 MHz for ^{13}C NMR (with carbon-proton interaction decoupling). UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer (Japan) and were in line with known standard values for thymidine derivatives.

5'-(Butylamino)carbonylphosphonyl-3'-azido-3'-deoxythymidine, ammonium salt 4. ^{1}H-NMR (D_2O): 7.65 (1H, s, H6), 6.22 (1H, t, J 6.5, H1'), 4.44 (1H, m, H3'), 4.18 (2H, m, H5'), 4.11 (1H, m, H4'), 3.20 (2H, dd, J 6.9 и 7.2, CH_2N (butyl)), 2.45 (2H, m, H2'), 1.88 (3H, c, 5-Me), 1.44 (2H, m, $\underline{CH_2}CH_2$N (butyl)), 1.26 (2H, m, $\underline{CH_2}CH_3$ (butyl)), 0.83 (3H, dd, J 6.5 и 6.9, CH_3 (butyl)). ^{31}P-NMR (D_2O): -1.27 s. ^{13}C-NMR (D_2O): 186.8 (d, $^{1}J_{C,P}$ 212.1, C(O)P), 169.2 (s, C4), 154.4 (s, C2), 140.1 (s, C6), 114.4 (s, C5), 87.7 (s, C1'), 85.6 (d, $^{3}J_{C,P}$ 7.2, C4'), 67.7 (d, $^{2}J_{C,P}$ 4.8, C5'), 63.0 (s, C3'), 41.4 (d, $^{3}J_{C,P}$ 6.4, $\underline{C}H_2$NHC(O)P), 39.1 (s, C2'), 33.3 (s, $\underline{C}H_2CH_2CH_3$), 22.2 (s, $CH_2\underline{C}H_2CH_3$), 15.7 (s, $CH_2CH_2\underline{C}H_3$), 14.4 (s, 5-Me).

Synthesis of 5'-aminocarbonylphosphonyl-3'-azido-3'-deoxy-[6-^{3}H]thymidine ([6-^{3}H]-1). Trimethylsilyl bromide (5 mmol, 650 μL) was added to triethylphosphonoformate **(**1 mmol, 190 μL) and the mixture stirred for 20 h at 22°C under argon. Anhydrous toluene (1 mL) was added, the solvent was evaporated in vacuum (1 mm Hg, 30°C) and a solution of $SOCl_2$ (10 mmol, 730 μL) in anhydrous CCl_4 (1 mL) was added to the residue. The mixture was refluxed for 2 h under argon; the solvent evaporated under vacuum and coevaporated with anhydrous benzene (2 mL). The residue was then dissolved in anhydrous benzene (1 mL) and a 10 μL (a.c. 10 μmol) aliquot of the solution was treated with [6-^{3}H]AZT (3 mCi, 14 Ci/mmol) and AZT (0.5 μmol, 133.5 μg). The mixture was stirred for 2 h at 22°C, evaporated under vacuum and 1M TEAB (1 mL) added to the residue. The solution was then stirred for 30 min at 45°C, evaporated under vacuum, and 32% NH_3/H_2O (100 μL) added to the residue. The mixture was stirred for 18 h at 0°C, the solvents removed under vacuum and the residue dissolved in H_2O (200 μL). The product was isolated by HPLC on a Lichrosorb RP-18 column (5μm, 4×150 mm) using a Gilson

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chromatograph (France) supplied with a digital GSIOC 506 controller (Gilson) and a Gilson-315 UV detector (254 nm). Solution A: 50 mM TEAB; solution B: 75% methanol. Gradient of B: 0 min at 0%, 10 min at 25%, 25 min at 25%, and 30 min at 100%. Retention times: 14.5 min for **1**, 20 min for AZT and 28 min for AZT 5'-ethoxycarbonyl phosphonate. The yield of product **[6-^{3}H]-1** was 2.6 mCi (87%), specific activity 4.2 Ci/mmol. The structure was confirmed using a nonradioactive reference standard by reverse-phase (for the conditions, see above) and ion-exchange (SynChropak AX300 column, 4.6 × 250 mm; solution A: water; solution B: 0.2 M KCl; retention time of **1** was 6.5 min) chromatography.

Antiviral activity of the synthesized compounds was studied in MT-4 cell culture infected with HIV-1, strain GKV-4046, using previously published procedures (Shirokova et al., 2004).

Cytotoxicity of the synthesized compounds was studied in MT-4 cell culture, using previously published procedures (Shirokova et al., 2004).

Cellular uptake

A suspension of MT-4 cells (0.7- 1 x 10^6 cells/mL) in the RPMI–1640 medium (50 mL) containing 2 mM *L*-Gln and 10% calf serum was incubated with 4 μM [6-^{3}H]AZT (200 μCi) or 4 μM **[6-^{3}H]-1** (300 μCi) at 37°C in the atmosphere containing 5% CO_2.

Incubation of the tested compounds with HL-60 cells was carried out as described previously (Yanvarev et al. 2007).

After 1, 3, 7 and 24 h incubation, two aliquots (2.5 mL) were removed and centrifuged at 1500 *g* for 10 min. The supernatants were used for HPLC analysis of the extracellular hydrolysis of phosphonate **1**. The precipitates were resuspended in PBS and washed three times (3 x 3 mL) with PBS. The final pellets were resuspended in water (200 μL), subjected to cryolysis, and methanol (400 μL) was added. The samples were kept at -18°C. Prior to HPLC analysis the samples were centrifuged at 1500 *g* for 10 min, the supernatant was evaporated under vacuum, the residue was dissolved in water (100 μL), and the standards (thymine, thymidine, AZT-MP, phosphonate **1** and AZT 5'-H-phosphonate, 5 μg each) were added.

The HPLC analysis was performed on a Nucleosil 100 C-18 column (5 μm, 4 x 150 mm) with UV detection at 254 nm using a Gilson chromatograph (see above); the flow rate 0.5 mL/min. System A: 50 mM ammonium bicarbonate; system B: 75% aqueous ethanol. Gradient: 0 min at 0%B, 10 min at 10%B, 50 min at 20%B, 55 min at 100%B. Retention times: 37 min for AZT; 25 min for **1**, 22 min for AZT-MP, 19 min for thymidine,12 min for thymine. The aliquots were taken out every 0.5 min (0.25 mL), scintillation liquid (Beckman ready value, 5 mL) was added, and radioactivity was measured using an LS-counter SL-4000 Intertechnique (France).

Based on the value of specific activity, the concentration of the radioactive compounds was calculated.

Chemical stability

Chemical stabilities of compounds **1-5** at the concentrations of 0.5 mM were studied in 0.1 M sodium phosphate buffer at 37°C and pH in the range of 5.5 - 8.5. The aliquots (10 μL) were taken out after certain intervals, frozen in liquid nitrogen, and the products analyzed on a liquid Gynkotek chromatograph (Germany) supplied with a UV detector (Model 320), at a wavelength of 265 nm. Chromatographic conditions: an Ultrasphere ODS - IP column, 5μm, 4.6 x 150 mm; mobile phase: acetonitrile-0,1% phosphoric acid containing 0,2% of triethylamine, pH 2.7-2.9. The ratios of the mobile phase components were individual for each compound (Table 1).

Stability in whole blood

Water solutions of the tested compounds (10-20 μL, 1 μg/μL) were placed into tubes with Na-heparin, fresh dog whole blood (1 mL) was added into each tube and the tubes were kept at 37°C. The aliquots were taken out every 1 h during 6 h and centrifuged for 10 min at 1500 *g* to separate plasma. Methanol (0.5 mL) was added to the supernatant (0.25 mL) and the mixture was shaken for 30 s and again centrifuged for 10 min at 1500 *g*. The supernatant was evaporated at 40°C. The dry residue was dissolved in water and analyzed as described in section "Chemical stability"

Animal experiments were carried out according to the protocols of Council Directive **86/609/EEC** of November 24, 1986, on the protection of animals used for experimental purposes.

