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5′-Hydrogenphosphonates of anti-HIV nucleoside analogues revisited: controversial mode of action

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Summary

The monomeric and symmetrical dimeric 5′-hydrogenphosphonate derivatives of AZT were prepared and evaluated for their inhibitory properties against HIV-1 in several cell lines. The synthesis of the compounds was achieved by reaction of AZT with in situ prepared phosphorus tris(imidazolide) or with phosphonic acid in the presence of pivaloyl chloride. The two title compounds showed in vitro anti-HIV activity similar to (but not better than) that of AZT in three cell lines which were not deficient in thymidine kinase. On the other hand they were inactive in CEM-TK$^-$ cells. Pharmacokinetic studies in several media corroborate the assumption that these compounds must not be considered as 'true antiviral agents', but that they act by releasing their nucleoside entity.

Human immunodeficiency virus; 5′-Hydrogenphosphonate nucleoside derivatives; Stability; Mode of action

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Introduction

Zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) is a nucleoside analogue widely used for the treatment of patients suffering from acquired immunodeficiency syndrome (AIDS) owing to its efficiency against the human immunodeficiency virus (HIV, the etiological agent of AIDS). As with other chemotherapeutic nucleoside analogues (De Clercq, 1992), the antiretroviral effect of AZT involves its conversion, through cellular kinases, to the corresponding triphosphate metabolite which competitively inhibits HIV reverse transcriptase or terminates the newly synthesized viral DNA chains. This dependence on kinase-mediated phosphorylation may limit the efficiency of AZT in a cellular environment low in nucleoside kinase activities as in macrophages (Richman et al., 1987) or in cells which have lost their ability to phosphorylate AZT after prolonged exposure to the drug (Stretcher et al., 1991). Therefore attempts have been made to circumvent this dependence through either the use of prodrugs of 5′-monophosphate AZT (AZTMP) (McGuigan et al., 1992; Henin et al., 1991; Puech et al., 1993) or the use of liposome- encapsulated AZTMP (Hostetler and Richman, 1989). With such strategies, AZT shows better in vitro biological responses and, furthermore, in the case of 2′,3′-dideoxyuridine (ddU) which is inactive due to its inability to be phosphorylated by cellular kinases (Hao et al., 1990), an anti-HIV effect can be observed owing to the intracellular delivery of its mononucleotide (Sastry et al., 1992; Puech et al., 1993; Zelphati et al., 1993).

As an original class of anti-HIV nucleotide analogues modified on the phosphate residue we decided to synthesize and to study 5′-hydrogenphosphonate derivatives of AZT. This choice was justified by the fact that 5 years ago we had found that 9-β-D-arabinofuranosyladenine 5′-hydrogenphosphonate retained, although to smaller extent than its parent nucleoside (araA), both antiviral activity (against DNA viruses) and cytostatic activity (Puech et al., 1988). In fact, concerning the possible mode of action of nucleoside hydrogenphosphonate analogues, it was thought that these compounds: (i) could be resistant to extra and/or intra-cellular dephosphorylation; (ii) might enter the cell due to their less polar nature; and (iii) might intracellularly either be converted into a pyrophosphorylhydrogenphosphonate derivative or be oxidized to the corresponding phosphate entity and further converted into the triphosphate. During the course of our work it was independently reported that the 5′-hydrogenphosphonate derivatives of several dideoxynucleosides exhibit potent anti-HIV activity with selectivity indices similar to or better than those of their parent nucleosides. The authors suggested that the observed activity was apparently due to an additional functionality present in these compounds (i.e., the H-phosphonate moiety) and should not be merely ascribed to releasing of parent nucleosides upon hydrolysis of the H-phosphonate derivatives (Krayevsky et al., 1992a,b). However, it was recently reported that conversion of another potent anti-HIV oxetane derivative of thymidine into the 5′-hydrogenphosphonate derivative did not improve its antiviral activity (O-Yang

et al., 1992).

This report describes the synthesis, biological evaluation and pharmacokinetic studies of the monomeric and dimeric 5'-hydrogenphosphonate derivatives of AZT designed as either potential anti-HIV agents or as prodrugs of AZT monophosphate.

Materials and Methods

Cell cultures and viruses

The origin of the human T-cell lines and virus isolates used was described previously (Puech et al., 1993).

