# Amino Acid Phosphoramidate Monoesters of 3′-Azido-3′-deoxythymidine: Relationship between Antiviral Potency and Intracellular Metabolism

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A series of phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT) bearing aliphatic amino acid methyl esters (**3a**, **3c**, **4a**, **4c**, **5**−**7**) and methyl amides (**3b**, **3d**, **4b**, **4d**) was prepared and evaluated for anti-HIV-1 activity in peripheral blood mononuclear cells (PBMCs). These compounds, which showed no cytotoxicity at concentrations of 100 $\mu$M, were effective at inhibiting HIV-1 replication at concentrations of 0.08−30 $\mu$M. Since the D-phenylalanine and D-tryptophan derivatives exhibited equivalent or enhanced antiviral activity compared to their L-counterparts, there appears to be no specific stereochemical requirement for the amino acid side chain. In addition, except for the D-phenylalanine derivatives, the methyl amides had greater antiviral activity than the corresponding methyl esters. On the basis of the observed antiviral activity of AZT phosphoramidate monoesters **3a** and **4a** in PBMCs and CEM cells, the mechanism of action of these two compounds was investigated. AZT-MP and substantial amounts of either phosphoramidate were detected in PBMCs and CEM cells treated with either **3a** or **4a**. Biological mechanistic studies demonstrated that **3a** and **4a** affect viral replication at a stage after virus entry and preceding viral DNA integration. Quantitation of the intracellular levels of AZT-TP in PBMCs and CEM cells treated with **3a** and **4a** in the presence and absence of exogenous thymidine correlated the intracellular levels of AZT-TP to the antiviral activity and suggested that AZT-TP was responsible for the activity observed. In addition, the reduced toxicity of **3a** and **4a** toward CEM cells relative to AZT correlated with reduced levels of total phosphorylated AZT and not AZT-TP. Stable carbamate analogues of **3a** and **4a** were prepared and shown to inhibit the production of AZT-MP from cell-free extracts of CEM cells, further suggesting that a phosphoramidate hydrolase may be responsible for intracellular P−N bond cleavage. Taken together, these results suggest that the biological activity and intracellular metabolism of nucleoside phosphoramidate monoesters are distinct from that of phosphoramidate diesters.

## Introduction

Nucleoside-based reverse transcriptase inhibitors (NRTIs), which function as DNA chain terminators and competitive inhibitors of viral reverse transcriptase (RT), have been widely used in treating HIV-1 infection. Their antiviral activity depends on the cellular uptake and conversion to the corresponding 5′-triphosphate by cellular kinases.[1] For example, thymidine kinase, thymidylate kinase, and nucleoside diphosphate kinase are responsible for the sequential phosphorylation of AZT (3′-azido-3′-deoxythymidine) and d4T (2′,3′-didehydro-3′-deoxythymidine) to the corresponding mono-, di-, and triphosphates, repectively.[2−4]

This dependence on cellular kinases has limited the efficacy of NRTIs in treating HIV-1 infection. Downregulation of thymidine kinase expression in patients on prolonged AZT therapy has been observed and may be a factor contributing to the emergence of resistance.[5,6] Furthermore, many NRTIs, such as d4T and ddA (2′,3′-dideoxyadenosine), are poorly phosphorylated, thus limiting the intracellular levels of nucleoside triphosphate and correspondingly their antiviral potency.

In an attempt to improve the therapeutic potential of the current NRTIs, various masked monophosphate derivatives, designed to act as prodrugs of the bioactive monophosphate form, have been reported.[7] Among these approaches, nucleoside phosphoramidate derivatives have shown promise as potent antiviral agents, since in several cases they have exhibited enhanced antiviral activity and reduced cytotoxicity when compared to the parent nucleoside.[7]

Previously, we have demonstrated that AZT phosphoramidate derivatives **3a** and **4a** exhibit nanomolar anti-HIV-1 activity in peripheral blood mononuclear cells (PBMCs) and CEM cells with no observable cytotoxicity at concentrations of 100 $\mu$M.[8−11] In addition, we have shown that nucleoside amino acid phosphoramidate monoesters are indefinitely stable in cell culture medium, water, and rat and human plasma (H. Song, C. L. Zimmerman, and C. R. Wagner, personal communication) and exhibit excellent pharmacokinetics in rats.[9−11] To further improve the efficacy, and to better understand the mechanism required for their activation, the antiviral potency of a set of structurally diverse AZT phosphoramidate monoester derivatives was investigated. Furthermore, because the antiviral potency of the

