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α,β- and β,γ-METHYLENE 5′-PHOSPHONATE DERIVATIVES OF 3′-AZIDO-2′,3′-DIDEOXYTHYMIDINE-5′-TRIPHOSPHATE

CORRELATION BETWEEN AFFINITY FOR REVERSE TRANSCRIPTASE, SUSCEPTIBILITY TO HYDROLYSIS BY PHOSPHODIESTERASES AND ANTI-RETROVIRUS ACTIVITY

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Abstract—A series of 5′-phosphorylated derivatives of 3′-azido-2′,3′-dideoxythymidine (AzddThd), including AzddThd 5′-mono- and 5′-triphosphate, α,β-methylene AzddThd-5′-diphosphate, α,β-methylene AzddThd-5′-trisphosphate, and β,γ-methylene AzddThd-5′-triphosphate, were evaluated for their cytostatic and anti-retrovirus properties, and their inhibitory effects on the reverse transcriptases of Moloney murine leukemia virus and human immunodeficiency virus. In contrast with the 5′-mono- and 5′-triphosphates of AzddThd, which showed cytostatic and anti-retrovirus activities comparable to those of AzddThd, the α,β-methylene 5′-phosphonates of AzddThd were considerably less cytostatic and also much less inhibitory to cell transformation by Moloney murine sarcoma virus and cytopathogenicity of human immunodeficiency virus. The decreased biological activity of the phosphonate derivatives of AzddThd is most likely due to the resistance of these compounds to phosphorolytic attack by phosphodiesterases and phosphatases, and the reduced affinity for the retrovirus-associated reverse transcriptase.

3′-Azido-2′,3′-dideoxythymidine (AZT, AzddThd) is a thymidine (dThd) analogue in which the 3′-hydroxyl group of the 2′-deoxyriboside moiety is replaced by an azido group (Fig. 1). This compound was first synthesized by Horwitz *et al.* in 1964 [1]. Ten years later, Ostertag *et al.* [2] found AzddThd to inhibit C-type murine retrovirus replication *in vitro*. De Clercq *et al.* in 1980 reported on the antiviral and cytostatic properties of AzddThd, demonstrating that this compound was not markedly active against a series of DNA and RNA viruses [3]. However, in this study, no retroviruses were included. In 1985, Mitsuya *et al.* demonstrated that AzddThd blocks the expression of the p24 *gag* protein of human immunodeficiency virus (HIV) by more than 90% at 1 μM, and completely inhibits the cytopathic effect of HIV for ATH8 cells at a concentration of 1–5 μM [4]. This concentration is about 10- to 20-fold lower than the cytotoxic concentration of AzddThd. Furthermore, AzddThd efficiently blocks HIV replication in normal peripheral blood mononuclear cells at a concentration of 0.5 μM, as monitored by reverse transcriptase activity in the supernatant of these cell cultures [4].

The conversion of AzddThd to its phosphorylated metabolites is similar in uninfected and HIV-infected H9 cells, indicating that no specific viral enzyme is involved in the activation or metabolism of the drug [5]. AzddThd is phosphorylated by cellular dThd kinase to AzddTMP. Its K_m for this enzyme nearly equals that of the physiological substrate dThd ($K_m \sim 3$ μM), and the K_i/K_m ratio of AzddThd for dThd kinase (as measured with radiolabelled dThd as the substrate) is 0.6 [5, 6]. These data suggest that AzddThd is an excellent substrate for dThd kinase. When evaluated for its substrate properties for thymidylate (dTMP) kinase (dTMP-K), AzddTMP has a K_m of 8 μM, that is 2-fold higher than the K_m of the physiological substrate dTMP. Yet, the V_{max} of AzddTMP for dTMP-K is only 0.3% of the V_{max} of dTMP for dTMP-K [5]. Thus, AzddTMP has to be considered as a potent alternative-substrate inhibitor of dTMP kinase. Although the substrate properties of AzddThd-5′-diphosphate (AzddTDP) for nucleoside 5′-diphosphate kinase (NDP-K) have not yet been determined or reported, dTMP kinase may, in all likelihood, be regarded as the rate-limiting step in the conversion of AzddThd to its active metabolite AzddTTP [5, 6]. Once converted to AzddTTP, AzddThd could in principle interfere with all cellular and viral DNA polymerases. However, AzddTTP has a very high affinity for the HIV reverse transcriptase: $K_i = 0.002$–0.04 μM [5, 7], and this may to a large extent explain the selectivity and potency of AzddThd as an antiretroviral agent.