Pharmacokinetic parameters were calculated using the Thermo Kinetika 4.4.1 program (Thermo electron corporation, USA). Pharmacokinetics following oral administration were studied by the extravascular noncompartmental model of the Thermo Kinetica program. For intravenous administration, the noncompartmental IV Infusion model was used.

Pharmacokinetics in dogs

The studies were performed following standard protocols (Kurlyandskii, 2000), with outbred dogs (males and females, 13.6 ± 2.6 kg body weight). The tested compounds were administered orally in the fasting state; the animals were fed 3 h after dosing (50 or 20 mg/kg body weight). Intravenous administration (1-5 mL of aqueous solution, 5 mg/kg) was performed in the antebrahial vein of anterior foot (*v. cephalica antebrachii*) for 2-5 min. Ten blood samples (not less than 3 mL) were taken out from the femoral vein 0-24 h after administration and placed into tubes containing heparin (5 μL, 5000 U/mL) and centrifuged for 10 min at 1500 *g*. The

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plasma samples were kept at –24°C. The samples were treated as described in section “Stability in blood” and analyzed under conditions given in section “Chemical stability”.

Bioavailability (F) in dogs was calculated by the following formula:

$F = (AUC_{p.os.} \times D_{i.v.})/(AUC_{i.v.} \times D_{p.os.}) \times 100\%$

where $D_{i.v.}$ is an intravenous dose in dogs;

$D_{p.os.}$ is an oral dose in dogs;

$AUC_{i.v.}$ is the area under the time-concentration curve for the intravenous dose

$AUC_{p.os.}$ is the area under the time-concentration curve for the oral dose

Pharmacokinetics in rabbits

Chinchilla rabbits (males, 3 ± 0.5 kg body weight) were kept in separated cages with free access to water and food and 12 h lighting. The animals did not receive food 14 h before the experiment and were fed 6 h after dosing of the tested compounds. In the case of single intragastric administration the animals were anaesthetized with a 10:1 ether-halotane mixture and a polyurethane gastrointestinal tube was introduced 15 cm deep. The tested compounds were administered as water solutions (12 mL) at concentrations of 7 or 70 mg/kg body weight.

In the case of intravenous administration (10 mg/kg body weight), the compounds were injected into the *Auricularis marginalis* vein in physiological solution (1 mL) for 1 min; the injection time was 1 min. At each day of the experiment, a single heparin dose (0.5 mL of water solution, 5000 U/mL) was injected to each rabbit prior to taking blood samples. The blood samples (1mL on average) were taken from the *Auricularis marginalis* vein into microtubes containing 5 μL of heparin (5000 U/mL). The tubes were shaken and aliquots of 0.5 mL were taken out and mixed with methanol (1 mL), rapidly cooled in liquid nitrogen and stored at -24°C. The control blood samples were taken before administration of the tested compounds.

The water-methanol mixture was treated as described in section "Cellular uptake". The samples were analyzed by HPLC on a Gilson chromatograph (see section “Cellular uptake”) on a Nucleosil 100 C-18 column (4 x 150 mm). The chromatographic conditions were as follows: solution A, 50 mM triethylammonium acetate; solution B, 75% methanol; gradient of B: 0 min at 0%B; 12 min at 10%B; 50 min at 20%B; 55 min at 100%B. The flow rate was 0.5 mL/min. Retention times: 32 min for AZT *H*-phosphonate; 37 min for compound **1**; 51 min for AZT.

Acute toxicity

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LD_{16}, LD_{50} and LD_{84} values were obtained according to the standard procedure (Khabriev, 2005). BALB/c mice (males and females, 19 ± 1 g body weight) were administered single intraperitoneal doses of the tested compounds dissolved in a sterile isotonic solution of sodium chloride. The control animals received the corresponding volume of the isotonic solution of sodium chloride. The animals were examined for 14 days.

The BALB/c mice were received from the laboratory animal hatchery of the Scientific Center of Biomedical Technologies of Russian Academy of Medical Sciences. The animals were kept in T-3 cages at artificial illumination (light and darkness, 12 h of each), mandatory 12-fold per hour ventilation, at 20-22°C and 50-65% relative humidity, on mats from wood chippings sterilized in an oven. The animals had a free access to water and food.

Blood samples of mice died after dosing of compound **1** at a dose of 6 g/kg were immediately treated and analyzed by HPLC as described in section "Pharmacokinetics in rabbits".

Cumulative effect

The experiments on the cumulative effect of phosphonate **1** were carried out on BALB/c mice (males, 17 ± 1 g body weight) using a standard scheme (Table 2) (Khabriev, 2005). The effect was estimated based on the cumulating coefficient C_c, which was calculated as follows:

$C_c = LD_{50n} / LD_{50\,1}$, where LD_{50n} is a mean lethal dose following n-fold administration, $LD_{50\,1}$ is a mean lethal dose following single administration (see section “Acute toxicity”).

$C_c > 1$, habituation; $C_c < 1$, cumulation.

Statistical Analysis

Statistical significance of comparisons between treatment groups was assessed by a two-tailed Student's t test. The values are expressed as mean ± SD (standard deviation). Each pharmacokinetic experiment was repeated at least thrice. Pharmacokinetic parameters were determined using the Thermo Kinetika 4.4.1 ("Thermo Electron Corporation") program. The lower limits of detection of AZT and amide **1** were 10 nmol for unlabelled compounds (UV detection, ε_{267} 9700) and 10 pmol for labeled compounds (radioactive detection, a specific activity of 4.2 Ci/mmol).

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RESULTS

Antiviral properties of the synthesized phosphonates were studied in MT-4 cells infected with HIV-1. The results given in Table 3 indicate that the compounds inhibited virus replication similarly to AZT *H*-phosphonate and by one order of magnitude lower than parent AZT. At the same time their toxicity (except phosphonate **2**) was considerably lower than those of AZT *H*-phosphonate and parent AZT. Compound **2** was excluded from further studies because of comparatively high toxicity. A higher CD_{50} of the phosphonates under study allowed better selectivity indexes SI (Table 3).

Cellular uptake was studied with radiolabelled phosphonate **1** (**[6-^{3}H]-1**) in two human lymphoid cell lines MT-4 and HL-60. As is seen in Fig.2, the efficacy of penetration of phosphonate **1** into HL-60 cells was tenfold higher than that for MT-4 cells at the same extracellular concentration of 4 μM.

Earlier we showed that penetration of phosphonate **1** into HL-60 cells was about tenfold less effective than that of AZT (Yanvarev et al., 2007). In a similar experiment with MT-4 cells the AZT penetration rate dropped only by 25-30%, if compared with its penetration into HL-60 cells, whereas in the case of phosphonate **1**, the reduction was considerably higher (about one order of magnitude). Thus the difference between penetration rates of phosphonate **1** and AZT into MT-4 cells exceeded 100 times.

The analysis of metabolic products in MT-4 cells after their incubation with phosphonate **1** showed the presence of AZT (25±10%), AZT-MP (55±10%) and starting **1** (12±4%) (Fig. 3). In addition, thymine (8±5%) was found. No other metabolites were detected in the mixture. Compositions of intracellular metabolites were similar for MT-4 and HL-60; variations did not exceed 10%. It is noteworthy that a degree of extracellular hydrolysis was only 5% after 24 h incubation.