Cytotoxicity assay on MT-4 cells

Replication of HIV-1 in MT-4 cells was measured by the reduction in viability of the cells, resulting from the infection (Génu-Dellac et al., 1991). 50% effective concentration (EC_{50}) was defined as the concentration of the compound required to achieve 50% protection of the infected cells.

Inhibition of virus production

Production of virus particles in CEM cells was evaluated by measurement of virion-associated reverse transcriptase activity in the culture supernatant as described previously (Puech et al., 1993). 50% effective concentration (EC_{50}) was defined as the concentration of drug that reduced the reverse-transcriptase activity by 50%. 50% cytotoxic concentration (CC_{50}) was determined by the MTT dye reduction assay as for MT-4 cells.

Preparation of cell extracts

Exponentially growing CEM-SS cells were collected by centrifugation, washed three times in phosphate-buffered saline, and resuspended in 10 mM Tris-HCl, 140 mM KCl (pH 7.4), at the concentration of 24×10^6 cells/ml. Cells were lyzed by ultrasonic treatment (15 s) and cellular debris were removed by centrifugation at $100\,000 \times g$, 1 h at 4°C. The soluble proteins were recovered in the supernatant (about 5.5 mg/ml) and analyzed for their enzymatic activities.

Decomposition studies of the 5'-hydrogenphosphonate derivatives of AZT

The method for determination of kinetics of decomposition of phosphate-modified nucleotides has recently been described (Pompon et al., 1993). Briefly, HPLC was performed on a Waters-Millipore instrument equipped with two Model 510 solvent delivery systems, a Model 680 solvent programmer, a Model 712 autosampler, and a Model 990 diode-array UV-detector. The column assembly consisted of an Internal-Surface Reversed-Phase (ISRP) precolumn (Ultrabiosep C_8, 10 × 4.6 mm id, 10 μm particle size) linked in series to a Reversed Phase (RP) analytical column (Nucleosil, C_{18}, 100 × 4.6 mm, 3 μm),

both columns purchased from SFCC/Shandon (Eragny, France). The analytical column was thermostated at 30°C, and a six-port 7000 Reodyne valve allowed to modify the flow path.

Starting materials

AZT was purchased from Intsel Marsing, France. 3'-Azido-2',3'-dideoxythymidine-5'-monophosphate (AZTMP) was synthesized by treating AZT with phosphorous oxychloride in trimethylphosphate (Yoshikawa et al., 1969) following the experimental protocol reported for the preparation of the 5'-monophosphate of ddU (Zelphati et al., 1993). General procedures and instrumentation used have been described previously (Périgaud et al., 1992). ^{31}P-NMR spectra were recorded at ambient temperature on a Bruker WP 200 SY spectrometer with proton heteronuclear decoupling.

Results

Chemical synthesis

3'-Azido-2',3'-dideoxythymidin-5'-yl hydrogen phosphonate (1). To a solution of imidazole (0.49 g, 7.2 mmol) in anhydrous acetonitrile (13.5 ml) were added, with stirring and cooling in an ice bath, phosphorus trichloride (0.19 ml, 2.2 mmol) and triethylamine (1.06 ml, 7.6 mmol). The mixture was stirred for 15 min, and then a solution of AZT (0.134 g, 0.5 mmol) in acetonitrile (13.5 ml) was added dropwise. After the addition of AZT was complete, the reaction mixture was stirred at room temperature for 4 h. Water (3.4 ml) was added and the solution was stirred for another 30 min. The solvent was removed under vacuum, and the residue was reevaporated with a mixture of pyridine/triethylamine (4:1, v/v) three times. Chromatography on a silica-gel column [eluent: stepwise gradient of methanol (0–30%) in methylene chloride] afforded pure **1** (0.130 g, 78%): UV (EtOH) λ_{max} 262 nm (ε, 9800), λ_{min} 233 nm (ε, 2700); ^{1}H-NMR (Me_2 SO-d_6) δ ppm 11.31 (*s*, 1H, NH-3), 7.74 (*s*, 1H, H-6), 6.62 (*d*, 1H, P-H; $J_{H,P}$ = 595 Hz), 6.12 (*t*, 1H, H-1'; J = 6.7 Hz), 4.47 (*m*, 1H, H-3'), 3.92 (*m*, 3H, H-4', 5' and 5''), 2.41 and 2.26 (2*m*, 1H each, H-2' and 2''), 1.80 (*s*, 3H, CH_3); ^{31}P-NMR (Me_2SO-d_6) 1.51 ppm; mass spectra (matrix = glycerol), FAB>0: 424 $(M + H + glycerol)^+$, 332 $(M + H)^+$, 127 $(BH_2)^+$; FAB<0: 330 $(M - H)^-$, 125 $(B)^-$.

