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AIDS dementia: synthesis and properties of a derivative of 3′-azido-3′-deoxythymidine (AZT) that may become 'locked' in the central nervous system

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In an attempt to provide a derivative of 3′-azido-3′-deoxythymidine (AZT) which might be sequestered in the central nervous system and release AZT, the dihydropyridine ester 5′-(1,4-dihydro-1-methyl-3-pyridinylcarbonyl)-3′-deoxythymidine, was synthesized in a three step sequence. This material showed potent anti-HIV-1 activity in MT-4 cells most probably by hydrolysis to the parent nucleoside, AZT. This dihydropyridine derivative of AZT could be easily oxidized to a positively charged pyridinium derivative of AZT in rat brain cytosol. In turn the pyridinium form could be hydrolyzed, non-enzymatically, to AZT.

HIV-1; Antiviral activity; Anti-retrovirus agent; Nucleoside; (Rat brain)

1. INTRODUCTION

While it has been recognized for some time that profound immune dysfunction is a hallmark of infection with human immunodeficiency virus type 1 (HIV-1), it is now also clear that serious neurologic dysfunction, the AIDS dementia complex, is an important cause of morbidity in many patients [1,2]. While it is presently not certain whether or not the pathology of AIDS dementia complex may be caused by a direct infection of the brain by HIV-1 or by an indirect mechanism (e.g. interference with neuroleukin [3–5]) [1,2], it is certain that brain macrophages, microglia as well as multinucleated cells can be productively infected with HIV-1 [6–10], and that the proviral DNA, viral nucleic acid, viral antigens, as well as HIV-1 virons are present in the brains of patients with AIDS dementia [2]. This invasion of the nervous system by HIV-1, coupled with the possibility that it could create a reservoir of persistent infection even if peripheral clearance were realized [11], has led to the general agreement that an ideal chemotherapeutic agent for HIV-1 should penetrate and be highly active in the CNS [12].

3′-Azido-3′-deoxythymidine [13] (AZT, zidovudine, Retrovir) is a nucleoside analog that inhibits HIV replication in vitro [14] and can decrease mortality and the frequency of opportunistic infections in selected patients with AIDS and AIDS-related complex [14–16]. It also leads to a marked improvement in neurological manifestations of AIDS dementia [1,17]. While most nucleosides as polar molecules do not readily cross the blood-brain barrier, AZT has been found in cerebrospinal fluid of patients on AZT therapy

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Part of this work was presented at the Second International Conference on Antiviral Research, Williamsburg, VA, April 10–14, 1988. At that meeting, a report containing similar findings was authored by M.E. Brewster, R. Little, V. Venkatraghavan and N. Bodor

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[15,18]. Some uncertainty regarding specific brain levels of AZT exists, since CSF levels may reflect transport through the choroid plexus. For instance, radioactive AZT was not significantly transported into the brain of rats [19]. On the other hand, AZT stands apart as a nucleoside analog in that it appears to cross the cell membrane mainly by nonfacilitated diffusion and not by means of a nucleoside transport system [20]. Regardless of the efficiency with which AZT crosses the blood-brain barrier, it is to be expected that it may leave the CNS by the same mechanism with equal facility. In view of the possibility that HIV-1 may replicate in the brain [1,2], it may be of interest to investigate the properties of an agent which might accumulate in the brain, thereby providing a sustained release of the antiviral to maintain a therapeutically effective concentration.

Bodor and colleagues have achieved brain-specific delivery of dopamine, phenylethylamine, *N*-pyridinium-2-carbaldoxime chloride, testosterone, estradiol, tryptamine and other pharmacological agents by means of a redox delivery system based on the chemistry of a dihydropyridine-pyridinium salt interconversion [21–24]. The basic concept is the generation of an adduct of the pertinent drug to a 1,4-dihydropyridine which is a neutral lipophilic molecule and may cross the blood-brain barrier [21,22]. In the brain, redox systems, such as dehydrogenases, may convert the dihydropyridine to a pyridinium form which, as a positively charged molecule, cannot readily exit the blood-brain barrier. This adduct may then be cleaved to yield the active drug. In this report, we describe the application of this principle to AZT and the resultant properties of such an adduct.