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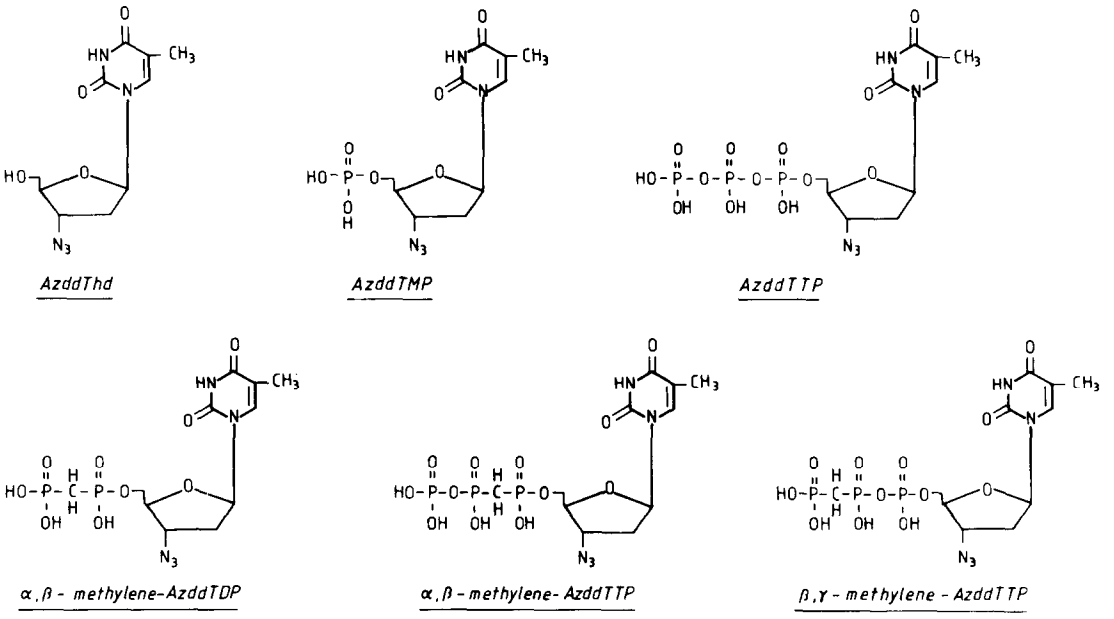

Fig. 1. Structural formulae of 3'-azido-2',3'-dideoxythymidine (AzddThd) analogues.

A marked decrease in the intracellular concentration of 2'-deoxythymidine 5'-triphosphate (dTTP) in H9 cells incubated in the presence of AzddThd has been observed [5]. Most likely, this decrease in dTTP levels is due to an inhibition of dTMP-K by AzddTMP. Since the entire flow from dTMP to dTTP has to go through this enzyme, it is obvious that a block at this level may result in a decrease of dTDP and dTTP pools. Consequently, starvation of dTTP following AzddThd treatment may lead to a shut off of DNA synthesis, a phenomenon that has been termed thymineless cell death [8–10]. Thus, the decreased dTTP pools may contribute to the cytostatic effects of AzddThd. In an attempt to avoid the inhibitory effects of AzddThd at the dTMP-K level, and the ensuing dTTP starvation and cytotoxicity, we now synthesized α,β-methylene AzddTDP, α,β-methylene AzddTTP and β,γ-methylene AzddTTP (Fig. 1). These methylene groups are highly resistant to enzymatic attack [11]. Since the "methylene phosphonate" derivatives are less polar than the corresponding phosphate derivatives, they may be taken up by the cells more efficiently than their phosphate counterparts. Assuming that the "methylene phosphonate" AzddThd derivatives enter the cells in an intact form, they do not need phosphorylation by dTMP-K, nor should they be able to interact with the dTMP-K, and this may make them less or not cytotoxic. We found that α,β- and β,γ-methylene AzddTTP and α,β-methylene AzddTDP were not only considerably less cytostatic for a series of murine and human cell lines, but they also proved less potent as anti-retrovirus agents. Although no formal proof was provided that these phosphonate derivatives of AzddThd actually penetrated the cells in an intact form, we showed that α,β-and β,γ-methylene AzddTTP lost their potency to inhibit the retrovirus-associated reverse transcriptase, and this may account for their poor anti-retrovirus activity.

MATERIALS AND METHODS

Compounds. AzddThd was synthesized according to Horwitz *et al.* [1]. The synthesis of AzddXyloT is described by Herdewijn *et al.* [12].

AzddTMP was synthesized from AzddThd and $POCl_3$ [13] (4 hr, 0°) and purified by chromatography on a XAD-2 column (1, H_2O; 2, H_2O–MeOH 75:25) (yield: 55%).

AzddTTP was prepared from the monophosphate as described by Hoard and Ott [14] (yield: 60%).

α,β-Methylene AzddTDP was prepared from AzddThd and methylene diphosphonic acid in the presence of DCC [15] followed by an acetic anhydride treatment in dry pyridine [16]. The product was purified on a DEAE cellulose column ($Et_3NH_2CO_3$) followed by an XAD-2 column and precipitated as sodium salt in acetone (without MeOH) (yield: 25%).

β,γ-Methylene AzddTTP was synthesized from AzddTMP (1 mmol) and methylene diphosphonic acid (monotributylammonium salt) (5 mmol) in the presence of carbonyl diimidazole (5 mmol) in anhydrous DMF. The product was purified on DEAE cellulose ($Et_3NH_2CO_3$) and precipitated as sodium salt in acetone (yield: 26%).

α,β-Methylene AzddTTP was synthesized by condensation of α,β-methylene AzddTDP with di(tributylammonium)phosphate in the presence of carbonyl diimidazole, purified by DEAE cellulose column and precipitated as sodium salt.

The products were controlled by ^1H NMR, UV, IR and by gel electrophoresis (acrylamide) with dTTP and dTMP as references. α,β-Methylene AzddTTP showed a purity of only 70%.

The other reagents used were of the highest quality available.

Cells. The origin and growth characteristics of the murine (L1210/0 and L1210/BdUrd) and human (Molt/4F, Raji/O, Raji/TK$^-$ and ATH8) cell lines used in this study have been described previously [4, 17, 18]. The human H9, MT4 and CEM cells were cultivated as reported for Molt/4F cells [18]. Both L1210/BdUrd and Raji/TK$^-$ represent dThd kinase-deficient cell lines [17].

Viruses. Human immunodeficiency virus (HIV) was obtained from the culture supernatant of a H9 cell line persistently infected with HTLV-III$_B$ [19].

