Pergamon

*Bioorganic & Medicinal Chemistry Letters*, Vol. 5, No. 16, pp. 1819-1824, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00302-9

# AROMATIC AMINO ACID PHOSPHORAMIDATE DI- AND TRIESTERS OF 3'-AZIDO-3'-DEOXYTHYMIDINE (AZT) ARE NON-TOXIC INHIBITORS OF HIV-1 REPLICATION

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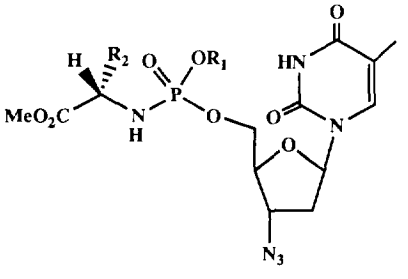
**Abstract.** A series of aromatic, water soluble and non-toxic amino acid phosphoramidate di- and triesters of zidovudine (AZT) were shown to inhibit the replication of HIV-1 in human peripheral blood mononuclear cells (PBMC). Cells treated with the active AZT diesters contained four fold more phosphorylated AZT than those treated with AZT, and no free AZT.

**Figure 1**



**1**, $R_1$ = Cyanoethyl, $R_2$ = Benzyl
**2**, $R_1$ = Methyl, $R_2$ = Benzyl
**3**, $R_1$ = Hydrogen, $R_2$ = Benzyl
**4**, $R_1$ = Cyanoethyl, $R_2$ = 3-Indolylmethyl
**5**, $R_1$ = Methyl, $R_2$ = 3-Indolylmethyl
**6**, $R_1$ = Hydrogen, $R_2$ = 3-Indolylmethyl

Chemotherapeutic nucleosides that function as inhibitors of polymerases must be phosphorylated intracellularly by endogenous nucleoside kinases to their respective mono-, di- and triphosphate derivatives. Unfortunately, biological targets, such as the herpes-virus or tumor cells, can readily become resistant to these nucleosides by either altering or removing the nucleoside kinase responsible for mono-phosphorylation. For example, the efficacy of antiviral nucleosides against HIV is reduced in monocytes and macrophages, since they contain only low levels of nucleoside kinases.[1-3] In addition, in quiescent lymphocytes, thymidine analogs, such as AZT, are poorly phosphorylated.[4] Because of these concerns, a number of approaches have been developed for the delivery of mono- and diphosphorylated nucleosides.[5-13]

Recently, several attempts have been made to increase the therapeutic index of AZT by delivering the phosphorylated compound in the form of a phosphate or phosphoramidate prodrug.[14] In particular, due largely to their reduced cytotoxicity, hydrophobic alkyl and aryl triesters of glycine, alanine, leucine, and phenylalanine phosphoramidates of AZT are highly effective and selective

inhibitors of HIV viral replication.[15-17] In a different approach, the mono-phosphate of AZT was effectively delivered to virally infected cells by coupling AZT monophosphate to a glycosylated carrier protein through exposed lysines.[18] Activation of the lysine phosphoramidate diesters of AZT was proposed to occur intracellularly via an uncharacterized process. We reasoned, therefore, that sufficiently non-polar but water soluble amino acid phosphoramidate diesters of AZT may be capable of delivering AZT-5'-monophosphate more effectively than the corresponding triesters, since the number of potential hydrolytic activation steps would be reduced.

**Table 1. Effect of Nucleoside Derivatives on HIV-1 Replication, Reverse Transcriptase Activity and Cytotoxicity in Human Cells.[a]**

| Compound[b] | $EC_{50}$ (μM) HIV-1 in PBMC | $IC_{50}$ (μM)[c] HIV-RT | $CC_{50}$ (μM) Toxicity toward PBMC | $CC_{50}$ (μM) Toxicity toward CEM cells |
|---|---|---|---|---|
| **1** | >1.0 | >100 | >100 | >100 |
| **2** | 0.09 | N.D. | >100 | >50 |
| **3** | 0.6 | >100 | >100 | >100 |
| **4** | 0.4 | >100 | >100 | >100 |
| **5** | 0.3 | N.D. | >50 | >50 |
| **6** | 0.01 | >100 | >100 | >100 |
| **AZT** | 0.08 | 0.01[d] | >100 | 14.3 |

[a]$EC_{50}$: 50% effective molar concentration required to inhibit the replication of HIV by 50%. Values were determined from a set of six values, as previously described.[20] [b]Prior to biological testing, the purities of the individual compound preparations were shown to be ≥ 99.3 % by HPLC.[19] [c]$IC_{50}$ values were determined as previously described with (rA)(dT).[18] [d]The $IC_{50}$ for inhibition of HIV-RT with AZT was determined with AZT triphosphate.