2. MATERIALS AND METHODS

AZT was purchased from Sigma (St. Louis, MO) or synthesized according to Horwitz et al. [25] and nicotinyl chloride was from Lancaster Synthesis (Windham, NH). HPLC was with a μ-Bondapak C_{18} column (3.9 × 300 mm) with a mobile phase of CH_3CN/0.01 M Na_2HPO_4 (1:1) with 0.001 M *n*-octane sulfonic acid at a flow rate of 1 ml/min. Detection was at 254 nm. Under these conditions, retention times were as follows: AZT, 4.0 min; compound **4**, 5.3 min; compound **3**, 12.5 min; nicotinic acid methiodide, 3.9 min. Infrared spectra were determined on a Beckman 4230 instrument, ultraviolet spectra on a Beckman DU8B spectrophotometer, NMR (300 MHz) on a Varian XL300 spectrometer, and mass spectra on either a Finnigan/Extrel 1015 (chemical ionization) or on a Finnigan thermospray (TSP46) instrument (positive ion thermospray).

Rat brain S_{10} was prepared by homogenization of brain tissue in 50 mM Tris buffer (pH 7.4) followed by centrifugation at 10000 × *g* for 10 min. The supernatant was used immediately for the assays.

2.1. *AZT nicotinate [5'-(3-pyridinylcarbonyl)-3'-azido-3'-deoxythymidine (2)]*

3'-Azido-3'-deoxythymidine (AZT, dried over P_2O_5, 3.1 g, 11.6 mmol) was dissolved in dry pyridine (20 ml). Then the pyridine was removed in vacuo. This was repeated three times to remove all traces of moisture. Finally, the AZT was taken up in anhydrous pyridine, and nicotinyl chloride (2.34 g, 13 mmol) was added. The mixture was maintained at 70°C for 1.5 h. Tlc $CHCl_3$/MeOH (9:1) showed a small quantity of unreacted AZT, so an additional amount of nicotinyl chloride (180 mg) was added. After a further 0.5 h at 70°C, no AZT remained. The reaction mixture was cooled and pyridine was removed in vacuo. Xylene was added and evaporated to aid in the removal of residual pyridine. The reaction mixture then was applied to a column of silica gel (100 g) and it was eluted with $CHCl_3$/MeOH (50:1). Appropriate fractions were combined to give, after evaporation, AZT nicotinate (**2**, 4.1 g, 95%). Trituration with cyclohexane produced a foamy powder. Recrystallization from acetone/hexane gave **2** as colorless needles: m.p. 136–137°C; UV λ_{max} (CH_3OH) 263 nm (ϵ 12800), 214 nm (ϵ 19100); IR (KBr) 2100 cm^{-1} (N_3), 1730 cm^{-1} (ester C=O); CI-MS, *m/z* 373 (M+H); NMR ($CDCl_3$) δ: 1.78 (s,3,5-CH_3); 2.54 (t*, 2, J = 7 Hz, 2'-H's), 4.18 (br dt, 1, J = 7 Hz, 4 Hz, 4'H), 4.43 (q*, 1, J = 7 Hz, 3'H), 4.61 and 4.69 (dd, each 1, J = 12 Hz, 4 Hz, 5'H's), 6.08 (t, 1, J = 7 Hz, 1'H), 7.15 (d, 1, J = 1 Hz, 6H), 7.45 (dd, 1, J = 8 Hz, 5 Hz, pyridine 5H), 8.34 (dt, 1, J = 8 Hz, 2 Hz, pyridine 4H), 8.86 (br d, 1, J = 4 Hz, pyridine 6H), 9.30 (br s, 1, pyridine 2H).

Anal. Calc. for $C_{16}H_{16}N_6O_5$: C, 51.61; H, 4.33; N, 22.57. Found: C, 51.69; H, 4.38; N, 22.52.

2.2. *AZT nitotinate methiodide [5'-(1-methyl-3-pyridinium-carbonyl)-3'-azido-3'-deoxythymidine(3)]*

AZT nicotinate (**2**, 1.35 g, 2.6 mmol) was dissolved in acetone (30 ml). Iodomethane (1.5 ml, 24 mmol) then was added to this solution and the reaction mixture was refluxed for 5 h. The reaction mixture was cooled and the yellow precipitate that formed was removed by filtration and dried in vacuo to give 1.85 g (3.6 mmol, yield 100%) of a yellow amorphous powder: UV λ_{max} (CH_3OH) 264 nm (ϵ 13100), 217 nm (ϵ 28000); IR(KBr) 2110 cm^{-1} (N_3), 1740 cm^{-1} (ester C=O); NMR (D_2O) δ: 1.80 (s, 3, 5-CH_3); 2.6–2.7 (m, 2, 2'H's); 4.34 (m, 1, 4'H), 4.51 (s, 3, NCH_3), 4.58 (q*, 1, J = 7 Hz, 3'H), 4.7–4.9 (m, 2, 5'H's), 6.19 (dd, 1, J = 7 Hz, 5 Hz, 1'H), 7.50 (s, 1, 6H), 8.24 (t*, 1, J = 7 Hz, pyridine 5H), 9.06 (br d, 2, J = 7 Hz, pyridine 4 and 6 H), 9.49 (s, 1, pyridine 2H).

