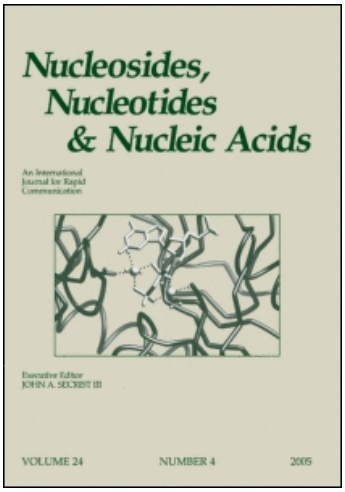


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# ANTIVIRAL NUCLEOSIDE DRUG DELIVERY VIA AMINO ACID PHOSPHORAMIDATES

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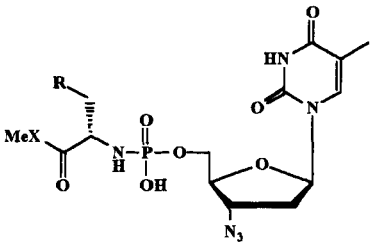
**ABSTRACT:** Stable and water soluble amino acid phosphomonoester amidates of AZT were synthesized and shown to have potent anti-HIV-1 activity. Intracellular and cell extract metabolism studies revealed that these compounds are likely to be enzymatically converted to the corresponding monophosphates. In addition, we have shown that the half life and tissue distribution of a phosphoramidate of AZT is 5 and 10-fold greater, respectively, than AZT.



Nucleosides have become an important class of agents for viral chemotherapy. Unfortunately, the relatively short plasma half-life and low tissue distribution of agents, such as AZT, have hampered efforts to achieve their maximal therapeutic effect. Furthermore, the repertoire of antiviral nucleosides is limited by the inability of some nucleosides to serve as substrates for thymidine kinase. Several approaches for the delivery of nucleotide monophosphates have been developed. [1] In particular, lipophilic phosphoramidate diesters of D-alanine methyl ester of d4T and water soluble human serum albumin conjugates of AZT-MP have exhibited potent antiviral activity.[2, 3] We hypothesized, therefore, that hydrophobic amino acid phosphoramidate monoesters of antiviral nucleosides would be more water soluble than phosphoramidate diesters, but would still able to cross the cellular membrane and exert antiviral activity. To test our hypothesis, we designed and synthesized phosphoramidate monoesters of AZT, evaluated their *in vitro* antiviral activity, conducted mechanistic experiments of their mode of action

**Table 1. Antiviral Activity of AZT Phosphoramidates**



| Compound | R = | X = | IC50 (μM) PBMCs Donor 1 | IC50 (μM) PBMCs Donor 2 |
|---|---|---|---|---|
| **1** | Phenyl | O | 30 | 15 |
| **2** | Phenyl | NH | 0.180 | 0.250 |
| **3** | 3-Indolyl | O | 0.300 | 0.350 |
| **4** | 3-Indolyl | NH | 0.700 | 1.80 |
| **5** | Isopropyl | O | 1.30 | 0.900 |
| **6** | Dimethyl | O | 1.30 | 1.00 |
| **7** | Hydrogen | O | 0.050 | 0.030 |
| **AZT** | --- | --- | 0.003 | 0.010 |



in peripheral blood mononuclear cells (PBMCs) and the T-leukemia cell line, CEM, and preliminarily characterized their pharmacokinetic behavior in the rat.

Phosphoramidates of AZT were constructed from either the methyl ester or methyl amide of aliphatic and aromatic amino acids by a previously reported procedure.[4] These compounds were shown to be highly water soluble and stable indefinitely in cell culture media and human blood. The viral growth inhibitory activity of the AZT phosphoramidates was determined by monitoring the infection of peripheral blood lymphocytes from two donors by LAI-HIV-1. (**Table 1**) Of the methyl ester phosphoramidates the alanine, **7**, ($IC_{50}$=30-50 nM) and tryptophan, **3**, ($IC_{50}$=300-350 nM) derivatives exhibited the greatest inhibitory activity of virus production. Interestingly, the activity of the methyl amide of the phenylalanine compound, **2**, was 60- to 166-fold greater than the corresponding methyl ester, **1**, while the methyl amide of the tryptophan compound, **4**, was 2- to 5-fold less active than the methyl ester, **3**. At concentrations of 100 μM or greater, none of the compounds proved to be cytotoxic to PBMCs or CEM cells.