Moloney murine sarcoma virus (MSV) was prepared from tumors induced by *in vivo* infection of 10-day-old NMRI mice according to a procedure described previously [20].

Cytostatic assays. The anti-proliferative effects of the compounds were measured according to previously published procedures [18, 21]. Briefly, cells were suspended in growth medium (containing 10% fetal calf serum) and seeded into microplate wells at a density of 5–7.5 × 10^4 cells/well in the presence of varying concentrations of the test compounds. After an incubation period of 48–72 hr at 37°, cells were counted in a Coulter counter. The ID_{50} was defined as the concentration of compound that reduced the number of viable cells by 50%.

Anti-retrovirus assays. Murine embryo fibroblast C3H cells were suspended in growth medium (containing 10% fetal calf serum) and seeded into 2.3 cm^2 wells of Tissue Culture Cluster plates at 5 × 10^4 cells/ml/well and grown to confluency. Cell cultures were then infected by 150 foci-forming units of Moloney murine sarcoma virus (MSV) during 90 min, whereafter the medium was replaced by 1 ml fresh culture medium containing different concentrations of the test compounds. After 6 days, transformation of the cell cultures was examined microscopically. The 50% effective dose (ED_{50}) was defined as the concentration of compound that inhibited cell transformation by 50%.

The procedure to measure anti-HIV activity in ATH8 cells has been described previously [4]. After 7 days incubation of HIV- or mock-infected ATH8 cells in growth medium (containing 15% fetal calf serum) with appropriate concentrations of the test compounds, the number of viable cells was determined in a blood cell counting chamber after trypan blue staining. The ED_{50} was defined as the concentration of compound that increased the cell number to 50% of the control cell number (based on viable cell counting).

Reverse transcriptase assays. Reverse transcriptase assays were carried out with partially purified Moloney murine leukemia virus (MLV) (Electronucleonics, Bethesda, MD) and HIV prepared from HIV-infected H9 cell cultures. Inhibition of reverse transcriptase by the particular compounds was measured within the time scale in which the reverse transcriptase activity occurred linearly (60 min for MSV- and 30 min for HIV-reverse transcriptase).

In the MLV reverse transcriptase assays, the endogenous viral RNA served as template. The reaction mixture (250 μl) contained 40 mM Tris–HCl (pH 7.8), 48 mM NaCl, 2 mM $MnCl_2$, 1.6 mM dithiothreitol, 0.012% (v/v) Triton X-100, 0.64 mM each of dATP, dCTP, and dGTP, 1 μM [*methyl*-^{3}H]dTTP (specific radioactivity 30 Ci/mmol) (40 μCi), 20 μl of virus stock suspension, and varying concentrations of the compounds. The reaction mixtures were incubated at 37° for 0, 30 or 60 min, at which time 50 μl aliquots were withdrawn and added to 1.0 ml calf thymus DNA (12 μg/ml) at 0°. Then, 1 ml cold TCA 16% was added to the tubes, the solutions kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity.

In the HIV reverse transcriptase assays, exogenous polyrA:oligodT_{12-18} served as template. The reaction mixture (50 μl) contained 50 mM Tris–HCl (pH 7.8), 5 mM dithiothreitol, 300 μM glutathione, 500 μM EGTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, 1 μM of [*methyl*-^{3}H]dTTP (specific radioactivity 30 Ci/mmol) (5 μCi), 0.01 unit of polyrA:oligo dT_{12-18}, 0.03% Triton X-100, 10 μl compound solution (containing varying concentrations of the compounds), and 10 μl of the reverse transcriptase preparation (partially purified by low centrifugation of the supernatant of a H9/HTLV-III$_B$ cell suspension, followed by filtration (0.45 μ) and ultracentrifugation (100,000 *g*, 2 hr)). The reaction mixtures were incubated at 37° for 0 and 30 min at which time 100 μl calf thymus DNA (150 μg/ml), 2 ml $Na_4P_2O_7$ (0.1 M in 1 N HCl) and 2 ml TCA (10% v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity.

Treatment of AzddThd derivatives with phosphodiesterase I and II, and inactivated foetal calf serum. Phosphodiesterase I and II were obtained from Merck (Darmstadt, F.R.G.). Fetal calf serum (FCS) was from Boehringer Mannheim (Mannheim, F.R.G.). For the phosphodiesterase I experiments, 5 μl enzyme (0.027 U/ml) was added to 20 μl compound solution (10 mM) and 25 μl 50 mM potassium phosphate buffer pH 6.5. After 0, 4 and 24 hr of incubation at 37°, 10 μl was spotted on Silicagel 60 F-254 and developed in a mixture of *n*-butanol : glacial acetic acid:water (2.5:1:1). For the phosphodiesterase II experiments, 5 μl enzyme (2 U/ml) was added to 20 μl compound solution (10 mM) and 25 μl 100 mM Tris–HCl pH 9.0. After 0, 4 and 24 hr of incubation at 37°, 10 μl was spotted on Silicagel 60 F-254 and developed as described above. For the FCS experiments, 25 μl compound (10 mM) was added to 25 μl 100% or 20% FCS and incubated for 0, 4 and 24 hr at 37°. The reaction was stopped by adding 50 μl TCA 20%, and the mixture was centrifuged for 2 min at 12,000 *g*. Then, 100 μl Freon : Tri-*n*-octylamine (4 : 1) was added, shaken for 20 min and centrifuged for 2 min at 12,000 *g*. Finally, 8 μl of the supernatant was spotted on Silicagel 60 F-254 and developed as indicated above.