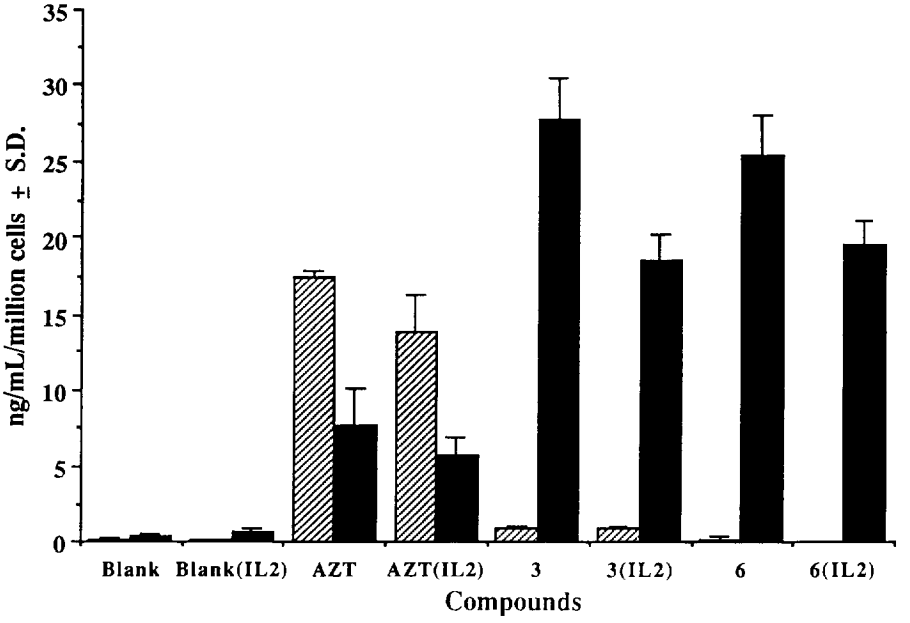
To test our hypothesis, a series of stable, water soluble aromatic amino acid di- and triester nucleoside phosphoramidates of AZT (Figure 1, **1-6**) were constructed.[19] The triesters, **1**, **2**, **4**, and **5** were shown to be a 1:1 mixture of two diastereomers by phosphorous NMR. The anti-HIV activities and toxicity of compounds **1-6** were determined by standard assay procedures and are listed in Table 1.[20]

With the exception of **1**, the phosphoramidate di- and triesters of AZT exhibited significant activity against HIV-1 in PBMC. Analysis of the inhibition data revealed a 1.1 to 13 fold increase in the $EC_{50}$ for compounds **1-5** relative to AZT. However, an eight fold decrease in the $EC_{50}$ value was observed for compound **6** (Table 1). In addition, at concentrations up to 100 μM, compounds **1-6** were found not to be cytotoxic to PMBC or CEM cells, whereas AZT was toxic to CEM cells ($CC_{50}$=14.3).

Comparison of the $EC_{50}$ values for the AZT di- and triesters revealed unique structure-activity requirements. First, despite the substantial difference in hydrophobicity due to the cyanoethyl moiety (data not shown), a modest 1.7 fold enhancement of the activity of the AZT phenylalanine diester, **3**, relative to the cyanoethyl triester, **1**, was observed. Nevertheless, the methyl triester, **2**, was nearly six fold more active than the diester, **3**. In contrast to the phenylalanine derivatives, a 40 fold enhancement of the activity of the AZT tryptophan diester, **6**, was observed relative to the cyanoethyl triester, **4**, and a 30 fold enhancement was observed relative to the methyl triester, **5**.