TABLE 1

Anti-HIV-1 activity of the monomeric 1 and dimeric 2 5'-hydrogenphosphonate derivatives of AZT in various cell cultures

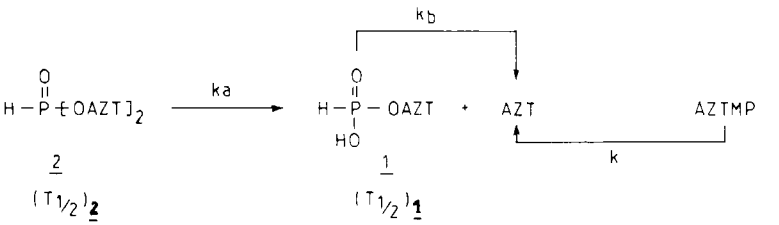
	MT-4			CEM-SS			CEM-X 174			CEM-TK		
	EC_{50}[a]	CC_{50}[b]	SI[c]	EC_{50}[a]	CC_{50}[b]	SI[c]	EC_{50}[a]	CC_{50}[b]	SI[c]	EC_{50}[a]	CC_{50}[b]	SI[c]
1	$7\ 10^{-9}$	$> 10^{-4}$ (26%)[d]	≥ 14300	$5\ 10^{-9}$	$> 10^{-4}$ (15%)[d]	≥ 20000	$3\ 10^{-9}$	$> 10^{-4}$ (0%)[d]	≥ 33300	$> 10^{-4}$ (0%)[e]	$> 10^{-4}$ (12%)[d]	< 1
2	$1.5\ 10^{-9}$	$2\ 10^{-5}$	13300	$2\ 10^{-9}$	$6\ 10^{-5}$	30000	$2\ 10^{-9}$	$> 10^{-4}$ (41%)[d]	≥ 50000	$> 10^{-4}$ (0%)[e]	10^{-4}	< 1
AZT	$4\ 10^{-10}$	$5\ 10^{-5}$	125000	$1.5\ 10^{-9}$	$> 10^{-4}$ (20%)[d]	≥ 66600	$8\ 10^{-10}$	$> 10^{-4}$ (20%)[d]	≥ 125000	$> 10^{-4}$ (14%)[e]	$> 10^{-4}$ (5%)[d]	≈ 1
AZTMP	10^{-9}	$> 10^{-5}$ (20%)[d]	≥ 10000	10^{-9}	$> 10^{-5}$ (13%)[d]	≥ 10000	$3\ 10^{-8}$	$> 10^{-5}$ (7%)[d]	≥ 3300	$\approx 10^{-5}$ (56%)[e]	$> 10^{-5}$ (0%)[d]	≥ 1

[a]50% effective molar concentration or molar concentration required to inhibit the replication of HIV by 50%.
[b]50% cytotoxic molar concentration or molar concentration required to reduce the viability of the cells by 50%.
[c]Selective index or ratio CC_{50}/EC_{50}.
[d]Percent reduction of viable cells at the indicated highest concentration tested.
[e]Percent inhibition of HIV production at the indicated highest concentration tested.