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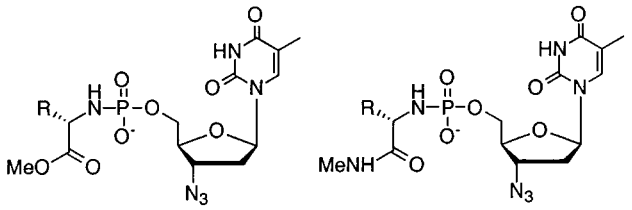
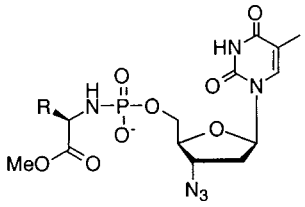
**Chart 1.** Amino Acid Phosphoramidates and Carbamates of AZT



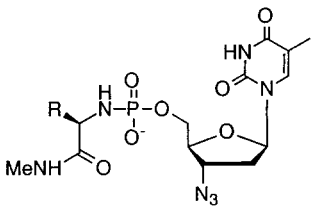
**3a** R = 3-indolyl-$CH_2$-
**4a** R = $PhCH_2$-
**5** R = $CH_3$-
**6** R = $(CH_3)_2CH$-
**7** R = $(CH_3)_2CHCH_2$-



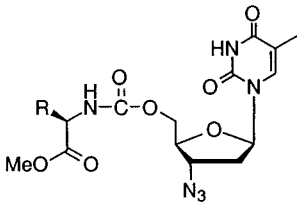
**3b** R = 3-indolyl-$CH_2$-
**4b** R = $PhCH_2$-



**3c** R = 3-indolyl-$CH_2$-
**4c** R = $PhCH_2$-



**3d** R = 3-indolyl-$CH_2$-
**4d** R = $PhCH_2$-



**8** R = 3-indolyl-$CH_2$-
**9** R = $PhCH_2$-

structurally similar phenylalanine and tryptophan AZT phosphoramidate monoesters, **3a** and **4a**, differ subtantially between PBMCs and the T-leukemia cell line, CEM, we have attempted to elucidate the rationale for this biological behavior by carrying out a series of biochemical and cellular studies.

## Results

**Chemistry.** A series of phosphoramidate monoesters of AZT bearing aliphatic amino acid methyl esters (**3a**, **3c**, **4a**, **4c**, **5**−**7**) and methyl amides (**3b**, **3d**, **4b**, **4d**) were prepared from AZT (**1**) and AZT H-phosphonate (**2**), as previously reported[12] (Chart 1). The carbamates **8** and **9** were synthesized from AZT (**1**), as previously described.[13]

**Antiviral Activity in PBMCs.** To probe the importance of phosphoramidate structure on antiviral activity in primary cells, the antiviral activity in PBMCs of a series of AZT phosphoramidate monoesters was evaluated. To control for inter- and intra-individual effects, two different donors (1 and 2) of PBMCs were used for these assays. As shown in Table 1, these compounds exhibited various levels of antiviral activity with no associated cytotoxicity. Changing the L-amino acid moiety from the bulky aryl group to L-valine (**6**) or L-leucine (**7**) led to a minor improvement in potency ($EC_{50}$ = 0.9−1.3 $\mu$M). Conversely, substitution by L-alanine (**5**) led to a substantial increase in potency ($EC_{50}$ = 0.03 $\mu$M) and suggested that decreasing the steric bulk of the amino acid side chain would lead to improved antiviral activity. However, the enhanced activity observed for the L-tryptophan derivative compared to the L-phenylalanine derivative suggests that the steric bulk of amino acid side chain alone was not the only determinant of antiviral activity. In addition, although previously it was suggested that the phosphoramidate monoesters may act by direct inhibition of HIV-1 RT, at a concentration of 100 $\mu$M none of the compounds inhibited HIV-1 RT inhibitor (data not shown).[8]

**Table 1.** Effect of AZT Phosphoramidate Monoester Derivatives on HIV-1 Replication and Cytotoxicity in PBMCs and CEM Cells[a]