Anal. calc. for $C_{17}H_{19}N_6O_5I$: C, 39.70; H, 3.72; N, 16.34; I, 24.68. Found: C, 39.86; H, 3.86; N, 16.19; I, 24.51.

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* This represents an apparent multiplicity resulting from either fortuitous nearly equivalent J values or J values approaching zero.

2.3. *HPAZT [5'-(1,4-dihydro-1-methyl-3-pyridinylcarbonyl)-3'-azido-3'-deoxythymidine(4)]*

The pyridinium salt (**3**, 100 mg, 0.2 mmol), sodium dithionite ($Na_2S_2O_4$, 174 mg, 1 mmol) and sodium bicarbonate (84 mg, 1 mmol) were combined as solids and stirred in a flask in an ice-bath. Cold deaerated water (10 ml) was added to the mixture. Then the ice-bath was removed and the reaction mixture was stirred for 20 min at ambient temperature and then filtered. The precipitate was dried in vacuo. The yield was 50 mg (0.13 mmol, 65%) of a pale yellow amorphous powder; UV λ_{max} (CH_3OH) 360 nm (ϵ 7520), 265 nm (ϵ 13200), 210 nm (ϵ 25600); IR (KBr) 2110 cm^{-1} (N_3); m/z = 389 (M + H); NMR ($CDCl_3$) δ: 1.93 (s, 3, 5-CH_3), 2.23 (dt, 1, J = 14.7 Hz, 2'-H_{ax}), 2.49 (m, 1, J = 14 Hz, 7 Hz, 2'H_{eq}), 2.94 (s, 3, N-CH_3), 3.10 (br s, 2, pyridine 4H), 4.13 (q*, 1, J = 4 Hz, 3'H), 4.22 (dt, 1, J = 7 Hz, 4 Hz, 4'H), 4.37, 4.40 (dd, 1 each, J = 12, 4 Hz, both 5'H), 4.79 (dt, 1, J = 8 Hz, 4 Hz, pyridine 5H), 5.64 (dd, 1, J = 8 Hz, 2 Hz, pyridine 6H), 6.16 (t*, 1, J = 7 Hz, 1'H), 7.02 (s, 1, pyridine 2H, 7.25 (s, 1, 6H), 8.83 (br s, 1, 3 NH).

Anal. calc. for $C_{17}H_{20}N_6O_5$ 1/2H_2O: C, 51.38; H, 5.33; N, 21.15; Found: C, 51.36; H, 5.29; N, 20.98.

2.3.1. Cells

Human lymphocyte MT-4 cells were kindly provided by Dr N. Yamamoto (Yamaguchi University, Yamaguchi, Japan). MT-4 cells were cultivated in RPMI 1640 medium (Gibco) and 2 mM Hepes buffer, 10% (v/v) inactivated fetal calf serum (FCS) (Gibco) and 2 mM L-glutamine (Flow Laboratories).

Murine C3H embryo fibroblasts were cultured in Eagle's minimum essential medium (Gibco) containing 10% (v/v) inactivated FCS and 2 mM L-glutamine.

2.3.2. Viruses

$HTLV\text{-}III_B$ (designated HIV) stock was derived from a pool of American patients with AIDS and obtained from the supernatant of HIV-infected H9 cell cultures [26].

Moloney murine sarcoma virus (MSV) stock was prepared from tumors induced upon intramuscular inoculation of 10-day-old NMRI mice with MSV, as described in [27].