O,O'-Bis(3'-azido-2',3'-dideoxythymidin-5'-yl) hydrogen phosphonate (2). Phosphonic acid (90 mg, 1.1 mmol) and AZT (535 mg, 2.0 mmol) were dried by evaporation of added pyridine, and the residue was dissolved in the same solvent (5 ml). Pivaloyl chloride (0.49 ml, 4.0 mmol) dissolved in pyridine (5 ml) was added dropwise during 20 min to the stirred reaction mixture and stirring was continued for 1 h. Within that time conversion of AZT to the corresponding symmetrical hydrogenphosphonate diester 2 was complete (TLC) and the reaction mixture was evaporated to near dryness. The residue was co-evaporated twice with acetonitrile in order to remove residual pyridine and chromatographed on a silica-gel column using as eluent a stepwise gradient of 2-propanol (0–12%) in chloroform. The fractions containing the pure compound were pooled, evaporated, dissolved in acetonitrile and evaporated to give 2 (372 mg, 66%) as a white foam: UV (EtOH): λ_{max} *263 nm (ε, 18100),* λ_{min} *233 nm (ε, 5000);* ^{1}*H-NMR (MeCN-d*$_3$*)* δ *ppm 7.33 (m,* 2H, 2 H-6), 6.92 (*d,* 1H, P-H; $J_{H,P}$ = 720 Hz), 6.11 (*t,* 2H, 2 H-1'; J = 6.6 Hz), 4.3 (*m,* 6H, 2 H-3',5',5''), *4.0 (m,* 2H, 2 H-4'), *2.4 (m,* 4H, 2 H-2',2''), *1.83 (d,* 6H, 2 CH_3; J = 1.1 Hz); ^{31}P-NMR (MeCN) 10.2 ppm; mass spectra (matrix = *m*-nitrobenzylalcohol), FAB>*0: 581 (M + H)*$^+$*, 332 (M − AZT + 2H)*$^+$*, 127 (BH*$_2$*)+; FAB<0: 579 (M − H)*$^-$*, 330 (M − AZT)*$^-$*, 125 (B)*$^-$.

Antiretroviral activity

The monomeric 1 and dimeric 2 5'-hydrogenphosphonate derivatives of AZT



Schema 2. Decomposition pathways of the 5'-hydrogenphosphonate derivatives of AZT.

TABLE 2

Rate constants (k) and half-life times ($T_{1/2}$) of the decomposition of AZTMP and of the 5'-hydrogenphosphonate derivatives **1**, **2** of AZT at a concentration of $5\ 10^{-5}$ M and 37 C in three different media (the constants k, k_a and k_b are related to the reactions depicted in the Scheme 2)

	RPMI	RPMI + 10% heat-inactivated foetal calf serum (Culture medium)	CEM cell extract
2	$k_a = 9.6\ 10^{-3}$ min^{-1}; $(T_{1/2})_{\mathbf{2}} = 72$ min $k_b = 1.7\ 10^{-5}$ min^{-1}; $(T_{1/2})_{\mathbf{1}} = 28$ day	$k_a = 6.7\ 10^{-2}$ min^{-1}; $(T_{1/2})_{\mathbf{2}} = 10$ min $k_b = 3.5\ 10^{-4}$ min^{-1}; $(T_{1/2})_{\mathbf{1}} = 33$ h	$k_a = 3.9\ 10^{-2}$ min^{-1}; $(T_{1/2})_{\mathbf{2}} = 18$ min $k_b = 1.0\ 10^{-2}$ min^{-1}; $(T_{1/2})_{\mathbf{1}} = 69$ min
1	$k_b = 2.2\ 10^{-5}$ min^{-1}; $(T_{1/2})_{\mathbf{1}} = 22$ day	$k_b = 4.9\ 10^{-4}$ min^{-1}; $(T_{1/2})_{\mathbf{1}} = 24$ h	$k_b = 1.7\ 10^{-2}$ min^{-1}; $(T_{1/2})_{\mathbf{2}} = 41$ min
AZTMP	ND	$k = 1.6\ 10^{-3}$ min^{-1}; $T_{1/2} = 7.2$ h	$k = 4.3\ 10^{-3}$ min^{-1}; $T_{1/2} = 2.7$ h

ND: not determined.

were evaluated for their inhibitory effects on the replication of HIV-1 in four different cell cultures (Table 1). These compounds proved markedly active in the three cell lines (MT-4, CEM-SS, CEM-X 174) not deficient in thymidine kinase. On the other hand they were devoid of any activity at a concentration as high as 10^{-4} M in the CEM-TK$^-$ cell line.