| | $EC_{50}$[b,e] | | | $IC_{50}$[c,e] | | |
|---|---|---|---|---|---|---|
| entry | donor 1 PBMCs | donor 2 PBMCs | CEM cells | PBMCs | CEM cells | log $P$[d] |
| **3a** | 2 | 0.35 | 0.004 | >100 | >100 | −0.686 |
| **3b** | 0.7 | 1.8 | nd | >100 | >100 | −1.382 |
| **3c** | 1 | 1 | nd | >100 | >100 | −1.364 |
| **3d** | 0.4 | 0.18 | nd | >100 | >100 | −1.894 |
| **4a** | 30 | 15 | <0.001 | >100 | >100 | −1.836 |
| **4b** | 0.18 | 0.25 | nd | >100 | >100 | −1.492 |
| **4c** | 0.089 | 0.08 | nd | >100 | >100 | −1.329 |
| **4d** | 0.9 | 0.1 | nd | >100 | >100 | −1.701 |
| **5** | 0.05 | 0.03 | nd | >100 | >100 | −1.252 |
| **6** | 1.3 | 0.9 | nd | >100 | >100 | −1.560 |
| **7** | 1.3 | 1 | nd | >100 | >100 | −1.498 |
| AZT | 0.006 | 0.007 | <0.001 | >100 | 14.2 | 0.225 |

[a] Values are in $\mu$M. [b] Effective concentration required to inhibit the replication of HIV-1 by 50%. PBMCs were from donors 1 and 2. The limit of quantitation was 0.001 $\mu$M. [c] Concentration required to kill 50% of the cells as compared to the untreated cultures. [d] Values taken from Iyer et al.[12] [e] Values represent an average of three experiments. The variance for the $EC_{50}$ and $IC_{50}$ was less than ±20%.

Substitution of the methyl amide for the methyl ester had little impact on the antiviral activity for the tryptophan derivatives (**3a**, **3b**). However, for phenylalanine derivatives (**4a**, **4b**), this substitution improved the potency of the phenylalanine AZT phosphoramidate 60−166-fold.

The effect of amino acid stereochemistry was also investigated. D-Phenylalanine and D-tryptophan derivatives exhibited similar or better antiviral potency compared to their L-counterparts (Table 1). In particular, the phenylalanine methyl ester **4c** was approximately 200-fold more active than **4a**. Compared to the methyl esters, the methyl amides of the D-tryptophan derivatives exhibited enhanced activity while the D-phenylalanine methyl amides were not as active as the methyl esters.

Analysis of the effect of the partition coefficients for the phosphoramidates on antiviral potency did not reveal a correlation between the log $P$ and $EC_{50}$ values for these compounds. A similar observation was made for these compounds when the partition coefficients and cytotoxicity toward MCF-7 cells was examined.[12]

**Comparison of Antiviral Activity in PBMCs and CEM Cells.** The antiviral activities of AZT phosphoramidate derivatives **3a**, **4a**, and AZT were examined in both HIV-1-infected PBMCs and CEM cells. The amount of virus in culture supernatants was determined by p24 ELISA (for CEM cells) at day 6 postinfection. As shown in Table 1, the activities for compound **3a** and **4a** in CEM cells were both in the nanomolar range (**3a**, $EC_{50}$ = 3.5 nM; **4a**, $EC_{50}$ < 1 nM), similar to the value for AZT ($EC_{50}$ < 1 nM). In both cases, the compounds were

**Table 2.** Antiviral Activity of AZT Phosphoramidates with Pretreated PBMCs, AZT-Resistant HIV-1, and Exogenous Thymidine

| | $IC_{100}$ ($\mu$M)[b] unactivated PBMCs | $EC_{50}$ ($\mu$M)[a] activated PBMCs | | |
|---|---|---|---|---|
| entry | no pretreatment | no pretreatment | 2-h pretreatment | AZT-r virus |
| **3a** | 0.100 | 0.350 | <0.010 | 30 |
| **4a** | >100 | 15 | 1.0 | >100 |
| AZT | 0.010 | 0.010 | 0.015 | 1.0 |

[a] The assay was carried out as previously described with LAI/HIV-1 and PBMCs from donor 2. [b] Concentration necessary to inhibit the production of proviral DNA.

90−30000-fold more potent in CEM cells than in PBMCs (Table 1). In contrast to AZT, neither compound exhibited cytotoxicity toward CEM cells at concentrations as high as 100 $\mu$M.[10]

**Effect of Pretreatment on Antiviral Potency.** Because we previously demonstrated that the uptake of amino acid phosphoramidates of AZT is slower than AZT, we hypothesized that pretreatment of cells with the phosphoramidates may enhance antiviral potency.[10] Pretreatment of PBMCs with compounds **3a** and **4a** for 2 h prior to infection increased the antiviral potency of **4a** by 15−30-fold and **3a** by 100-fold (Table 2). The antiviral potency of AZT was not affected. While the effect on potency is due primarily to the slow internalization of **3a** and **4a**, relative to AZT, it is also possible that differences in the rate of intracellular conversion to AZT-MP may also be a factor.