Stability

All the synthesized compounds were chemically stable with half-lives in buffer solutions (pH 5.5-8.5) considerably greater than 48 h. They also demonstrated high stability in 100% human blood serum at 37^{o}C ($T_{1/2}$ >> 6 h) (data not given). None of the tested phosphonates were hydrolyzed in dog whole blood ($T_{1/2}$ >> 6 h) (data not given).

Pharmacokinetic parameters

Some pharmacokinetic parameters were evaluated in dogs following oral administration of 480-560 mg of phosphonates **1, 3, 4,** and **5** (50 mg/kg). In the case of phosphonate **1**, the analysis of dog blood serum demonstrated the presence of both AZT and **1** (Fig. 4). The maximum concentration of released AZT was observed 4 h after dosing. The concentration of phosphonate **1** was approaching zero at this time point.

For compounds **3-5,** the only metabolite detected in plasma was AZT. The peak AZT concentration (0.9 ±0.1 mg/L serum) was achieved approximately 3 h after dosing of phosphonates **4** and **5**. For compound **3** it was more than twofold higher but an hour earlier. The AZT plasma concentration curves following single oral doses of phosphonates **3-5** (50 mg/kg body weight) in dogs are shown in Fig. 5.

Pharmacokinetic parameters of phosphonate **1** and AZT released from compounds **1, 3-5** are given in Table 4. As is seen in the table, aminocarbonylphosphonate **1** is the most effective AZT donor among the tested phosphonates: AUC for AZT was nearly three times as high as that of compounds **3-5.**

At a dose of phosphonate **1** decreased to 20 mg/kg, AZT was detected as a single product (Fig. 6a), unlike at the dose of 50 mg/kg, when starting **1** was also found in blood (Fig. 4).

Pharmacokinetic parameters of AZT after administration of phosphonate **1**, AZT *H*-phosphonate or AZT itself were compared following oral doses of the above mentioned agents equivalent to administration of 20 mg AZT per kg of dog weight (Fig. 6a,b and Table 5). As observed in Table 5, the peak plasma AZT concentration for phosphonate **1** was about 2.5 and 13 times lower than that of AZT *H*-phosphonate and AZT, respectively. However, the time for peak concentration was twice as long as that of AZT and one hour longer than that of AZT *H*-phosphonate. Thus, AZT accumulation in blood and its elimination proceeded slower for phosphonate **1**.

When phosphonate **1** was injected intravenously (5 mg/kg), its plasma concentration in dogs gradually reduced and in 20 min was by half lower. In 2 h its quantity in plasma was negligible (Fig. 7). It is noteworthy that unlike oral dosing, we failed to detect AZT following intravenous administration and the injected phosphonate **1** was the only product within the total experimental time (up to 24 h).

Bioavailability of compound **1** was calculated using the data from Figs. 6a and 7 and Tables 5 and 6. Pharmacokinetic parameters of reference AZT *H*-phosphonate and AZT itself were obtained using the same protocols (data not given). Total bioavailability of compound **1** in dogs was 3.5% and that of AZT generated by **1** was 8%. Oral bioavailability of AZT from phosphonate **1** was considerably lower in respect to AZT following oral administration of AZT (49%), but it was close to that of AZT from AZT *H*-phosphonate (14%).

Plasma concentration curves of phosphonate **1** and generated AZT after an intragastric dose of 70 mg/kg in rabbits are shown in Fig. 8. Following this route of administration,

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phosphonate **1** circulated in blood flow for more than 24 h gradually releasing AZT. The peak AZT concentration was observed 2 h after dosing.

Similar experiments with a lower dose of tested **1** (7 mg/kg rabbit weight) were performed using a tritium-labeled analogue **[6-^{3}H]-1**. Tenfold reduction of the dose was shown to affect neither the curve shapes nor the AZT:**1** ratio. Both of the products were detected in blood up to 48 h time (data not shown). Peak concentrations dropped linearly as it was also shown for dogs; time to peak concentrations only insignificantly changed.

As in dogs, AZT was not found in plasma following intravenous administration in rabbits at a dose of 10 mg/kg. Phosphonate **1** was the only product and could be detected as long as 1h after dosing.

Acute toxicity

Toxicity was studied in BALB/C mice following a single intraperitoneal dose of compound **1** in the range of 4000 - 8000 mg/kg. The data given in Table 8 show that phosphonate **1** was at least twice less toxic than AZT *H*-phosphonate and about four times less toxic than AZT. Toxic effects were exhibited as hypodynamia turning into adynamia; death came after 3-10 h.

The mouse blood analyzed after intraperitoneal administration of phosphonate **1** at a dose of LD_{84} (6000 mg/kg weight) contained both intact **1** (3.6 mmol/L, 1.35 g/L) and AZT (0.126 mmol/L, 0.047 g/L).

Cumulative properties of phosphonate 1

Disorders of motor activity were registered in mice at the doses of phosphonate **1** close to LD_{50}. They were observed 2-3 h after administration, however, all motor activity returned completely. During the course of the experimental period the tested compound was well tolerated with negligible intoxication symptoms. After 28 days the total dose of compound **1** was 12.8 LD_{50} for 8 animals from the experimental group of 10. Of ten animals, one mouse died on day 19 (the total dose was 4.34 LD_{50}, C_c 4.34), another mouse died on day 26 (the total dose was 9.46 LD_{50}, C_c 9.46), and for the remaining mice, C_c was 12.8.

DISCUSSION

Antiviral experiments in HIV-infected MT-4 cells demonstrated that the compounds inhibited the viral replication by one order of magnitude less effectively than parent AZT. At the same time their toxicity, with the exception of compound **2**, was also considerably lower than that of AZT. The same trend was observed for Nikavir®, the anti-HIV drug approved in the Russian Federation for the treatment of AIDS, which also contains a phosphonate fragment. Therefore we proposed that, similarly to Nikavir, the phosphonates from this investigation can generate AZT, i.e., are depot forms of AZT, but as a result of a lower content of AZT released at each time point they are less toxic.

Stability is a very important characteristic for depot forms, as it determines the rate of releasing the active core. In that regard, all of the synthesized phosphonates proved to be stable in biological fluids (human blood serum and dog's whole blood).

The ability of the phosphonates to penetrate into cells was also studied with labeled **[6-^{3}H]-1**. Earlier it was synthesized from [6-^{3}H]AZT and aminocarbonylphosphonic acid in the presence of DCC (Yanvarev et al., 2007). In this work [6-^{3}H]AZT was coupled with etoxycarbonylphosphonic dichloride and the resulting 5'-ethoxycarbonyl-3'-azido-3'-deoxy-[6-^{3}H]-thymidine was treated with 32% ammonium solution. The total yield of the radioactive product was 75-90%. If compared with the earlier described method, the current procedure provided a substantial increase in the phosphonylation rate (by more than 10 times) and a fourfold increase in product yield.

The efficacy of penetration of aminocarbonylphosphonate **1** into cells and its intracellular transformations were studied in two human lymphoid cell lines MT-4 and HL-60. It is noteworthy that the difference in the efficacy of the [6-^{3}H]-**1** uptake by these cells achieved about one order of magnitude. A degree of intracellular hydrolysis of compound **1** was close to 90% for both cell cultures and the composition and proportions of intracellular metabolites were very similar. In both cases, the major metabolite was AZT-MP (nearly 55% of the total product contents), which may result from phosphorylation of the AZT formed in the process of intracellular hydrolysis of aminocarbonylphosphonate **1.** The hydrolysis may occur in either a one-step or a multistep mode, with an intermediate formation of AZT *H*-phosphonate, since it was earlier found that breakdown of phosphonate **1** in the buffer at pH 2 and 37°C yielded a 3:2 mixture of AZT and AZT *H*-phosphonate (Yanvarev et al., 2007). Of other metabolites detected, AZT dominated (about 25% of the total product contents). In addition, the starting phosphonate **1** and thymine

were found. Thus, the intracellular hydrolysis of phosphonate **1** and subsequent phosphorylation of the formed AZT proceeded effectively in the tested cell cultures.