RESULTS

Cytostatic effects of AzddThd derivatives

A series of AzddThd derivatives, including the *threo* derivative of AzddThd (in which the 3'-azido group is in the "up" position) (AzddXyloT), AzddThd 5'-mono- and triphosphate, and the

Table 1. Inhibitory effects of AzddThd derivatives against murine and human tumor cell proliferation

Compound	50%-Inhibitory dose (μM)							
	L1210/0	L1210/BdUrd	Raji/0	Raji/TK$^-$	Molt/4F	MT4	CEM	H9
AzddThd	525 ± 166	>1000	57 ± 28	>1000	114 ± 5.6	60 ± 13	400 ± 10	814 ± 274
AzddXyloT	>500	>500	>500	>500	>500	>500	>500	>500
AzddTMP	542 ± 22	>1000	83 ± 33	>1000	148 ± 6.3	65 ± 13	621 ± 219	>1000
AzddTTP	>1000	>1000	125 ± 44	>1000	190 ± 8.6	78 ± 5.7	>1000	>1000
α,β-Methylene AzddTDP	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
α,β-Methylene AzddTTP	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
β,γ-Methylene AzddTTP	>1000	>1000	114 ± 45	>1000	192 ± 14	74 ± 10	>1000	>1000

phosphonate analogues α,β-methylene AzddTDP, α,β-methylene AzddTTP and β,γ-methylene AzddTTP (Fig. 1) were evaluated for their inhibitory effects on the proliferation of murine and human tumor cell lines (Table 1).

The cytostatic effects of AzddThd ranged from 57 μM (for Raji/0) to 814 μM (for H9), depending on the cell line examined; AzddThd was totally devoid of any antiproliferative action against the dThd kinase-deficient mutant tumor cell lines L1210/BdUrd and Raji/TK$^-$. The *threo* derivative of AzddThd did not show any cytostatic effect at 500 μM, irrespective of the cell line evaluated. The 5′-monophosphate and 5′-trisphosphate derivatives of AzddThd proved slightly less inhibitory to cell proliferation than the parent compound AzddThd (Table 1).

In contrast, the α,β-methylene AzddTDP and α,β-methylene AzddTTP were totally devoid of any cytostatic activity at a concentration as high as 1000 μM. On the other hand, the antiproliferative action of β,γ-methylene AzddTTP closely resembles that of AzddTTP in all cell lines that were evaluated (Table 1).

Inhibitory effects of AzddThd derivatives on the replication of Moloney murine sarcoma virus (MSV) and human immunodeficiency virus (HIV)

The anti-retrovirus activities of the AzddThd derivatives were determined in both a murine and a human cell model. In the murine model, the inhibitory effect of the compounds was evaluated on the transformation of mouse embryo fibroblast C3H cells by MSV. In the human model, the effects of the compounds were examined on the cytopathogenicity of HIV for human ATH8 lymphocytes.

AzddThd had a remarkably strong anti-MSV effect (Table 2). At 0.015 μM, it inhibited MSV-induced transformation of C3H cells by 50%. The phosphate derivatives AzddTMP and AzddTTP, and the phosphonate analogue β,γ-methylene AzddTTP were almost as active as the parent compound. However, AzddXyloT, α,β-methylene AzddTDP and α,β-methylene AzddTTP were 400- to 1000-fold less potent than AzddThd in their inhibitory effects on C3H cell transformation (Table 2). Similar observations were made when these compounds were examined for their anti-HIV activity in ATH8 lymphocytes (Table 2). As a rule, all compounds, including the parent compound AzddThd, proved 30–500-fold less inhibitory to HIV replication in ATH8 cells than MSV replication in C3H cells. AzddTMP, AzddTTP and β,γ-methylene AzddTTP, whose anti-MSV activity was almost identical to that of AzddThd, were 10- to 40-fold less protective than AzddThd against HIV-infected ATH8 cells, while the α,β-methylene derivatives even showed a >400-fold decrease in anti-HIV potency.

Inhibitory effects of AzddThd derivatives on the activity of Moloney murine leukemia virus (MLV)- and human immunodeficiency virus (HIV)-associated reverse transcriptase

The AzddThd derivatives were evaluated for their inhibitory effects on the reverse transcriptase derived from Moloney murine leukemia virus (MLV) and

Table 2. Inhibitory effects of AzddThd derivatives on the replication of Moloney murine sarcoma virus (MSV) and human immunodeficiency virus (HIV)

Compound	Anti-MSV activity (50%-effective dose) (μM)	Anti-HIV activity (50%-effective dose) (μM)
AzddThd	0.015	0.5
AzddXyloT	>200	>200
AzddTMP	0.048	14
AzddTTP	0.046	19
α,β-Methylene AzddTDP	11.7	>200
α,β-Methylene AzddTTP	12.2	>200
β,γ-Methylene AzddTTP	0.032	7

human immunodeficiency virus (HIV). The initial concentration of radiolabelled substrate dTTP in the reaction mixture was 1 μM. The nucleosides AzddThd and AzddXyloT, and the 5'-monophosphate of AzddThd did not inhibit MSV or HIV reverse transcriptase reaction at a concentration as high as 200–1000 μM (Table 3). In contrast, AzddTTP was strongly inhibitory to the reverse transcriptase. Its 50% inhibitory concentration (IC_{50}) was in the range of 0.5–1.1 μM for the MLV reverse transcriptase and 0.023 μM for the HIV reverse transcriptase, respectively. None of the phosphonate derivatives showed a potent inhibitory effect on reverse transcriptase. These compounds proved, at an average, 100- to 300-fold less effective than AzddTTP (Table 3, Figs 2 and 3).