**Antiviral Activities in HIV-1-Infected Resting PBMCs.** To investigate the possibility of using **3a** and **4a** to treat HIV-1 infection in resting PBMCs, freshly prepared PBMCs were infected with HIV in the absence of any standard activation signal (PHA or IL-2) and treated with various concentrations of compounds **3a** and **4a**. PHA-stimulated cultures were also prepared and infected in parallel. At the end of 6 days, the culture supernatants for both PHA-stimulated and resting PBMC cultures were assayed for viral RT activities. Viral RT activity was only detectable for PHA-stimulated cultures (data not shown). This was consistent with the previous results suggesting that HIV-1 replicates more efficiently in dividing activated cells than in nondividing resting cells.[14] To directly observe the infection in resting cells, the proviral DNA content of these cells was determined by polymerase chain reactions (PCR) using primers located in the *gag* region. The amount of template used for individual PCR amplifications was standardized according to amplification of a chromosomal DNA control, interferon-$\beta$ DNA. As shown in Table 2, up to 10 $\mu$M, compound **4a** did not exhibit any appreciable inhibitory effect on proviral DNA accumulation in resting cells. In contrast, AZT and compound **3a** both showed almost 100% inhibitory effects on proviral DNA formation at concentrations of 0.01 and 0.1 $\mu$M, respectively.

**Antiviral Activity against AZT-Resistant HIV-1.** To probe the mechanism of action of compounds **3a** and **4a**, studies were designed to measure their antiviral activity on the replication of AZT-resistant HIV-1 in PBMCs. The AZT-resistant HIV-1 strain used for these studies has threonine-215 mutated to tyrosine and is regarded as an AZT intermediate isolate.[15] As shown in Table 2, the $EC_{50}$ values for compounds **3a**, **4a**, and AZT increased approximately 85-, 6,- and 143-fold for PBMCs from donor 2, respectively. Because compounds **3a** and **4a** were unable to overcome resistance to AZT, we concluded that the antiviral activity associated with these two compounds relies on inhibition of HIV-1 RT. Although the difference in the increase for **4a** was smaller than for **3a** and AZT, an alternative explanation for the lower effect on potency is not warranted, since the highest concentration used for these experiments did not exceed 100 $\mu$M.

**Time of Addition Studies.** To determine the targeting stage of these AZT derivatives in the HIV-1 replication cycle, time of addition studies were carried out as described previously.[16] In these studies, test compounds were added to the infected cultures at various times postinfection and the amount of p24 antigen in the culture supernatants was determined at the end of one HIV-1 replication cycle (~29 h). The p24 ELISA, instead of RT assays, was used because of greater sensitivity to detect low-level production of virus particles at 29 h postinfection. In principle, compounds added after their targeting stage should not inhibit the progression of HIV-1 infection and the production of progeny virions. For example, AZT, which is well-known to target the reverse transcription stage of HIV-1 replication, must be added to the culture within the first 6 h of infection to exhibit antiviral activity, while dextran sulfate, an inhibitor of HIV-1 entry, must be present at the beginning of the infection to stop virus production. Protease inhibitors, such as Ro31-8959, can be added as late as 12 h postinfection and still retain antiviral activity.[16,17] Compound **3a** and AZT exhibited similar inhibition patterns, regardless of the donor of PBMCs. The inhibitory effects for both compounds were only observed when compound addition occurred within the first 6 h postinfection. This suggested that compound **3a** targets a stage after virus entry but before provirus integration. At a concentration of 30 $\mu$M, no inhibitory effect was observed for compound **4a**.

**Inhibitory Effect on Chronic HIV-1 Infection in U1 Cells.** On the basis of the results from the time of addition studies, AZT phosphoramidate monoester derivatives appeared to act on a stage prior to provirus integration but after virus fusion. To verify this, the antiviral activity of compounds **3a** and **4a** in U1 cells was evaluated. The U1 cell line is chronically infected with HIV-1, and these cells can be stimulated with TNF-$\alpha$ to produce HIV-1 virions.[18] Since HIV-1 proviral DNA is already integrated into the cellular genome in U1 cells, compounds targeting steps before integration will not exhibit antiviral activity following HIV-1 induction. Based on the results, the $EC_{50}$ values for compounds **3a**, **4a**, and AZT were 40, 25, and 25 $\mu$M, respectively. The increased $EC_{50}$ values in U1 cultures for compound **3a** and AZT, compared to HIV-1-infected PBMCs, implied that **3a** and AZT have little inhibitory effect on HIV-1 production in U1 cells and suggest that both compounds targeted stages before proviral DNA integration. The parallel decline of virus production and numbers of viable cells, as the concentration of compounds increased, suggests that the decrease in virus production is due to a decrease in the number of viable cells and not due to direct inhibition on virus production.