The efficacy of cellular uptake of aminocarbonylphosphonate **1** was 10-100-fold lower than that of AZT and approximately 6 times as low as that of AZT *H*-phosphonate (Skoblov et al., 2004). Its antiviral activity in MT-4 cell culture was about 16 and 4.5 times lower if compared with AZT and AZT *H*-phosphonate, respectively. Its toxicity dropped even to a greater extent (34- and 15-fold, respectively). This leads us to believe that reduction in anti-HIV activity and toxicity of phosphonates of this type in MT-4 cells, if compared with these indices for AZT and AZT *H*-phosphonate, can be related to a decreased efficacy of penetration into these cells, i.e, there is direct dependence between the uptake efficacy and antiviral properties for aminocarbonylphosphonate **1**, AZT and AZT *H*-phosphonate.

Based on the analysis of the results obtained we performed pharmacokinetic studies in experimental animals of phosphonates **1** and **3-5**. Methylaminocarbonylphosphonate **2** was excluded from further studies because of relatively high toxicity in MT-4 cells. Pharmacokinetics in dogs following oral administration of the tested phosphonates showed that all the compounds could generate AZT. The curves "plasma concentration-time" were similar in all the cases; Cmax values of the AZT released from phosphonates **3, 4-5** and **1** were 2.0, 0.8-0.9 and 3.7 mg/kg, respectively.

As concluded from these experiments, phosphonate **1** was the most effective AZT donor and was therefore the focus of more comprehensive studies. Further analysis of pharmacokinetic data demonstrated that aminocarbonylphosphonate **1**, following oral administration in dogs, was an effective AZT depot form at doses of both 50 and 20 mg/kg weight and the amount of AZT generated by aminocarbonylphosphonate **1** was dose-dependent. At the same time, following intravenous administration, only **1** was detected in dog blood. Taking into account the high stability of compound **1** in whole blood, we proposed that hydrolysis of aminocarbonylphosphonate **1** could occur in the course of absorption from the gastrointestinal tract, perhaps in epithelial cells.

Comparison of phosphonate **1** with AZT and AZT *H*-phosphonate following oral administration in dogs showed that the peak plasma AZT concentration was lower, whereas AZT accumulation and elimination times were longer in the case of compound **1**. Pharmacokinetic parameters of the latter were rather close to those of AZT *H*-phosphonate (being 2.5 times inferior to C_{max} and less than two times to AUC and surpassing in T_{max} and other parameters).

Bioavailability of AZT following oral administration of **1** was 8%, which is only twice less than that of AZT *H*-phosphonate, which is an effective anti-HIV drug with considerably lower toxicity and a longer time of appearance of viral resistant strains if compared with AZT (Skoblov

et al., 2004; Kukhanova and Shirokova; 2005, Machada, 1999). Bioavailability of AZT following oral administration of AZT itself was six times as high as that of AZT from **1**. However, in the case of AZT, a high AUC value is reached due to a very large maximum plasma concentration and short elimination half-life. A very high plasma concentration of AZT, which drops very fast, is associated with toxicity and the rapid appearance of drug resistant viral strains. We anticipate that the lower bioavailability of phosphonate **1** would not cause essential reduction of antiviral activity but rather noticeably decrease the toxicity due to a gradual accumulation of AZT in blood and the absence of a sharp difference between C_{max} and C_{min}, unlike AZT (Cato III et al., 1998).

It is noteworthy that the $T_{1/2}$ and T_{max} values of AZT in dogs increase in the order AZT < AZT *H*-phosphonate < phosphonate **1** (see Table 6)**,** which implies that compound **1** can be considered an extended release depot form of AZT.

Pharmacokinetic experiments in rabbits with aminocarbonylphosphonate **1**, following intragastric administration of its aqueous solution, also supported its efficacy as an AZT depot form, although the plasma ratio of starting **1** and AZT differed from that in dog plasma after oral dosing. This difference may be related to metabolic variations between dogs and rabbits and/or different routes of administrations. Following intravenous administration in rabbits as well as in dogs, AZT was not found and phosphonate **1** was the only product detected. This corroborates the hypothesis that AZT formation occurs in the process of transition from gastrointestinal tract into blood.

The results of determination of acute toxicity in mice supported the assumption that the gradual accumulation in blood of AZT released from aminocarbonylphosphonate **1** and the corresponding slower elimination, as compared with AZT itself and AZT *H*-phosphonate, may result in decreased toxicity. Indeed, LD_{50} of compound **1** was approximately 5 g/kg mouse weight versus 1.5 and 2.3 g/kg mouse weight for AZT and AZT *H*-phosphonate, respectively. Thus, phosphonate **1** proved to be considerably less toxic in mice and offered an obvious advantage over approved drugs Zidovudine and Nikavir. It is also noteworthy that following intraperitoneal administration of phosphonate **1** at a dose of 6 g/kg mouse weight, 3.5% AZT was found in addition to the starting compound. To summarize, aminocarbonylphosphonate **1** generates AZT following various routes of administration (oral, intragastric or intraperitoneal) in different experimental animals (mice, rabbits and dogs) within a wide range of doses (7 to 6000 mg/kg weight).

Cumulating experiments were based on the analysis of animal deaths following repeated dose administration. This method also allows for evaluation of drug addiction. The calculated cumulating coefficients C_c were > 1 in all the cases, which implies the absence of cumulating

properties of the tested **1**, i.e., the animal accommodates the drug and does not develop toxic reactions.

In conclusion, we have shown that AZT 5'-aminocarbonylphosphonate analogues, particularly, aminocarbonylphosphonate **1,** can be regarded as depot forms of Zidovudine® (AZT) widely used in the treatment of HIV infections. These analogues slowly release AZT following oral administration and penetrate efficiently into cells. Moderate efficacy of cellular uptake is not a serious drawback for these depot forms because they can still effectively generate AZT in a variety of animals. These compounds exhibit low toxicity in experimental animals and lack cumulative effects. Although this is only the first step of the studies, the data obtained are encouraging as the lead phosphonate **1** was noticeably less toxic and had a longer half-life in organisms than parental AZT.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Structural formulas of the synthesized phosphonates

Fig. 2. Cellular uptake of phosphonate **[6-^{3}H]-1** (overall with its metabolites).

Fig. 3. HPLC identification of intracellular radioactive metabolites of **[6-^{3}H]-1** with detection by radioactivity (upper chromatogram) and of reference standards [thymine (Thy), thymidine (T), AZT 5'-phosphate (AZT-MP) and **1**] with UV detection.

Fig. 4. The time-concentration dependences of phosphonate **1** and AZT at a single oral dose of phosphonate **1** (50 mg/kg body weight) in dogs (n=6).

Fig. 5. The time-concentration dependence of AZT following single oral doses of phosphonates **3-5** (50 mg/kg body weight) in dogs (n=3).

Fig 6. The AZT time-concentration dependence following a single oral dose of phosphonate **1 (A),** AZT *H*-phosphonate and AZT itself **(B)** at doses equivalent to 20 mg AZT/kg body weight in dogs (n=6).

Fig. 7. Time-concentration curve of phosphonate **1** following intravenous administration in dogs (n=6) at a dose of 5 mg/kg body weight.