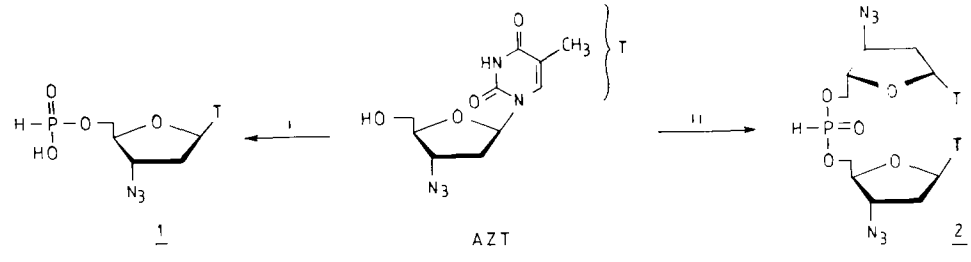
Stability studies

The kinetics of decomposition of the monomeric 1 and dimeric 2 5'-hydrogenphosphonate derivatives of AZT and the decomposition of AZTMP were determined under three experimental conditions by using an adapted 'on-line ISRP cleaning' HPLC methodology previously described for the degradation studies of oligonucleotides (Pompon et al., 1992). The observed degradation pathways and the kinetic data are reported in Scheme 2 and Table 2. The dimeric compound 2 was rapidly and selectively converted into the corresponding monomeric derivative 1 and into AZT in all media. On the other hand, 1 was quite stable in RPMI, but it was significantly hydrolyzed to AZT in culture medium and this transformation was about 30 times faster in cell extract. No AZTMP was detected and the only observed metabolite was AZT.

Discussion

Chemistry

The procedure applied for synthesizing the monomeric 1 and dimeric 2 hydrogenphosphonates of AZT (Scheme 1) were based on procedures published previously in other series and slightly modified. Thus, phosphorus trichloride was allowed to react with imidazole and triethylamine in acetonitrile to form phosphorus tris(imidazolide) (Garegg et al., 1986) which was used directly in a reaction with AZT to give 1. On the other hand, when AZT was allowed to react in pyridine with 0.5 equivalent of phosphonic acid in the presence of an excess of pivaloyl chloride as a sterically hindered activating agent (Sekine et al., 1988; Stawinski and Thelin, 1990), the symmetrical (5 → 5')



1 2

Scheme 1. Synthesis of the monomeric 1 and dimeric 2 5'-hydrogenphosphonate derivatives of AZT. Reaction conditions: (i) PCl_3, imidazole, $(C_2H_5)_3N/CH_3CN$; (ii) PO_3H_3, $(CH_3)_3CCOCl \cdot C_5H_5N$.

hydrogenphosphonate 2 was obtained. The reaction conditions have been modified to maximize the yield of 2, and this approach can thus be considered as a method of choice for a simple and fast one-pot preparation of symmetrical H-phosphonate diesters. Compounds 1 and 2 were purified by silica-gel column chromatography. Their structures were confirmed by spectral methods (^{1}H- and ^{31}P-NMR, FAB Mass spectra) and their purity was asserted by HPLC analyses. The latter method showed that 1 and 2 were free of their parent nucleoside, AZT (detection limit <0.5%).

Biological properties and stability

When evaluated against HIV-1 replication in several cell cultures, it was found that the 5'-hydrogenphosphonate derivatives 1 and 2 were markedly