**Table 2. Antiviral Activity of AZT Phosphoramidates with Pretreatment, AZT resistant HIV-1 and Exogenous Thymidine**

| Compound | IC50 (μM) a PBMCs 0 hr Preincubation | IC50 (μM) a PBMCs 2 hr Preincubation | IC50 (μM)b PBMCs AZT-r virus | IC50 (μM) a,b PBMCs + 50 μM Thymidine |
|---|---|---|---|---|
| **1** | 15 | 1.0 | >100 | >100 |
| **3** | 0.350 | <0.010 | 30 | 30 |
| **AZT** | 0.010 | 0.015 | 1.0 | 0.300 |

[a] The assay was carried out as previously described with LAI-HIV-1 and PBMCs from Donor 2, [b] Compounds and virus were simultaneously added.



Pretreatment of the PBMCs with compounds **1** and **3** for two hours prior to infection significantly enhanced the antiviral activity of these compounds by 35-fold, indicating that either cellular uptake or conversion of the phosphoramidates to phosphorylated AZT was slower than that for AZT. (**Table 2**) Mechanistically, the phosphoramidates appear to act similarly to AZT, since their activity is greatly reduced in the presence of AZT resistant virus (AZT-r) and exogenous thymidine. (**Table 2**) Time of addition studies demonstrated that the phosphoramidates interrupt the viral life cycle after viral fusion, but before protein translation. In addition, neither compound **1** nor **3** appreciably inhibited HIV-1 protease, integrase, reverse transcriptase or RNase H.

Intracellular and cell-free extract metabolism studies of the phosphoramidates **1** and **3** were carried out in order to more fully understand their mechanism of action. PBMCs and CEM cells were incubated with 100 μM of AZT, **1**, or **3** and the total amounts of phosphorylated AZT (mono-, di-, and triphosphate) and phosphoramidate was determined by a coupled radioimmunoassay-reverse phase HPLC (RIA-RPHPLC).[5] (**Table 3**) CEM cells treated with AZT contained approximately 7-fold more phosphorylated AZT than those treated with either **1** or **3**.

In contrast, 50- to 230-fold more phosphorylated AZT was observed in CEM cells treated with either AZT or compounds **1** and **3** compared to the amounts observed in PBMCs. However, similar amounts of phosphorylated AZT were generated in PBMCs

**Table 3. Intracellular Amounts of Phosphorylated AZT and Phosphoramidates of AZT**

| Compound[a] | Phosphorylated AZT[b] | | Phosphoramidate[b] | | IC50 (μM) | |
|---|---|---|---|---|---|---|
| | CEM | PBMC | CEM | PBMC | CEM[c] | PBMC |
| **AZT** | 810.9 | 3.5 | ------ | ------ | <0.001 | 0.010 |
| **1** | 120.7 | 2.4 | 7.8 | 62.8 | <0.001 | 15 |
| **3** | 115.8 | 2.0 | 14.6 | 66.2 | 0.003 | 0.350 |

[a] Cells were incubated with 100 μM compound for 17 hr and the intracellular amounts determined by RPHPLC-RIA

[b] Values are expressed in pmol/million cells. [c] Antiviral activity determined at the end of 6 days with p24 ELISA assay (Ref. 6).



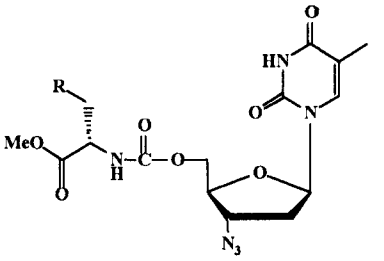
incubated with AZT and either **1** or **3**. The amount of intracellular phosphoramidate, however, was found to be 4.5- to 8.0- fold higher in treated PBMCs relative to CEM cells. Nevertheless, although cellular uptake of the phosphoramidates was observed, the antiviral activity of the phosphoramidates could not be correlated with the relative amounts of intracellular phosphorylated AZT or phosphoramidate.[5, 7] Studies carried out at lower concentrations of the phosphoramidates may yet reveal a correlation.