Fig. 8. **A.** The time-concentration curve of phosphonate **1** and released AZT following intragastric administration of compound **1** in rabbits (n=9) at a dose of 70 mg/kg body weight. **B.** A large-scale fragment of the curve.

TABLES

Table 1. Compositions of mobile phases and retention times in the HPLC analysis of stability experiments

Tested compound	Acetonitrile, %	0,1% phosphoric acid + 0,2% triethylamine in water, %	Retention time, min	
			Tested compound	AZT
1	7	93	4.4	14.6
3	25	75	8.1	1.8
4	15	85	5.8	3.5
5	20	80	4.1	2.2

Table 2. A scheme of cumulation experiments in toxicological studies in mice

Days of dosing	Animal number	LD_{50} portion
1-4	10	0.1 LD_{50} - 536.5 mg/kg
5-8	10	0.15 LD_{50} - 804.8 mg/kg
9-12	10	0.22 LD_{50} - 1180.3 mg/kg
13-16	10	0.34 LD_{50} - 1824.1 mg/kg
17-20	10-9	0.5 LD_{50} - 2682.5 mg/kg
21-24	9	0.75 LD_{50} - 4023.8 mg/kg
25-28	9-8	1.12 LD_{50} - 6008.8 mg/kg

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Table 3. Anti-HIV properties of phosphonates **1-5** in MT-4 cells infected with HIV-1 (strain GKV-4046)

Compound	CD_{50}[a], μM	ID_{50}[b], μM	SI[c]
1	2700	0.60	4500
2	260	0.23	1130
3	1500	0.11	13600
4	1970	0.48	4100
5	1080	0.20	5400
AZT	80	0.037	2200
AZT *H*-phosphonate	180	0.131	1400

[a]compound concentration required to reduce cell viability by 50%

[b]compound concentration required to inhibit HIV replication by 50%

[c]$SI = CD_{50}/ID_{50}$

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Table 4. Pharmacokinetic parameters of phosphonate **1** and AZT generated by phosphonates **1, 3-5** following single oral administration in dogs (n=6 for compound **1**, n=3 for compounds **3-5**) at a dose of 50 mg/kg body weight.

Compound	C_{max}, mg/L	T_{max}, h	AUC, mg·h/L	$T_{½}$, h	MRT, h	CL, L/h
1	0.633±0.033	1.5	1.35±0.06	3.06±0.14	1.87±0.21	333.91±34.13
AZT from **1**	3.700±0.526	4.0	21.02±0.3	9.79±0.32	13.10±0.2	21.41±0.36
AZT from **3**	2.032±0.091	2.0	6.71±0.29	19.10±0.44	18.61±0.4	74.50±0.89
AZT from **4**	0.872±0.045	3.0	6.40±0.44	6.28±0.37	9.36±0.20	78.12±0.47
AZT from **5**	0.816±0.013	3.0	6.03±0.06	11.63±0.48	12.33±0.2	82.90±1.87

***P<0.001

C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC, area under concentration-time curve from 0 to ∞; CL, total clearance; MRT, mean residence time; $T_{1/2}$, elimination half-life.

Table 5. Pharmacokinetic parameters of AZT following a single oral dose of phosphonate **1,** AZT *H*-phosphonate or AZT itself at doses equivalent to 20 mg AZT/kg in dogs (n=6).

Administrated compound	Pharmacokinetic parameters of AZT					
	$C_{max,}$ mg/L	$T_{max,}$ h	AUC, mg·h/L	$T_{½}$, h	MRT, h	CL, L/h
1	0.74±0.03	5.0	9.2±0.2	9.6±0.2	13.9±0.2	27.0±2.6
AZT *H*-phosphonate	1.89±0.07	4.0	16.6±0.3	7.2±0.3	10.4±0.5	15.0±0.7
AZT	9.77±0.30	2.5	58.8±1.1	5.2±0.5	7.5±0.4	4.2±0.3

***P<0.001

For designations, see Table 4.

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Table 6. Pharmacokinetic parameters of phosphonate **1** following intravenous administration in dogs (n=6) at a dose of 5 mg/kg body weight**.**

Comp	$C_{max,}$ mg/L	$T_{max,}$ h	AUC, mg·h/L	$T_{½}$, h	MRT, h	CL, L/h
1	9.129±0.23	0.08	4.46±0.17	0.46±0.05	0.63±0.08	11.21±0.40

***P<0.001

For designations, see Table 4.

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Table 7. Pharmacokinetic parameters of phosphonate **1** and AZT following intragastric administration of **1** in rabbits (n=9) at a dose of 70 mg/kg body weight.

Compound	$C_{max,}$ mg/L	$T_{max,}$ h	AUC, mg·h/L	$T_{½}$, h	MRT, h	CL, L/h
1	2.96±0.09	0.5	26.92±2.44	25.6±3.6	34.56±4.03	9.3±1.5
AZT	0.95±0.01	2.0	4.80±0.37	5.2±0.4	8.21±1.1	52.1±3.7

***P<0.001

For designations, see Table 4.

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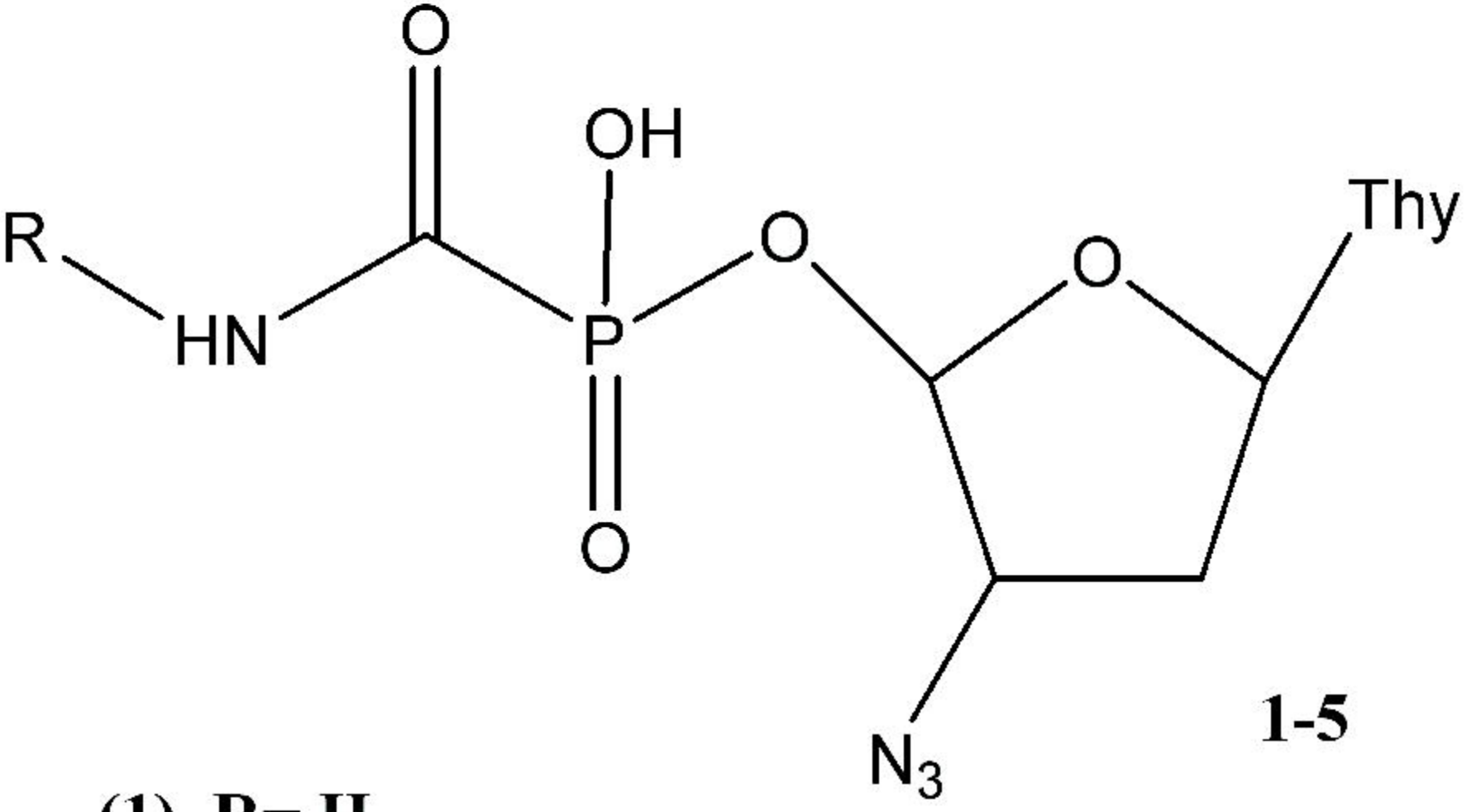
Table 8. Single dose toxicity in BALB/C mice (n=10) of compound **1**.