2.3.3. Anti-HIV assays

MT-4 cells (5×10^5 cells/ml) were suspended in fresh culture medium and infected with 200 $CCID_{50}$ (cell culture infective dose – 50) HIV per ml cell suspension. Then 100 μl of the infected cell suspension was added to 100 μl of an appropriate dilution of test compound in 200 μl microplate wells (i.e. 20 $CCID_{50}$ HIV/200 μl well/5×10^4 cells), and further incubated at 37°C. After incubation for 5 days, viable cell counts were determined for both virus-infected cell cultures and non-infected cell cultures. The 50% effective dose (ED_{50}) and 50% cytotoxic dose (CD_{50}) were defined as the compound concentrations which caused a 50% reduction in the number of viable cells (as compared to the untreated uninfected cell cultures) in the virus-infected and non-infected cell cultures, respectively.

2.3.4. Transformation of C3H mouse embryo fibroblasts by Moloney murine sarcoma virus (MSV)

C3H cells were seeded at 20000 cells per ml into wells of Costar Tissue Culture Cluster plates (48 wells per plate). 24 h later, cell cultures were infected with 80 foci-forming units of MSV for 90 min, whereupon the culture medium was replaced by 1 ml fresh medium containing different concentrations of the test compounds. After 6 days, transformation of the cells was examined microscopically.

2.4. *Chemical oxidation studies*

2.4.1. Oxidation by H_2O_2

HPAZT (**4**, 5 mg) was dissolved in CH_3CN (1 ml) and this solution was added to H_2O_2 (3%, 2 ml). The reaction mixture was maintained at 40°C and aliquots were removed at various times for HPLC analysis. At 30 min, partial oxidation to compound **3** had occurred together with hydrolysis to *N*-methylnicotinic acid and AZT. After 3 h, HPAZT had disappeared from the reaction mixture. After 24 h, all of compound **3** was converted to *N*-methylnicotinic acid and AZT. When compound **3** was added to 3% H_2O_2 solution at 40°C, hydrolysis at AZT was 30% complete in 1 h.

2.4.2. Oxidation by $AgNO_3$

HPAZT (**4**, 5 mg) was dissolved in saturated $AgNO_3/CH_3OH$ (1 ml). The reaction mixture was stirred for 15 min at room temperature and then filtered. Analysis by HPLC showed quantitative conversion to compound **3** with no evidence of hydrolysis.

2.4.3. Stability of HPAZT(**4**)

Two separate solutions of HPAZT (**4**) (1 mg/ml in 10% DMSO) were prepared. One was kept at 37°C overnight while the other was stored at −20°C. The latter was unchanged, however, the solution kept at 37°C resulted in oxidation and conversion to an unidentified product (retention time 4.2 min).

3. RESULTS AND DISCUSSION

3.1. *Chemistry*

The target compound (HPAZT, **4**; fig.1) was synthesized according to the general approach successfully employed previously by Bodor and coworkers [21,22]. AZT (**1**) was acylated at the 5'-hydroxyl with nicotinyl chloride in pyridine to give the ester, **2**, in nearly quantitative yield. Quaternization of compound **2** with methyl iodide provided a quantitative yield of the trigonellinate ester (**3**). Reduction of **3** was accomplished with basic sodium dithionite which routinely reduces substituted pyridinium salts to 1,4-dihydropyridines [25]. Under these conditions the azido group was not affected. HPAZT (**4**) was obtained in 65% yield. The product, HPAZT, showed the bands in its ultraviolet spectrum typical for a similarly substituted 1,4-dihydropyridine [21–24,28]. In the NMR spectrum, the dihydropyridine N-CH_3 group underwent a 1.57 ppm upfield shift relative to the N-CH_3 group of **3**. The remainder of the NMR spectrum, including the appearance at 3.1 ppm of the two dihydropyridine C-4 hydrogens, cor-

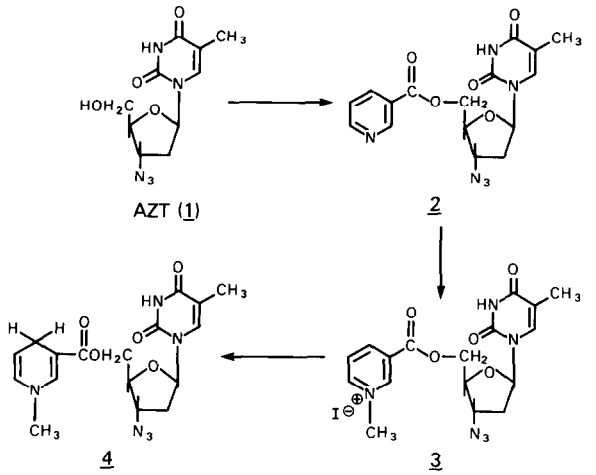

Fig.1. Synthetic scheme for the preparation of 5'-(1,4-dihydro--1-methyl-3-pyridinylcarbonyl)-3'-azido-3'-deoxythymidine (HPAZT, 4).

roborated the 1,4-dihydropyridine structure.