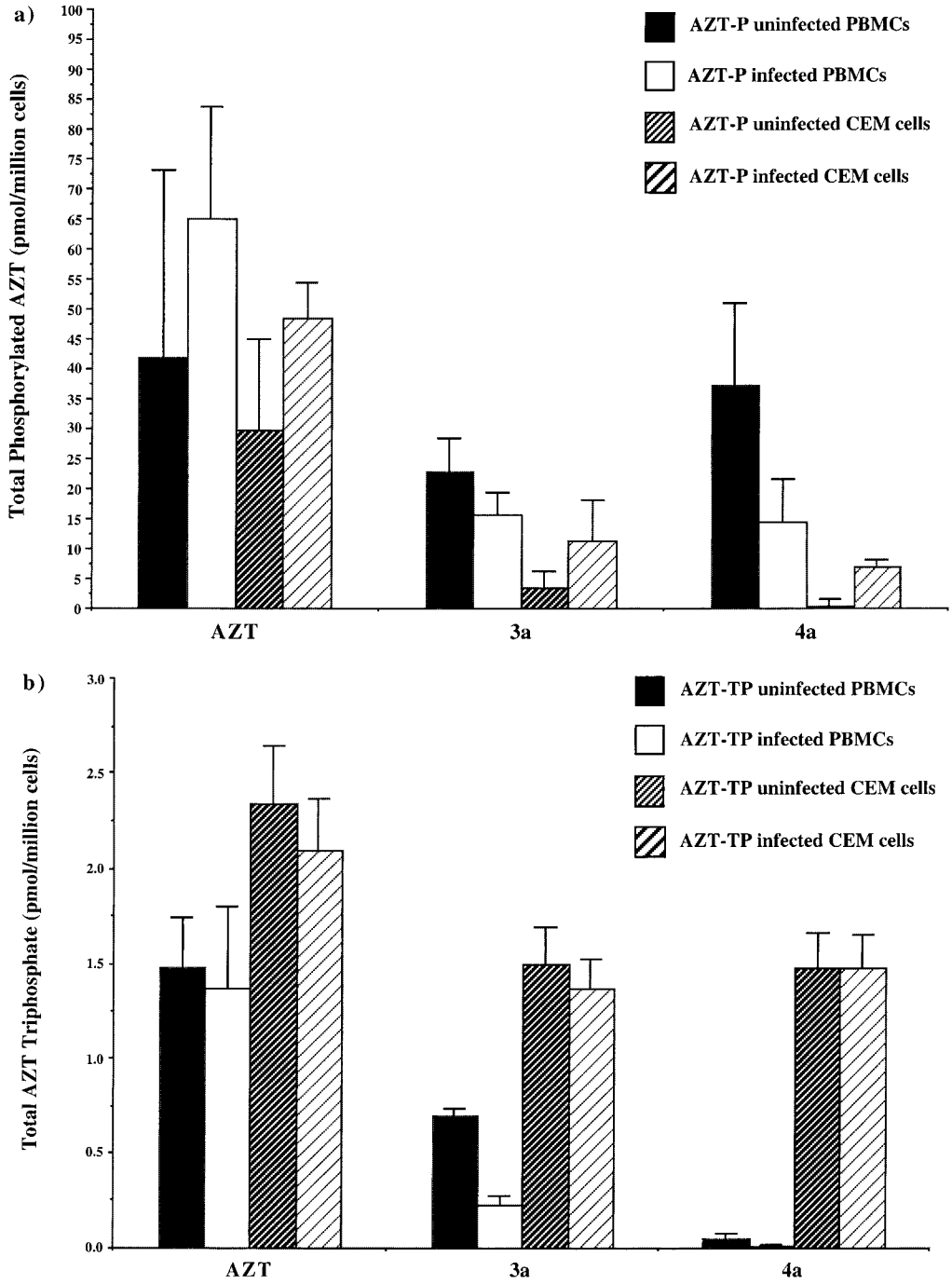



**Figure 1.** Intracellular concentration of (a) total AZT phosphate and (b) AZT triphosphate in uninfected and infected PBMCs and CEM cells treated with 10 $\mu$M AZT, **3a**, or **4a** for 18 h at 37 °C.