Compound	LD_{16}[a]	$LD_{50}\pm m$[a]	LD_{84}[a]
1	5090	5365 ± 200	6000
AZT	1300	1500 ± 70	1700
AZT *H*-phosphonate	2000	2300 ± 100	2500

**P<0.01

[a]Lethal doses causing the death of 16% (LD_{16}), 50% (LD_{50}), or 84% (LD_{84}) mice

Figure 1



1-5

(1), R= H

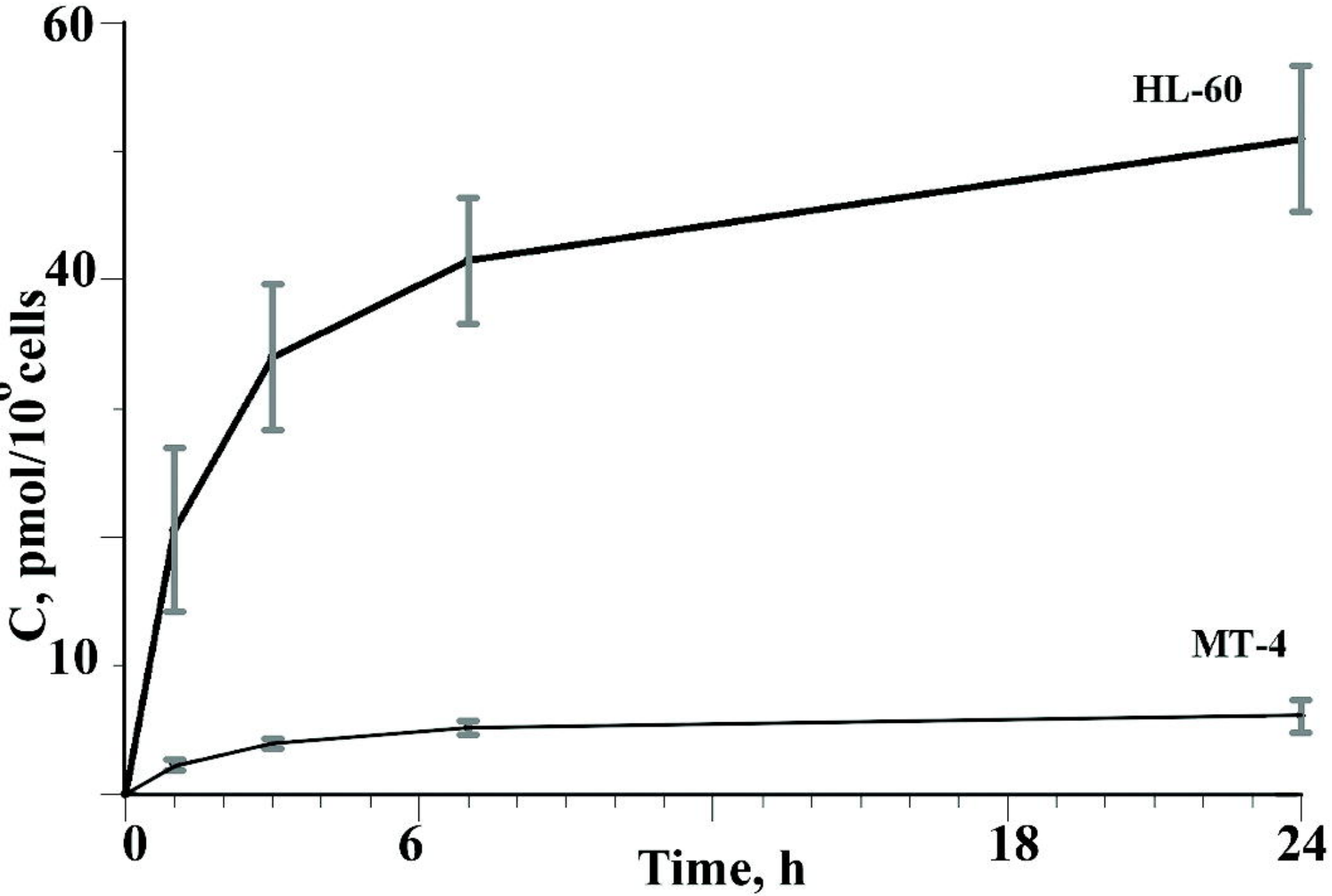
(2), R= CH_3

(3), R= $CH_3(CH_2)_5$

(4), R= $CH_3(CH_2)_3$

(5), R=$Ph(CH_2)_2$

Figure 2



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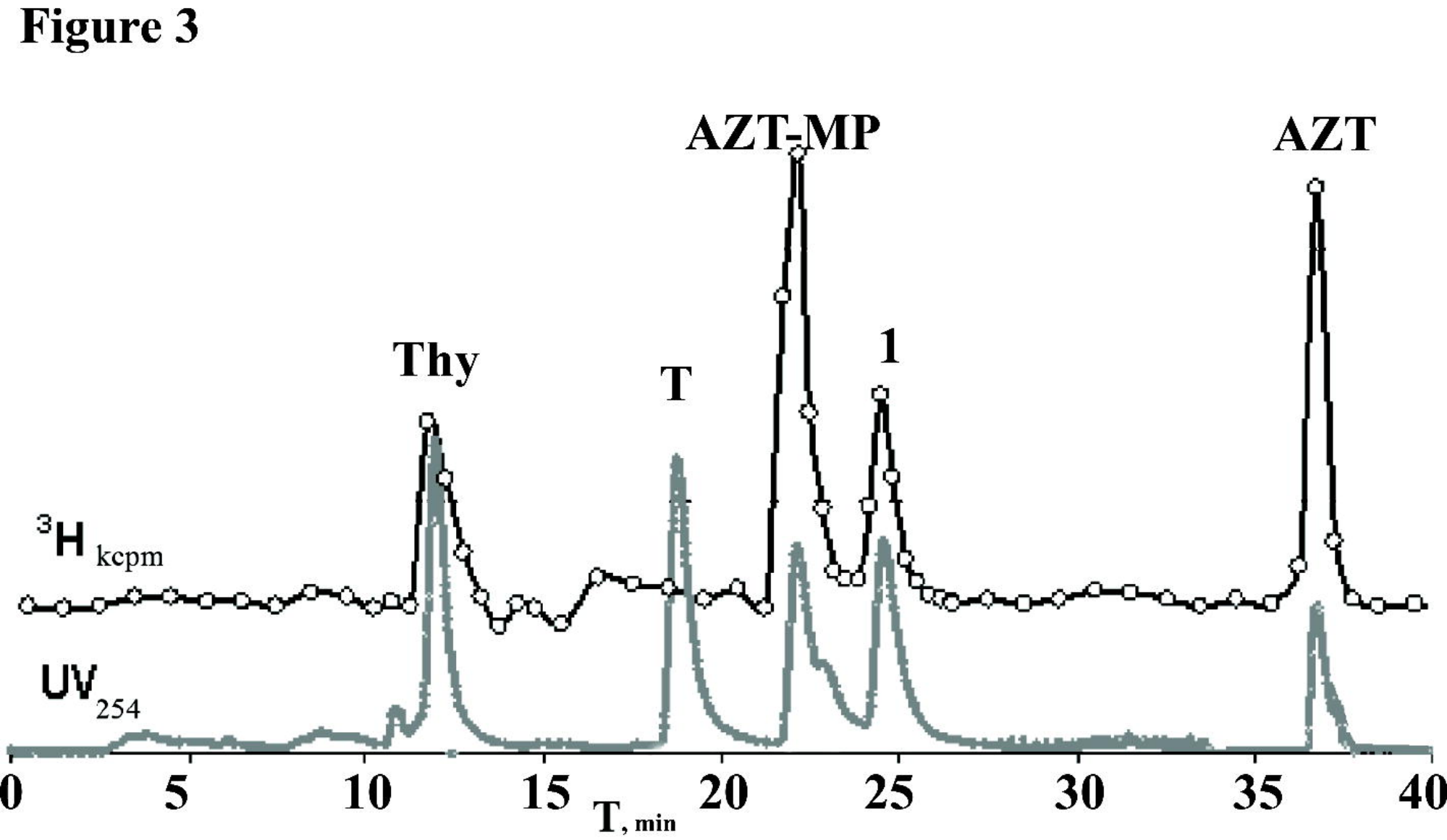