Enzymatic hydrolysis of AzddThd derivatives

All AzddThd derivatives, listed in Fig. 1, were subject to treatment with phosphodiesterase I, phosphodiesterase II and 50% or 10% inactivated fetal calf serum. Incubations were done with 4–5 mM of the test compounds for 0, 4 and 24 hr at 37°. Hydrolysis products were separated by thin layer chromatography (TLC) and characterized by running appropriate standards in parallel (Table 4).

Phosphodiesterase I converted AzddTTP to AzddTMP upon 4 hr of incubation, while traces of AzddThd were found after 24 hr of incubation, which is most likely due to contaminating phosphatase activity in the preparation. Phosphodiesterase II converted AzddTTP slightly to AzddTDP after 4 hr and more extensively after 24 hr (Table 4). Fetal calf serum was also able to convert AzddTTP to AzddTDP and AzddTMP. In contrast, none of the enzyme preparations were able to hydrolyse, α,β-methylene AzddTDP and α,β-methylene AzddTTP within a 24-hr incubation period. However, β,γ-methylene AzddTTP was hydrolyzed to AzddTMP by phosphodiesterase I within 4 hr and fetal calf serum within 24 hr, while trace amounts of AzddThd were detected after 24 hr. Phosphodiesterase II was not able to digest β,γ-methylene AzddTTP within the 24-hr incubation period.

DISCUSSION

The cytostatic effects of AzddThd varied from one cell line to another. Of the human lymphoid cell lines B-lymphoblast Raji/0 and T-lymphocyte MT4 cells were most sensitive to the antiproliferative effects of AzddThd (ID_{50}:60 μM), whereas the T-lymphocyte cell lines CEM and H9 were the least sensitive (ID_{50}: 400 and 814 μM, respectively). The biochemical basis for these marked differences has not yet been elucidated. Recently we found no apparent correlation between the cytostatic effect of AzddThd on different cell lines and its extent of phosphorylation to the 5'-triphosphate [22]. The fact that AzddThd is devoid of any cytostatic activity against the dThd

Table 3. Inhibitory effects of AzddThd derivatives on the activity of Moloney murine leukemia virus (MLV)- and human immunodeficiency virus (HIV)-associated reverse transcriptase*

	50%-inhibitory concentration (μM)		
	MLV		HIV
Compound	30 min assay	60 min assay	30 min assay
AzddThd	>1000	>1000	>1000
AzddXyloT	>1000	>1000	200
AzddTMP	>1000	>1000	>1000
AzddTTP	1.15 ± 0.4	0.51 ± 0.28	0.023 ± 0.004
α,β-Methylene AzddTDP	274 ± 36	244 ± 8	14 ± 4
α,β-Methylene AzddTTP	>200	242	4.6 ± 1.1
β,γ-Methylene AzddTTP	156	56.5 ± 9.2	7.9 ± 0.2

* Reverse transcriptase activity of MLV and HIV proceeded linearly during 60 min and 30 min, respectively.

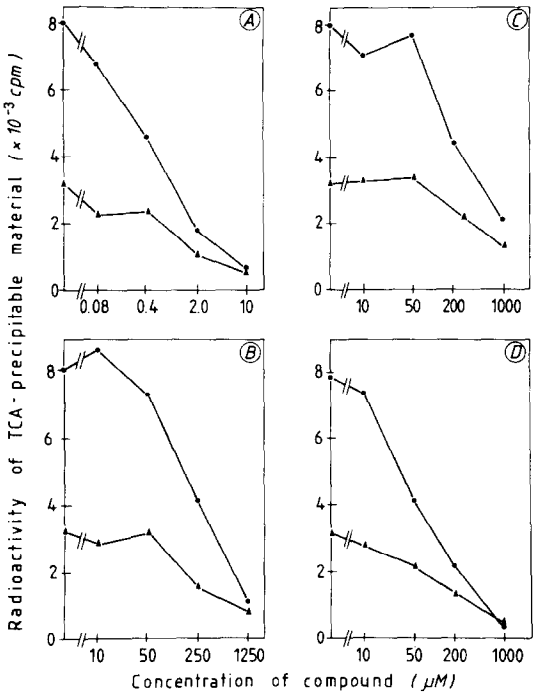

Fig. 2. Inhibitory effect of AzddTTP (Panel A), α,β-methylene-AzddTDP (Panel B), α,β-methylene-AzddTTP (Panel C) and β,γ-methylene-AzddTTP (Panel D) against MLV reverse transcriptase. Incubation of the compounds with the enzymes occurred at 30 min (▲) and 60 min (●). AzddTTP was incubated at 0.08, 0.4, 2.0 and 10 μM; α,β-methylene-AzddTDP at 10, 50, 250 and 1250 μM; α,β-methylene-AzddTTP at 10, 50, 200 and 1000 μM; and β,γ-methylene-AzddTTP at 10, 50, 200 and 1000 μM.