**Inhibitory Effect on HIV-1 Attachment.** To investigate the possibility of these AZT derivatives inhibiting HIV-1 replication at a stage prior to virus entry, the inhibitory effects on HIV-1 attachment were investigated.[16] Pretreatment of virus with compounds **3a**, **4a**, and AZT up to 100 $\mu$M concentrations had no effect on the attachment of the virus, as quantitated by the binding of p24 to the surface of CEM-SS cells, whereas dextran sulfate, a known virus entry inhibitor, produced nearly complete inhibition of binding at a concentration of 100 $\mu$g/mL (data not shown). These results were consistent with the previous time of addition studies that pointed to events after viral entry, and before proviral integration, as the target for the AZT phosphoramidates **3a** and **4a**.

**Intracellular Concentration of Phosphorylated AZT.** On the basis of the above results for compounds **3a** and **4a**, we postulated that after being metabolized, AZT monophosphate and/or AZT were generated and further metabolized to the corresponding triphosphate, which is then responsible for the observed antiviral activity. To determine the correlation between cellular metabolism and antiviral potency, we carried out intracellular metabolite studies of compounds **3a** and **4a** with PBMCs and CEM cells. PBMCs and CEM cells were incubated with 10 $\mu$M of either AZT, **3a**, or **4a** for 18 h at 37 °C and then assayed for the total amount of phosphorylated AZT (including mono-, di-, and triphosphates) using a coupled radioimmunoassay−reverse-phase HPLC (RIA−HPLC) method.[8] As can be seen in

Figure 1a, PBMCs treated with AZT generated approximately 2- and 4-fold more phosphorylated species in uninfected and infected cultures, respectively, compared to cultures treated with compound **3a** or **4a**. The amounts of phosphorylated AZT generated in PBMCs treated with either compound **3a** or **4a** were similar. For CEM cells, treatment with AZT produced at least 8-fold (uninfected) and 4-fold (infected) more phosphorylated species compared to cells treated with compounds **3a** and **4a**. Treatment with compound **3a** resulted in 2-fold (infected) and 8-fold (uninfected) more phosphorylated AZT than treated with compound **4a**. In contrast, almost no intracellular phosphorylated AZT was observed in uninfected cultures of CEM cells treated with compound **4a**. Overall, more phosphorylated AZT accumulated in PBMCs than CEM cells, regardless of infection. In addition, infection seemed to lead to a reduction in the levels of phosphorylated AZT in PBMCs and an increase in CEM cells treated with either **3a** or **4a**.

The intracellular AZT-TP concentrations in PBMCs and CEM cells treated with AZT, **3a**, or **4a** were determined by RT assays (Figure 1b). [19] Uninfected and infected PBMCs treated with AZT generated approximately 2.1- and 6.2-fold more AZT-TP, respectively, compared to compound **3a** and 22.8- and 139-fold more than cultures treated with compound **4a**, respectively. Infection of PBMCs with HIV-1 appeared to cause a 3.2- and 5-fold reduction in the production of AZT-TP in cultures treated with compound **3a** or **4a**, respectively, while not significantly affecting a AZT-TP levels in cells treated with AZT. In CEM cultures, cells treated with compound **3a** or **4a** generated similar levels of AZT-TP and infection did not appreciably affect the metabolism of these two compounds. AZT-treated CEM cultures generated slightly more AZT-TP compared to compound **3a**- or **4a**-treated cultures. Overall, higher levels of AZT-TP were generated in CEM cells than in PBMCs treated with either AZT, **3a**, or **4a**.

**Thymidine Competition.** To confirm the role of AZT-TP in HIV-1-infected cultures treated with compounds **3a** and **4a**, antiviral activity assays were conducted in the presence of 50 $\mu$M thymidine over a 6-day period. The addition of exogenous thymidine in the cultures should (1) lower the levels of AZT-MP by competing as a substrate for thymidine kinase and (2) increase the intracellular concentration of dTMP which would then compete with AZT-MP for thymidylate kinase and nucleoside diphosphate kinase for the synthesis of their corresponding triphosphate.[20] Therefore, if AZT-TP is the active species for compounds **3a** and **4a**, the addition of exogenous thymidine to the culture media should result in a reduction in their antiviral potency. As shown in Table 3, the addition of 50 $\mu$M thymidine indeed increased the $EC_{50}$ values for both compounds and AZT, suggesting that AZT-TP was the common active species.