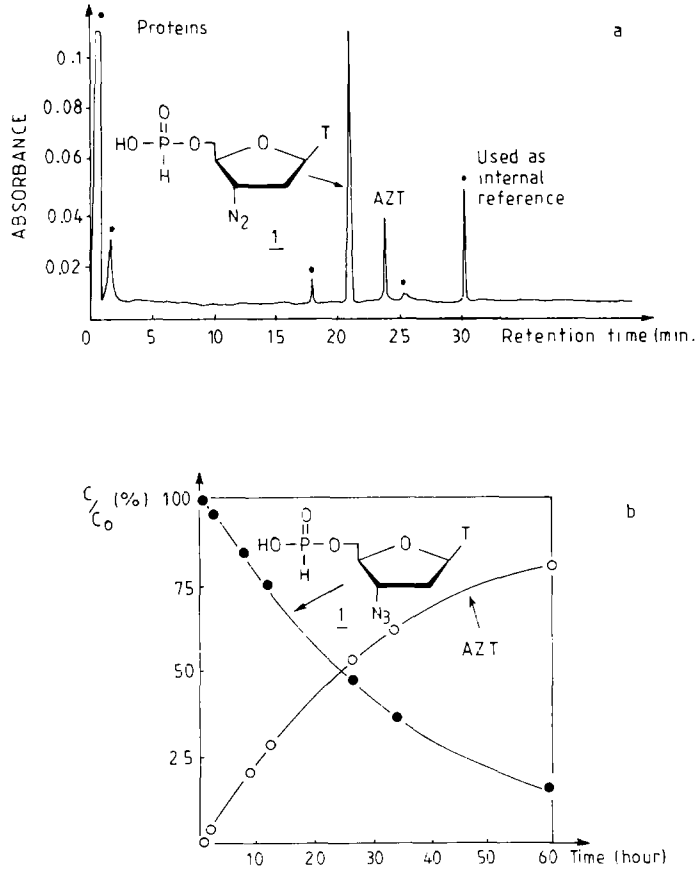


Fig. 2. Stability study of AZT 5'-hydrogenphosphonate 1 (initial concentration C_o = 5 10^{-5} M) at 37 C in cell culture medium using a 'on-line ISRP cleaning' HPLC methodology. (a) Typical HPLC chromatogram of 1 after 7 h of incubation. The peaks surmounted by a dot arise from the culture medium. (b) Decomposition curve of 1.

active in MT-4, CEM-SS and CEM-X 174 cell lines. However their selectivity indices were always lower than that of AZT. Furthermore, similar to AZT and AZTMP, compounds **1** and **2** showed no anti-HIV activity in a CEM cell line deficient in thymidine kinase (Table 1). These results strongly indicate that hydrogenphosphonates of AZT cannot deliver into a cell a monophosphorylated derivative able, by itself or after being metabolized, to inhibit HIV reverse transcriptase.

In order to substantiate this assumption the decomposition studies of compounds **1** and **2** in an enzyme-free medium (RPMI), in cell culture medium (RPMI containing 10% heat-inactivated foetal calf serum) and in a CEM cell extract were undertaken using a 'on-line ISRP cleaning' HPLC approach (Pompon et al., 1992; 1993). A typical chromatogram obtained for **1** in the cell culture medium and its decomposition curve in the same medium are shown in Figs. 2a and 2b, respectively.

The determined kinetic data (Table 2) deserve the following comments: (i) the symmetrical (5′ → 5′) hydrogenphosphonate **2** was very unstable, especially in cell culture medium ($T_{1/2}$ = 10 min), giving only AZT and the monomeric hydrogenphosphonate **1**; (ii) compound **1** was rather stable in RPMI ($T_{1/2}$ = 22 days) but it was rapidly metabolized in cell culture medium ($T_{1/2}$ between 24 and 33 h) and even faster in cell extract ($T_{1/2}$ between 41 and 69 min). It should be also pointed out that the only observed metabolite arising from **1** was the parent nucleoside AZT. Furthermore, since the half-life time of AZTMP is about three times higher than that of **1** in cell extract, it is unlikely that in this medium **1** could be oxidized into the 5′-monophosphate (otherwise this nucleotide would be observed). The transformation of **1** into AZT might result from its hydrolysis by phosphatases or other dephosphorylating enzymes present in the cell culture medium and in the cell extract.

From the current results it appears that the monomeric **1** and the dimeric hydrogenphosphonate **2** (whether they may or not penetrate cell membrane) most likely exert their action via releasing the parent nucleoside. This assumption was corroborated by the study of the same hydrogenphosphonate derivatives of ddU which showed that these compounds have no inhibitory effect on HIV-1 replication at a concentration as high as 10^{-4} M in all cell culture lines used (data not shown). Thus, the previous reports on the possible mode of action of dideoxynucleoside hydrogenphosphonates as 'true anti-HIV agents' (Krayevsky et al., 1992a,b) need to be reconsidered. It seems apparent from this study that hydrogenphosphonate derivatives cannot be used in order to overcome the resistance to nucleoside analogues, whether this resistance is due to a mutation of the target enzyme (reverse transcriptase) or a deficiency in the enzymes required for anabolic phosphorylations.

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