Figure 4

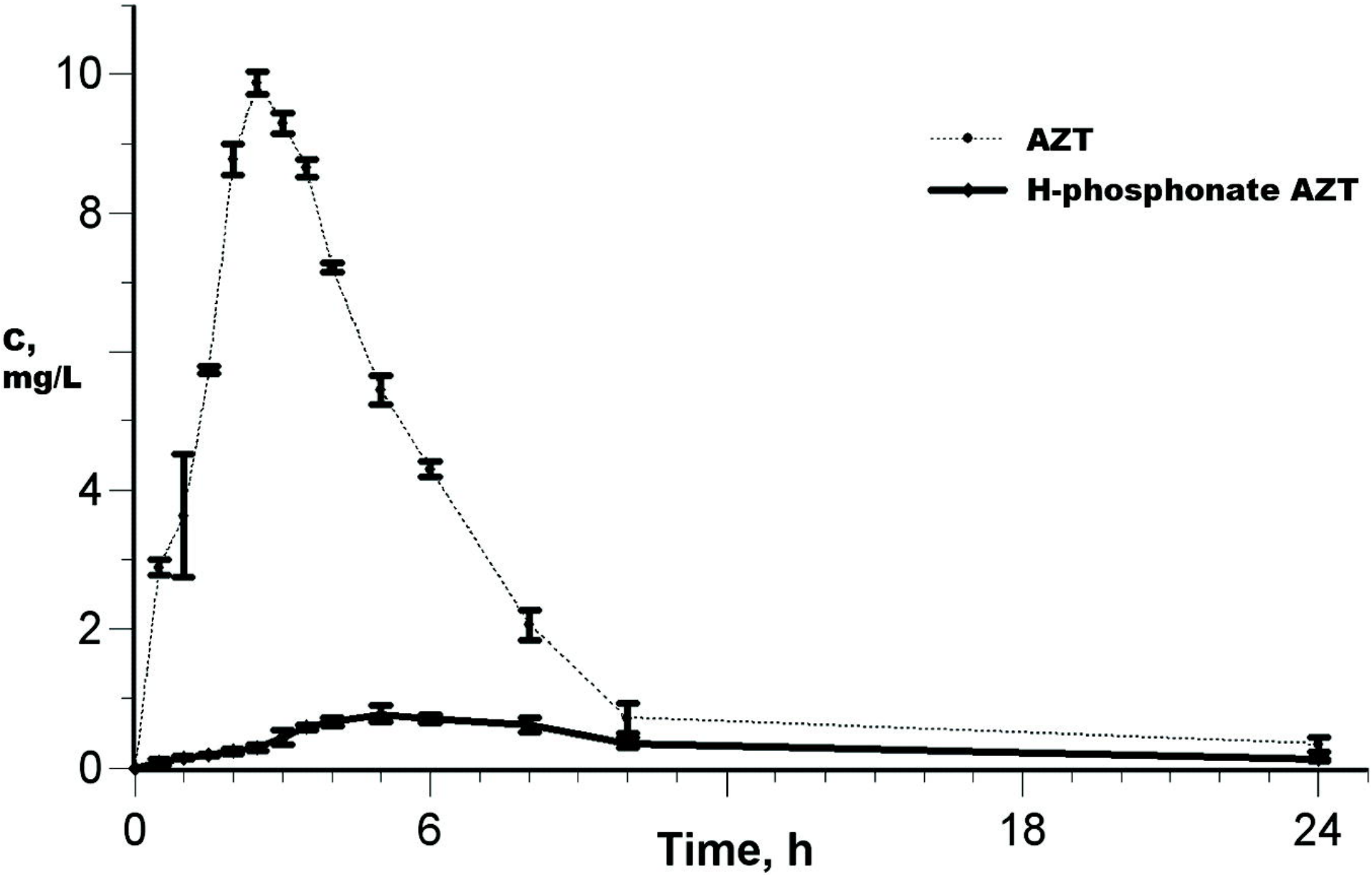


Figure 5

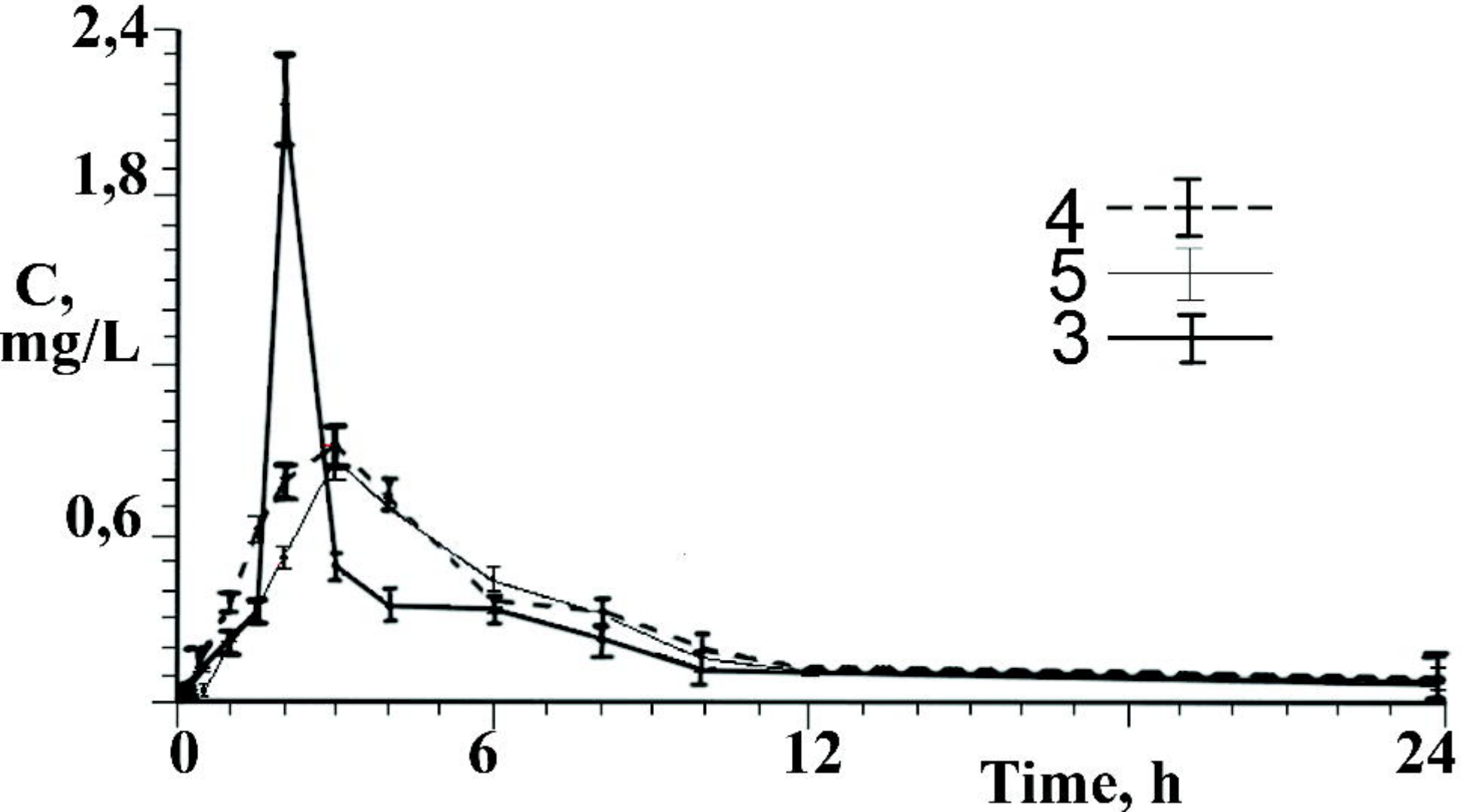
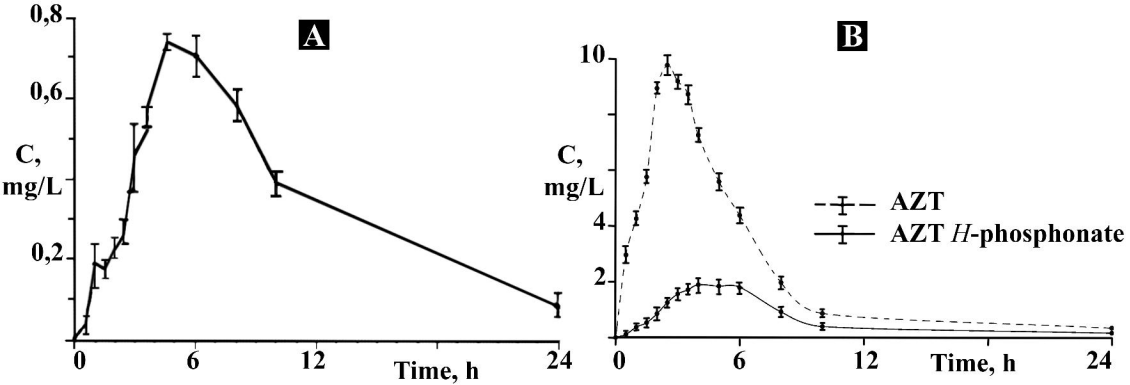


Figure 6


A
0,8
0,6
0,2
C,
mg/L
0
6
12
24
Time, h
B
10
4