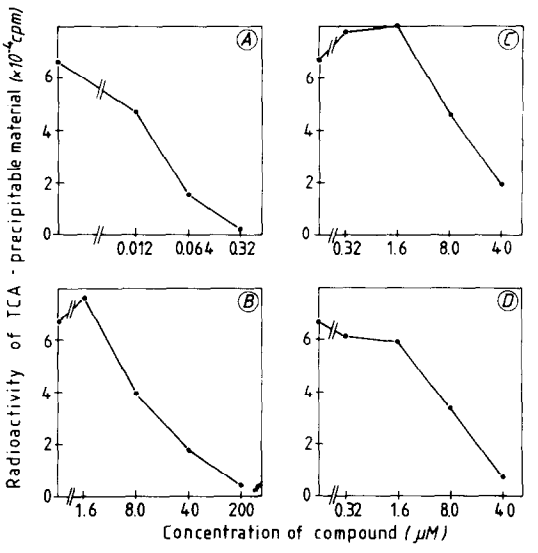

Fig. 3. Inhibitory effect of AzddTTP (Panel A), α,β-methylene-AzddTDP (Panel B), α,β-methylene-AzddTTP (Panel C) and β,γ-methylene-AzddTTP (Panel D) against HIV reverse transcriptase. Incubation of the compounds with the enzymes occurred at 30 min (●). AzddTTP was incubated at 0.012, 0.064 and 0.32 μM; α,β-methylene-AzddTDP at 1.6, 8.0, 40 and 200 μM; α,β-methylene-AzddTTP at 0.32, 1.6, 8.0 and 40 μM; and β,γ-methylene-AzddTTP at 0.32, 1.6, 8.0 and 40 μM.

kinase-deficient cell lines ($ID_{50} > 1000$ μM for L1210/BdUrd and Raji/TK$^-$ cells) clearly indicates that AzddThd needs to be activated by dThd kinase to exert its antiproliferative effects. This activation is also required for the anti-retrovirus activity of AzddThd. The lack of anticellular and anti-retrovirus activity of the *threo* derivative of AzddThd may be explained by its decreased affinity for dThd kinase ($K_i/K_m > 1000$) (unpublished data), and, furthermore, the apparent lack of anti-retrovirus activity of *threo* AzddThd may reside in the failure of its 5'-triphosphate to interact at the reverse transcriptase level, as has been shown with both HIV- and avian myeloblastosis virus-associated reverse transcriptase [7, 23].

As a rule, AzddThd and its phosphorylated derivatives are considerably more inhibitory to MSV-induced C3H cell transformation than HIV-induced cytopathogenicity in ATH8 cells (Table 2). In contrast, AzddTTP and its α,β- and β,γ-methylene AzddTTP derivatives are more inhibitory to HIV reverse transcriptase than MLV reverse transcriptase *in vitro*. This apparent contradiction can be easily explained by our observations that phosphorylation of AzddThd to its 5'-triphosphate metabolite occurs substantially more effective in murine (i.e. L1210) cells than human (i.e. ATH8, Molt/4F) cells (difference > 30-fold) [22]. Indeed, in human lymphoid cells AzddThd accumulates predominantly as its 5'-monophosphate AzddTMP, whereas in murine cells it is readily metabolised to the 5'-triphosphate AzddTTP. Thus, unlike the higher affinity of AzddTTP for reverse transcriptase from HIV than MLV, the rapid conversion of AzddThd to AzddTTP in murine cells may explain why AzddThd has a marked activity against MSV-induced transformation of murine C3H cells *in vitro*, being ~30-fold more pronounced than against HIV-induced cytopathogenicity in human ATH8 cells.

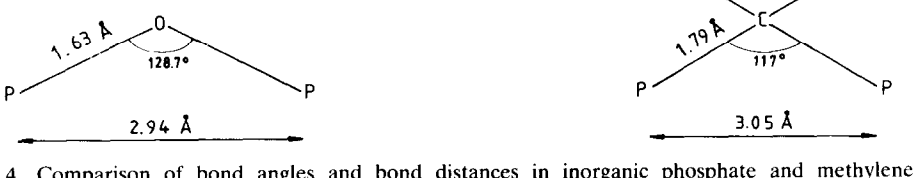

Fig. 4. Comparison of bond angles and bond distances in inorganic phosphate and methylene diphosphonate.

Table 4. Hydrolysis of AzddThd derivatives by phosphodiesterase I and II, and inactivated fetal calf serum

Compound	Formation of hydrolysis products upon incubation by							
	Phosphodiesterase I		Phosphodiesterase II		50% fetal calf serum		10% fetal calf serum	
	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
AzddThd	—	—	—	—	—	—	—	—
AzddTMP	—	AzddThd (+)	—	—	—	AzddThd (++)	—	AzddThd (+)
AzddTTP	AzddTMP (+++)	AzddTMP (+++) AzddThd (+)	AzddTDP (+)	AzddTDP (++) AzddTMP (+)	AzddTDP (+)	AzddTDP (++) AzddTMP (+)	AzddTDP (+)	AzddTDP (+) AzddTMP (+)
α,β-Methylene AzddTDP	—	—	—	—	—	—	—	—
α,β-Methylene AzddTTP	—	—	—	—	—	—	—	—
β,γ-Methylene AzddTTP	AzddTMP (++++)	AzddTMP (++++) AzddThd (+)	—	—	AzddTMP (++)	AzddTMP (+++) AzddThd (+)	AzddTMP (+)	AzddTMP (+) AzddThd (+)

—, No hydrolysis of the parent compound detectable by UV. (+): slight appearance of AzddThd derivative; (++): significant appearance of AzddThd derivative; (+++): predominant presence of AzddThd derivative; (++++): total conversion of AzddThd derivative.