To correlate the observed antiviral activity with AZT-TP, intracellular levels of AZT-TP were determined in the presence of 50 $\mu$M thymidine and 10 $\mu$M compound **3a**, **4a**, or AZT.[19] PBMC cultures treated with AZT alone accumulated approximately 2-fold more AZT-TP than cultures treated with AZT and thymidine. In contrast, cells treated with **3a** accumulated 22-fold more AZT-TP than cultures treated with **3a** and thymidine, while cells treated with **4a** and thymidine did not accumulate measurable amounts of AZT-TP. When compared to **3a** and **4a**, the concentration of AZT-TP in PBMCs treated with AZT and thymidine was at least 70-fold higher. Consequently, the increase observed in the $EC_{50}$ values observed for cells treated with AZT, **3a**, or **4a** is correlatable to the substantially lower levels of intracellular AZT-TP.

**Table 3.** Inhibitory Effect of AZT Phosphoramidates on Replication of LAI/HIV-1 in PBMCs and Intracellular AZT-TP Concentrations in LAI/HIV-1-Infected PBMCs in the Presence and Absence of Thymidine[a]

| | $EC_{50}$ ($\mu$M) | | AZT-TP (pmol/$10^6$ cells)[b] | |
|---|---|---|---|---|
| entry | alone | +dT | alone | +dT |
| **3a** | 0.35 | 30 | 0.22 ± 0.05 | 0.01 ± 0.00 |
| **4a** | 15 | >100 | 0.01 ± 0.01 | 0.00 ± 0.01 |
| AZT | 0.007 | 0.3 | 1.37 ± 0.43 | 0.69 ± 0.24 |

[a] $EC_{50}$: effective concentration required to inhibit the replication of HIV-1 by 50% in the absence and presence of 50 $\mu$M thymidine. [b] 10 $\mu$M compound **3a**, **4a** or AZT was incubated with infected PBMCs from donor 2 for 18 h then the intracellular AZT-TP concentrations were determined by radioactive RT assays as previously described.[10] Variance is reported as standard deviations.

**Table 4.** Inhibition of AZT-MP Production by Cell-Free Extracts of CEM Cells: Phosphoramidase Activity with AZT L-Amino Acid Carbamates[a]

| entry | nmol AZT-MP/mg protein/mL/45 min | % inhib[b] |
|---|---|---|
| **3a** | 6.39 | |
| **4a** | 6.30 | |
| **3a** + **8** | 2.56 | 59.96 |
| **4a** + **9** | 0.03 | 99.59 |

[a] CEM whole cell lysate was used for the assay. [b] Inhibition was determined based on the amount of AZT-MP generated in each sample compared to the no-carbamate control. Values represent an average of two experiments. The variance of the values was less than ±12%.

**Evidence of Phosphoramidase Activity in Lymphocyte Cell Lines.** Since the enzymatic conversion of amino acid phosphoramidates of nucleosides by a phosphoramidate hydrolase (phosphoramidase) has been reported, we attempted to determine the potential for lymphocyte cell lines to harbor a phosphoramidase, which may contribute to the biological activity of this class of compounds.[9,10,21,22] The phosphoramidase activity in CEM cells was first examined with a coupled RIA−RP-HPLC assay.[10] AZT-MP was readily detectable in CEM cell lysates incubated with 1 mM compound **3a** and **4a**, but not with 1 mM AZT (Table 4). In control experiments, no AZT-MP was observed for lysates incubated with just AZT. These results suggested the presence of phosphoramidase activity in CEM cells. Similar levels of AZT-MP were generated in CEM cell lysates incubated with either **3a** or **4a** suggesting that both compounds are substrates for the phosphoramidase.

To further probe the enzymatic character of the P−N bond cleavage in CEM cell lysates, we prepared carbamate analogues **8** and **9** of phosphoramidates **3a** and **4a** (Chart 1). Incubation of the phosphoramidates **3a** and **4a** with their corresponding carbamate analogues **8** and **9**, respectively, inhibited the production of AZT-MP by 21−99% (Table 4). These results provided further support for the presence of phosphoramidase activity

in CEM cell lysates and suggested that **8** and **9** were inhibitors of this enzyme.