HPAZT (4) was reasonably stable considering its dihydropyridine nature. It was unchanged when stored overnight at −20°C in an aqueous DMSO solution; however, maintenance of such a solution overnight at 37°C led to extensive decomposition. Under proper conditions, HPAZT (4) was readily oxidized back to the pyridinium form. Methanolic silver nitrate gave 3 (with nitrate rather than iodide as anion) in quantitative yield. Hydrogen peroxide also could oxidize 3, but in this case some hydrolysis of the ester linkage also took place.

3.2. *Anti-retrovirus effect*

3'-Azido-3'-dideoxythymidine (AZT, 1) and its esterified counterparts 2, 3 and 4 were evaluated for their inhibitory effects on the transformation of mouse embryo C3H fibroblast cells by Moloney murine sarcoma virus (MSV) (table 1). With an ED_{50} of 0.034 μM, AZT was the most potent inhibitor, whereas PAZT (3, ED_{50} 0.07 μM), HPAZT (4, ED_{50} 0.159 μM) and 2 (ED_{50} 0.24 μM) showed a 2-, 5- and 7-fold higher ED_{50}, respectively. None of the compounds showed any cellular toxicity at 10 μM as recorded microscopically.

When examined for their inhibitory effect on the HIV-induced cytopathogenicity in MT-4 cells, AZT again proved the most powerful inhibitor of HIV replication (ED_{50}: 0.007 μM), followed by HPAZT (4), PAZT (3) and 2 (table 2). Their 50% cytotoxic doses (CD_{50}) were 12, 28, 47, and 99 μM, respectively.

Table 1

Inhibitory effects of 5'-substituted 3'-azido-3'-deoxythymidine derivatives on MSV-induced transformation of murine embryo C3H fibroblasts

Compound	ED_{50}[a] (μM)	MCD[b] (μM)
AZT (1)	0.034	>10
2	0.214	>10
PAZT (3)	0.070	>10
HPAZT (4)	0.159	>10

[a] 50% antiviral effective dose

[b] Minimum cytotoxic dose or dose required to cause a microscopically detectable alteration of normal cell morphology

Consequently, the selectivity index of AZT was about 1700, and that of 2, PAZT (3) and HPAZT (4) 2700, 3600, and 2800, respectively.

Thus, in both the human and murine retrovirus system, the AZT derivatives exhibited an anti-retrovirus potency and selectivity that were similar to those of AZT. Therefore, one may assume that the AZT derivatives behave as prodrugs of AZT, and thus release the free nucleoside derivatives in the extra- or intracellular environment during incubation of the derivatives with the cells.

Since phosphorylation of AZT by thymidine (dThd) kinase is a prerequisite for its anti-retrovirus activity [29,30], the addition of high concentrations of dThd should prevent the activity of the AZT derivatives if their activity is, as presumed, due to the release of AZT. Indeed, addition of 250 μM dThd (in the presence of 500 μM 2'-deoxycytidine to avoid cytotoxicity of dThd) resulted in a decrease of the anti-HIV activity of AZT by more than 3 orders of magnitude, and, likewise, the anti-HIV activity of the AZT derivatives was decreased to the same extent (not shown). Furthermore, the cytotoxicity of all compounds for MT-4 cells was completely annihilated (CD_{50} > 400 μM) following addition of dThd (not shown). Thus, from the reversing effects of dThd on both the anti-HIV activity and cytotoxicity of the AZT derivatives one may infer that the latter act via the intermediary release of AZT.

3.3. *Biochemistry and pharmacology*

To address the question of whether or not HPAZT (4) could undergo the requisite series of

Table 2

Inhibitory effect of 5'-substituted 3'-azido-3'-deoxythymidine derivatives on HIV-induced cytopathogenicity in MT-4 cells

Compound	ED_{50}[a] (μM)	CD_{50}[b] (μM)
AZT (1)	0.007	12
2	0.037	99
PAZT (3)	0.013	47
HPAZT (4)	0.010	28

[a] 50% antiviral effective dose
[b] 50% cytotoxic dose

reactions necessary to fulfill its potential as a prodrug form which may be transported into the central nervous system and oxidized to the positively charged pyridinium form, the metabolic fate of HPAZT (4) was examined in vitro in cytoplasmic extracts of rat brain. Incubation of HPAZT at 37°C in a Tris buffer with 10000 × *g* supernatant from rat brain homogenate revealed that over a period of 2 h, HPAZT was converted gradually to the pyridinium form, PAZT (3) (fig.2). In conjunction with the disappearance of HPAZT from the incubation mixture, the concentration of PAZT (3) increased to an apparent steady state level and then began to decrease. Finally, after a lag phase the concentration of AZT (1) itself increased. These results demonstrate clearly that HPAZT can be converted readily to the pyridinium form PAZT by the enzymes present in rat brain.