Our observations that the 5′-mono- and 5′-triphosphate derivates of AzddThd are only slightly less cytostatic (Table 1) and only slightly less potent as anti-MSV agents (Table 2) than the parent nucleoside AzddThd suggest that they follow the same pathway(s) in their biological action. Most likely, the phosphorylated derivatives of AzddThd are converted to the nucleoside by phosphodiesterases and/or phosphatases present in the medium (serum) and/or associated with the outer cell membrane before the compounds are taken up by the cells. This hypothesis is supported by the lack of cytostatic activity of AzddTMP and AzddTTP against dThd kinase-deficient cell lines (Table 1) and the prompt conversion of AzddTMP and AzddTTP by fetal calf serum (containing phosphodiesterase and phosphatase activity) to their parent nucleoside (Table 4). The observation that AzddTMP and AzddTTP are 30-fold less protective than AzddThd against HIV-infected ATH8 cells, while only slightly less inhibitory to MSV-induced transformation of murine C3H cells (Table 2), may point to differences in the hydrolyzing enzymes associated with the human ATH8 cell membranes and murine C3H cell membranes. This would make the release and subsequent penetration of AzddThd more efficient in C3H cells than ATH8 cells. Similarly, ddTMP and ddCMP were found considerably less protective than ddThd and ddCyd against HIV-infected ATH8 cells, while ddAMP and ddAdo proved equally protective [24]. The fact that β,γ-methylene AzddTTP also showed a similar biological activity spectrum as AzddTTP (Tables 1, 2) indicates that this phosphonate analogue may also first be converted to AzddTMP and AzddThd before, during or after penetration into the cells. This hypothesis is in keeping with our findings that this compound is readily converted to its AzddTMP and AzddThd by phosphodiesterase I and 50% or 10% fetal calf serum, the latter concentration of serum being present in the culture medium of the cells (Table 4). Most likely, the phosphodiesterase splits the phosphodiester bond between the α- and β-phosphor of β,γ-methylene AzddTTP, thus resulting in the release of pyrophosphonate and AzddTMP, the latter being subject to phosphatase activity. It remains to be determined whether this enzymatic action takes place inside and/or outside the cells.

The lack of antiproliferative activity of α,β-methylene AzddTDP and α,β-methylene AzddTTP, and their substantially decreased anti-retrovirus effects (compared to AzddThd and AzddTTP) indicate that the α,β-methylene derivatives are not readily converted to AzddTMP or AzddThd (Tables 1 and 2). This is in agreement with our observations that the two α,β-methylene phosphonate analogues of AzddThd are resistant to enzymatic attack, since incubation of these compounds with the phosphodiesterases I and II for 24 hr leaves them essentially unchanged (Table 4). Thus, the α,β-methylene phosphonate analogues of AzddThd, in contrast with β,γ-methylene AzddTTP, cannot be considered as efficient prodrugs of AzddThd. Whether the α,β-methylene AzddThd phosphonates are as such taken up by the cells is an interesting possibility that is worth pursuing. However, it is clear from our reverse

transcriptase experiments with AzddTTP, α,β-methylene AzddTTP and β,γ-methylene AzddTTP that the phosphonate derivatives of AzddThd have a markedly decreased affinity for the MSV- and HIV-associated reverse transcriptases compared to AzddTTP, and this may explain why α,β-methylene AzddTTP, if it were shown to be taken up as such by the cells, has virtually no anti-retrovirus activity. A comparison of bond angles and bond distances in inorganic phosphate and methylene diphosphonate reveals that the bond angle of P–O–P differs considerably from the bond angle in P–CH_2–P (128.7° and 117°, respectively) [11, 25] (Fig. 4). However, because of the longer P–C bond, the P–P distances are essentially the same in both compounds. Another characteristic in which the β,γ-methylene derivative of AzddTTP appears to differ from the normal 5'-triphosphate derivatives is its effect on the dissociation constant (p*K*a) of the secondary phosphate group [25]. It has also been noted that, if equipped with a β,γ-methylene phosphonate, ATP binds divalent cations with higher affinity than unsubstituted ATP does [11]. At least one, if not several, of these features must be detrimental to the molecular interaction of these compounds with the active site of the reverse transcriptase, thus resulting in a considerable loss of affinity for the enzyme.

This is the first report on the affinity of nucleoside-5'-phosphonate analogues for the reverse transcriptase, and our findings are of major importance in the future design of new 2',3'-dideoxynucleoside derivatives as anti-retrovirus agents. From our data, it has become apparent that α,β- and β,γ-phosphonate derivatives of AzddThd, and most likely of other 2',3'-dideoxynucleosides as well, are not adequate substitutes for the parent nucleosides, as they are markedly less active, if not inactive, as inhibitors of retrovirus replication.

In conclusion, the potential advantage of 2',3'-dideoxynucleoside 5'-di- or -triphosphates in which one of the phosphate–phosphate bonds is resistant toward enzymatic attack (i.e. by conversion to α,β- or β,γ-methylene phosphonates), seems to be counteracted by their reduced affinity for the target enzyme reverse transcriptase. It does not seem justified to recommend the α,β- or β,γ-methylene phosphonate approach in the design of more effective anti-HIV agents, even if the compounds could be delivered directly into the cells, i.e. through permeabilisation of the cells, or through the aid of protein carriers or liposomes.