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C,
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AZT
AZT H-phosphonate
0
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12
24
Time, h

Figure 7

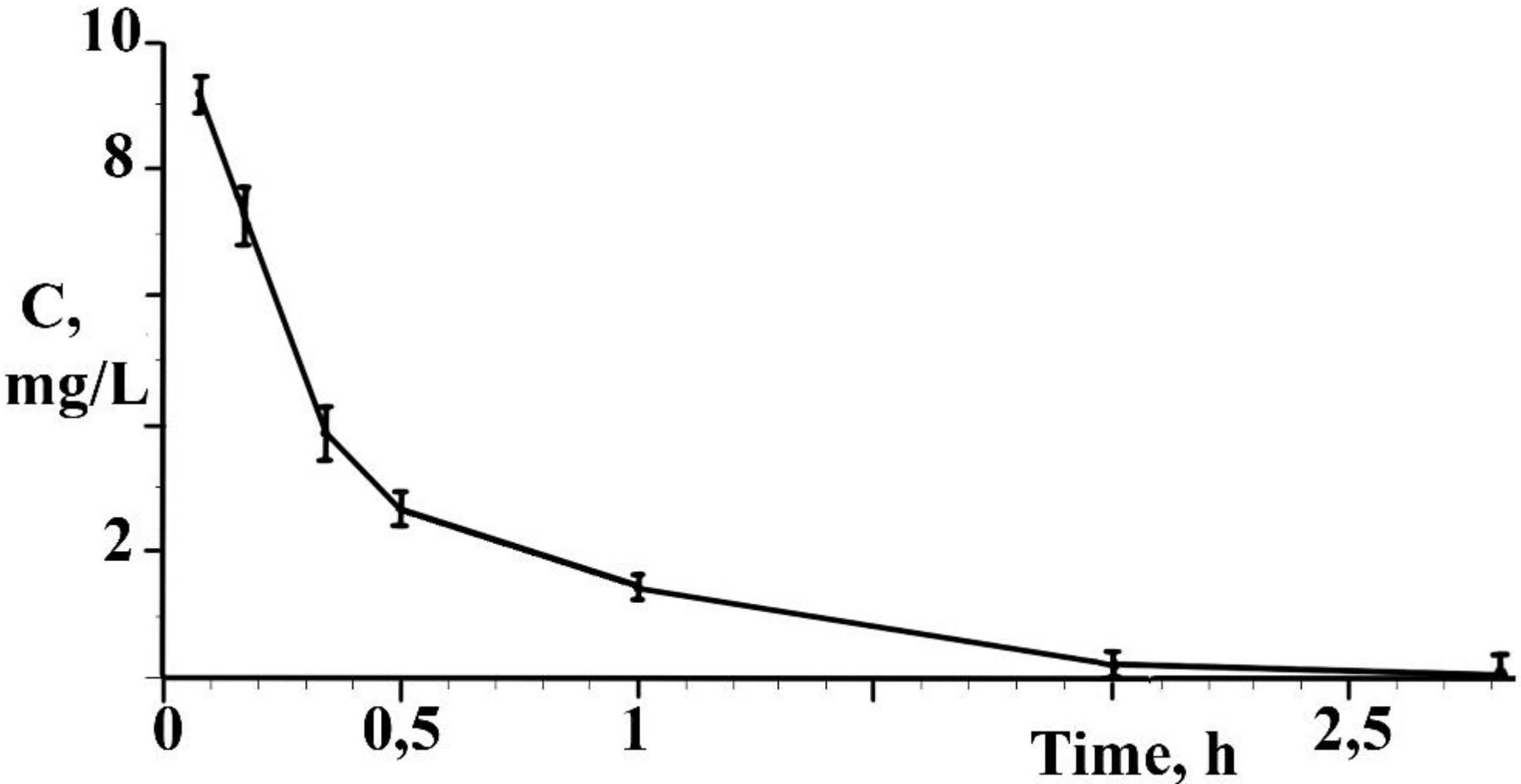

Figure 8