The AZT that forms during the course of the incubation of fig.2 most probably arises nearly exclusively from the newly formed PAZT (3) itself rather than HPAZT (4). The neutral ester linkage of the nicotinic acid esters seems relatively resistant to premature hydrolysis. The simple nicotinate ester (2) was quite stable during the course of a 2-h incubation under conditions identical to that of fig.2 (not shown). In Tris buffer or in rat blood, HPAZT (4) itself also was stable during incubation at 37°C. Conversely, PAZT (3) underwent a rapid hydrolysis to AZT (1) upon incubation with brain extract as in fig.2 or in Tris buffer. The half-life of PAZT under such conditions was approx. 40 min.

Preliminary in vivo experiments have been carried out to determine if the above conversion of HPAZT to PAZT might occur in an intact animal. Male Sprague-Dawley rats were injected intravenously (tail vein) with HPAZT (20 mg/kg) in 0.1–0.2 ml of DMSO. 1 h after injection, the animals were killed, the brains homogenized with acetonitrile/water (3:1) and, after centrifugation, the supernatants were analyzed by HPLC using the same system as employed for the experiments of fig.2. Under such conditions PAZT (3) was detected readily in the brain at levels of 1–3 μg/g brain. Thus far no experiments have been conducted to attempt to optimize these levels.

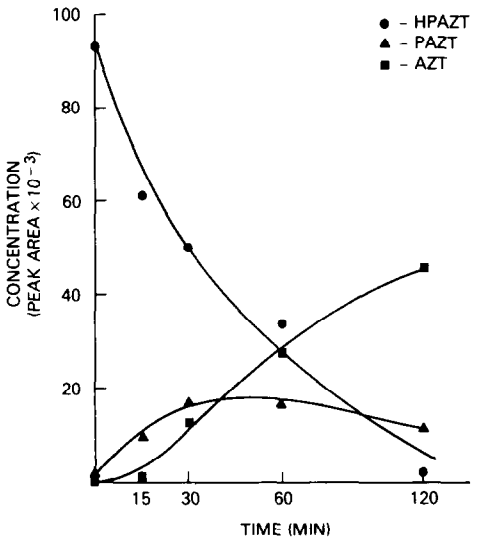

Fig.2. Metabolism of HPAZT (4) in rat brain cytosol. HPAZT was incubated at a final concentration of 2×10^{-4} M at 37°C in a reaction mixture final volume of 500 μl of a 1×10^{-3} M solution of HPAZT in 10% DMSO 50 mM bis, pH 7.4. At various time intervals aliquots were removed and diluted directly into 5 vols of ice-cold acetonitrile. These samples were kept at −20°C until the conclusion of the reaction at which point all samples were centrifuged for 10 min at 10000 × *g* and the supernatant carefully removed. Analysis was by injection of a 20 μl aliquot into the HPLC column and elution was as described in the experimental section. In the absence of brain extract, no pyridinium salt was formed nor was any hydrolysis of HPAZT detected under the same incubation conditions.

In summary, an efficient synthesis of a prodrug form of AZT has been described. This dihydropyridine derivative (HPAZT, 4) shows a high antiviral activity against HIV in MT-4 cells as well as prevention of transformation of mouse embryo fibroblasts by Moloney murine sarcoma virus. This activity is prevented by thymidine and most probably is mediated by hydrolysis to AZT.

HPAZT (4) is readily converted to the quaternary pyridinium salt PAZT (3) in rat brain cytosol. In turn PAZT (3) is readily hydrolyzed by a non-enzymatic pathway to AZT. Preliminary experiments have shown that upon injection of HPAZT in the periphery of the rat, the pyridinium salt (3) can be isolated from the brain. These results suggest that HPAZT (4) should be investigated further as a means of delivering AZT to the central nervous system to inhibit HIV replication.

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