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REFERENCES

1. Horwitz JP, Chua J and Noel MJ, Nucleosides. V. The monomesylates of 1-(2'-deoxy-β-D-lyxofuranosyl)-thymine. *J Org Chem* **29**: 2076–2078, 1964.
2. Ostertag W, Roesler G, Krieg CJ, Kind J, Cole T, Crozier T, Gaedicke G, Steinheider G, Kluge N and Dube S, Induction of endogenous virus and of thymidine kinase by bromodeoxyuridine in cell cultures transformed by Friend virus. *Proc Natl Acad Sci USA* **71**: 4980–4985, 1974.
3. De Clercq E, Balzarini J, Descamps J and Eckstein F, Antiviral, antimetabolic and antineoplastic activities of 2'- or 3'-amino or -azido-substituted deoxyribonucleosides. *Biochem Pharmacol* **29**: 1849–1851, 1980.
4. Mitsuya H, Weinhold KJ, Furman PA, St. Clair MH, Nusinoff Lehrman S, Gallo RC, Bolognesi D, Barry DW and Broder S, 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc Natl Acad Sci USA* **82**: 7096–7100, 1985.
5. Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff Lehrman S, Bolognesi DP, Broder S, Mitsuya H and Barry DW, Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci USA* **83**: 8333–8337, 1986.
6. Balzarini J and Broder S, Principles of antiretroviral therapy for AIDS and related diseases. In: *Clinical Use of Antiviral Drugs* (Ed. E De Clercq). Martinus Nijhoff, Norwell, in press.
7. Vrang L, Bazin H, Remaud G, Chattopadhyaya J and Oberg B, Inhibition of the reverse transcriptase from HIV by 3'-azido-3'-deoxythymidine triphosphate and its *threo analogue*. *Antiviral Res.* **7**: 139–149, 1987.
8. Balzarini J, Cooney DA, Broder S and Johns DG, Biochemical basis for the cytostatic effects of the potent anti-HIV (human immunodeficiency virus) drug 3'-azido-2',3'-dideoxythymidine (AZT). *Arch Int Physiol Biochim* **95**: B-52, 1987.
9. Cohen SS, On the nature of thymineless death. *Ann NY Acad Sci* **186**: 292–301, 1971.
10. Bjursell G and Reichard P, Effects of thymidine on deoxyribonucleoside triphosphate pools and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. *J Biol Chem* **248**: 3904–3909, 1973.
11. Scheit KH, Nucleotides with modified phosphate groups. In: *Nucleotide Analogs. Synthesis and Biological Function*, pp. 96–141. John Wiley, New York, 1980.
12. Herdewijn P, Balzarini J, De Clercq E, Pauwels R, Baba M, Broder S and Vanderhaeghe H, 3'-Substituted 2',3'-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. *J Med Chem* **30**: 1270–1278, 1987.
13. Yoshikawa M, Kato T and Takenishi T, Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull Chem Soc Jpn* **42**: 3505–3508, 1969.
14. Hoard DE and Ott DG, Conversion of mono- and oligodeoxyribonucleotides to 5'-triphosphates. *J Am Chem Soc* **87**: 1785–1788, 1965.
15. Myers TC, Nakamura K and Danielzadeh AB, Phosphonic acid analogs of nucleoside phosphates. III. The synthesis of adenosine-5'-methylene-diphosphonate, a phosphonic acid analog of adenosine-5'-diphosphate. *J Org Chem* **30**: 1517–1520, 1965.
16. Khorana HG, Vizsolyi JP and Ralph RK, Studies on polynucleotides. XII. Experiments on the polymerization of mononucleotides. A comparison of different polymerizing agents and a general improvement in the isolation of synthetic polynucleotides. *J Am Chem Soc* **84**: 414–418, 1962.
17. Balzarini J, De Clercq E, Torrence PF, Mertes MP,

Park JS, Schmidt CL, Shugar D, Barr PJ, Jones AS, Verhelst G and Walker RT, Role of thymidine kinase in the inhibitory activity of 5-substituted-2′-deoxyuridines on the growth of human and murine tumor cell lines. *Biochem Pharmacol* **31**: 1089–1095, 1982.

18. Balzarini J, Kang G-J, Dalal M, Herdewijn P, De Clercq E, Broder S and Johns DG, The anti-HTLV-III (anti-HIV) and cytotoxic activity of 2′,3′-didehydro-2′,3′-dideoxyribonucleosides: a comparison with their parental 2′,3′-dideoxyribonucleosides. *Mol Pharmacol* **32**: 162–167, 1987.
19. Popovic M, Sarngadharan MG, Read E and Gallo RC, Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**: 497–500, 1984.
20. De Clercq E and Merigan TC, Moloney sarcoma virus-induced tumors in mice: inhibition or stimulation by (poly rI).(poly rC). *Proc Soc Exp Biol Med* **137**: 590–594, 1971.
21. De Clercq E, Balzarini J, Torrence PF, Mertes MP, Schmidt CL, Shugar D, Barr PJ, Jones AS, Verhelst G and Walker RT, Thymidylate synthetase as target enzyme for the inhibitory activity of 5-substituted 2′-deoxyuridines on mouse leukemia L1210 cell growth. *Mol Pharmacol* **19**: 321–330, 1981.
22. Balzarini J, Pauwels R, Baba M, Herdewijn P, De Clercq E, Broder S and Johns DG, The *in vitro* and *in vivo* anti-retrovirus activity, and intracellular metabolism of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine are highly dependent on the cell species, *Biochem Pharmacol* **37**: 897–903, 1988.
23. Eriksson B, Vrang L, Bazin H, Chattopadhyaya J and Öberg B, Different patterns of inhibition of avian myeloblastosis virus reverse transcriptase activity by 3′-azido-3′-deoxythymidine 5′-triphosphate and its *threo* isomer. *Antimicrob Agents Chemother* **31**: 600–604, 1987.
24. Kim C-H, Marquez VE, Broder S, Mitsuya H and Driscoll JS, Potential anti-AIDS drugs. 2′,3′-Dideoxycytidine analogues, *J Med Chem* **30**: 862–866, 1987.
25. Yount RG, Babcock D, Ballantyne W and Ojala D, Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P–N–P linkage. *Biochemistry* **10**: 2484–2489, 1971.