Although intracellular amounts of AZT-phosphates were observed, the role of direct P-N bond cleavage by an intracellular phosphoramidase could not be ascertained since intracellular uptake by the thymidine kinase deficient cell line, CEM $TK^-$ was not observed. However, incubation of the phosphoramidates **1** and **3** at a concentration of 1 mM (37°C, pH 7.5, 45 min) with cell free extracts of CEM cells demonstrated that the compounds were readily converted to AZT-MP.

To probe the enzymatic character of the P-N bond cleavage, the analogs **8** and **9** were synthesized in which a carbamate linker was substituted for the phosphoramidate moiety. Incubation of the phosphoramidates with a comparable amount of the carbamate analogs, **8** amd **9**, inhibited the production of AZT-MP by 60 to 99 %.

Although the phosphoramidase activity of the CEM cell lysates could not be correlated with the antiviral activity, the concentrations used during the metabolism

**Figure 1. Structure of Carbamate Analogs**



**8** R = Phenyl
**9** R = 3-Indolyl



experiment, however, maybe well above the $K_m$ for either phosphoramidate. The increased efficacy of the phenylalanine carbamate **8** relative to **9** maybe a reflection of this phenomenon. On going experiments attempting to isolate the putative phosphoramidase should shed light on these inconsistiencies.

Because the phosphoramidates of AZT have been shown to be stable and effective antiviral agents, pharmacokinetic studies of these compounds in rats were initiated. Five female Sprague-Dawley rats were used. Three of the rats were used in a crossover study in which they first received an iv dose of 19 μmol/kg AZT, had blood samples collected over 24 hours, were rested for a day, then received 19 μmol/Kg of compound **1** as an iv dose, with blood samples taken for a day. Pharmacokinetic parameters were calculated by standard non-compartmental means.[8] There was no difference in the pharmacokinetics of **1** in the animals that had received AZT first, or animals that had received only **1**, so the pharmacokinetic data of **1** in these five animals were combined. (**Table 4**)

**Table 4. Rat Plasma Pharmacokinetics of AZT and Phosphoramidate 1**

| Parameter | AZT | 1 |
|---|---|---|
| **Half life (min)** | 24.5 ± 9.97 | 121.0 ± 46.8 |
| **CL (mL/min)** | 14.99 ± 5.25 | 35.3 ± 10.1 |
| **Vds (L)** | 1.71 ± 1.31 | 16.7 ± 7.98 |

The phosphoramidate level in plasma after iv administration of **1** was 1.6 μM at 15 min and decreased with a half-life of 121.3 ± 46.8 min. In contrast, when AZT was administered, its half-life was 24.8 ±10.0 min, and the limit of quantitation was reached within 4 hours. The half-life of AZT was similar to that in previous reports. [9] The half-life of **1** was significantly longer than that of AZT (unpaired t-test, $p<0.05$). Surprisingly, the total body clearance in plasma of the phosphoramidate **1** was significantly increased (35.3± 10.1 ml/min) over AZT (unpaired t-test, $p<0.05$). However, the volume of distribution for **1** was 16.7 ± 8.0 L vs. 1.70 ± 1.31 L for AZT (statistically significant at $p < 0.05$). This indicates that **1** has a 9-fold greater ability to distribute outside the plasma than does AZT. The large volume of distribution of **1** outweighs its higher CL, thus producing a significantly longer half-life than AZT and greater access to tissue space than AZT. Metabolism studies of **1** in 100% rat plasma revealed that unlike human plasma ($t_{1/2}$= >6 days), the methyl ester is subject to slow hydrolysis ($t_{1/2}$= 4.12 hr), which may be an important factor governing the plasma half-life of this compound in rats.

In conclusion, phosphoramidate monoesters of antiviral nucleosides are water soluble, highly stable in plasma and potent antiviral agents. Moreover, these compounds were shown to be converted intracellularly to the corresponding antiviral nucleotides and to have enhanced their pharmacokinetic parameters. The generality of this delivery procedure for other antiviral and antitumor nucleotides is currently under investigation and will be reported in due course.[10]



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