When the influence of the tryptophan and phenylalanine moieties were directly compared, a modest 2.5 fold increase in activity was demonstrated for the tryptophan cyanoethyl AZT triester, **4**, over the phenylalanine derivative, **1**. In contrast, the phenylalanine methyl triester, **2**, was three fold more active than the tryptophan derivative, **5**. When the phenylalanine and tryptophan diesters were compared, a 60 fold increase in the activity of the tryptophan AZT diester, **6**, over the phenylalanine diester, **3**, was observed, despite their similar lipophilicities (data not shown). Consequently, the antiviral activity of the 3-indolylmethyl moiety at position $R_2$ is enhanced by a hydrogen at $R_1$, while the antiviral activity of the benzyl moiety at $R_2$ is enhanced by a methoxy moiety at $R_1$.

**Figure 2. Quantitation of Free AZT and Phosphorylated AZT in Activated PBMC with or without added IL-2.[a]**




[a]Striped blocks, ▨, refer to the amount of free AZT nucleoside determined in the sample, while the solid blocks, ■, refer to the amount of phosphorylated AZT measured in the sample. The notation (IL2) refers to the results of experiments in which interleukin-2 (IL2) was added in addition to PHA. Blank samples refer to untreated PBMC grown under identical conditions.

Due to the reasonable antiviral activity and low toxicity of the phosphoramidates, mechanistic studies were initiated. Preliminary experiments revealed that compounds **1-6** were not inhibitors of HIV reverse transcriptase (Table 1) nor were they substrates for phosphodiesterase I or alkaline phosphatase (data not shown). However, **3** and **6** were found to be substrates for acid phosphatase.

Consequently, we undertook to determine and compare the amount of AZT and total phosphorylated AZT (i.e., mono-, di- and triphosphorylated AZT and AZT phosphoramidate) in PBMC treated with either AZT, **3**, or **6**, since their $EC_{50}$ values differed by 60 fold. Typically, after incubation of the compounds with activated PBMC, the lysates were divided in two and the concentration of AZT determined with a radioimmunoassay (Incstar, Corp. (Stillwater, MN)) (Figure 2).[21] Furthermore, the concentration of total phosphorylated AZT was quantitated with this assay by treating cell free extracts with acid phosphatase.[21]

As had previously been observed by Gao and coworkers, treatment of PBMC with AZT results in the production of a significant amount of phosphorylated AZT (Figure 1).[4] In contrast, although little or no free AZT was observed, PBMC incubated with **3** or **6** contained nearly four fold more phosphorylated AZT. Furthermore, the total amount of AZT for cells treated with AZT, **3** or **6** was essentially equivalent, suggesting little difference in their ability to cross the cell membrane. Regardless of whether they were treated with AZT, **3** or **6**, approximately 30% less intracellular AZT was observed for cells in which IL-2 had been added to the growth media (Figure 2).

Taken together, these results suggest that the 60 fold difference between the ability of **3** and **6** to inhibit HIV replication may rely on the preference of a phosphoramidase to convert the typtophan phosphoramidate diester to AZT monophosphate, relative to the phenylalanine phosphoramidate diester. It remains to be determined whether the triesters are first converted to the corresponding phosphoramidates or phosphate esters, before being processed to the monophosphate or free nucleoside. Nevertheless, because of the modest enhancement in antiviral activity and reduced toxicity of the tryptophan phosphoramidate of AZT relative to AZT and the inability of the phosphoramidates to inhibit HIV-RT, it is reasonable to conclude that **6** maybe impeding the function of a cellular or viral molecular species, other than reverse transcriptase, that plays an essential role in viral replication. To further clarify the mechanism of action of these compounds, we are currently attempting to determine the amount of mono-, di- and triphosphate AZT in uninfected and infected PBMC treated with the phosphoramidate derivatives. The results of these studies should provide further insights into a potentially useful phenomenon.

**Acknowledgements.** The authors wish to acknowledge partial funding of these studies through NIH grants (CA61909)(CRW) and (AI-25899) (RFS) as well as the University of Minnesota (CRW) and the Department of Veterans Affairs (RFS). We thank Ms. Angela McMillan and Irena Lieberman for excellent technical assistance and Dr. Rory Remmel and Dennis Weller for their expert guidance with the RIA assay.

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(*Received in USA* 16 *January* 1995; *accepted* 3 *July* 1995)