## Discussion

Previously, we demonstrated that AZT phosphoramidate monoesters of L-aromatic amino acid methyl esters have potent antiviral activity. To further delineate the role of the amino acid in governing antiviral potency, a series of AZT amino acid phosphoramidates was synthesized and their antiviral activity was evaluated. In contrast to nucleoside aryl phosphoramidates, their antiviral potency was not found to be rigidly dependent on amino acid stereochemistry.[23] For example, the antiviral activity of the phenylalanine methyl ester-based phosphoramidate of AZT preferred the D-configuration, while the tryptophan methyl ester phosphoramidate preferred the L-configuration. Whether this preference applies to other amino acids, especially alanine, the most potent derivative in this class, has not been established. In addition, the methyl amides, which are resistant to hydrolysis by carboxyesterases, were shown to be as potent or more potent than the corresponding methyl esters. Consequently, in contrast to aryl nucleoside amino acid phosphoramidates, the mechanism of action is unlikely to proceed by conversion to the corresponding carboxylic acid, followed by P−N bond hydrolysis. This observation is consistent with our inability to detect the carboxylic acid metabolite intracellularly in this and previous studies.[10]

The antiviral activity of the amino acid phosphoramidates of AZT is strongly associated with the intracellular levels of AZT-TP, and not AZT-MP. The amino acid moiety of the phosphoramidates appears to dictate, in a tissue-specific manner, the extent of conversion to AZT-MP and AZT-MP to AZT-TP. In CEM cells, reduced cytotoxicity of **3a** and **4a** relative to AZT correlated with the intracellular levels of total phosphorylated AZT, but not AZT-TP, suggesting that AZT-MP is responsible for the toxicity toward CEM cells associated with AZT.[24] Previously, we demonstrated that FUdR phosphoramidate monoesters bearing tryptophan and phenylalanine substituents are capable of delivering FUdR-MP through direct P−N bond cleavage by an unknown nucleoside phosphoramidate hydrolase.[25] Consequently, we investigated the possibility that a similar enzymic reaction may be responsible for the activation of AZT phosphoramidate monoesters. [10,11] Our observation of phosphoramidate hydrolase activity in cell-free extracts of CEM cells and the feasibility of using carbamate analogues of the phosphoramidates to inhibit AZT-MP release both support this hypothesis. The observed amino acid side chain preference, as well as the stereochemical preference, may thus reflect the enzymatic characteristics of this hydrolase. The idiosyncratic nature of the amino acid stereochemical preference on the activity on the AZT phosphoramidates contrasts with the phosphoramidase studies of Shabarova and co-workers.[21,22] In the analysis of a partially purified rabbit liver ribonucleoside 5′-phosphoramidase, a strong preference for ribonucleoside phosphoramidates, but not deoxyribonucleoside phosphoramidates, was observed. In addition, a preference for substrates containing D-amino acids was also noticed. Since we observed a substantial increase in antiviral potency for the D- over the L-phenylalanine methyl ester AZT derivative, the human lymphocytic and rabbit liver phosphoramidase may have similar substrate specificities.[21,22] However, the effect of amino acid stereochemistry was not observed for tryptophan. On the basis of these results, we postulate that the enzymes responsible for the direct P−N bond cleavage of AZT phosphoramidate monoesters by human lymphocytes and the ribonucleoside 5′-phosphoramidates by rabbit liver are fundamentally different in nature. Whether differences in the antiviral activity of amino acid phosphoramidate monoesters of AZT result from differences in the substrate specificity of the putative phosphoramidase, as well as modulation of nucleotide kinase behavior, remains to be determined.

## Experimental Section

**Culture of Human PBMCs.** Human PBMCs were isolated using Ficoll-Paque (Pharmacia Biotech, NJ) density sedimentation and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), streptomycin (100 $\mu$g/mL).[26] Cultures were supplemented with phytohemagglutinin (PHA; Sigma, MI) (10 $\mu$g/mL) and IL-2 (10 U/mL) (Boehringer Mannheim, IN) where noted. Incubations were done at 37 °C in a 10% $CO_2$−90% air environment, at a concentration of $10^6$ cells/mL.

**Antiviral Activity Assay.** The procedures for antiviral activity assays in human PBMCs have previously been reported.[27] CEM cell line experiments were carried out by a similar protocol. Briefly, uninfected PHA-stimulated CEM cells were counted using the trypan blue dye exclusion method and centrifuged at 1500 rpm, at room temperature for 10 min. Infection was carried out at 37 °C for 3 h with 10 000 disintegrations of RT activity/min/$10^6$ cells (DPM/$10^6$ cells) of LAI/HIV-1 for CEM cells. The virus inoculum was prepared from infected CEM cultures, and cell-free virions were quantitated by measurement of RT in the supernatant 6 days postinfection. At the end of a 3-h adsorption period, unbound virus was removed and the cells were washed three times with 15 mL of Hank's balanced salt solution (Gibco/BRL, NY) using centrifugation (1500 rpm, 10 min, room temperature). The cells were resuspended to $2.5 \times 10^5$ cells/mL in RPMI 1640 medium (10% FBS, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 10 U/mL IL-2) and cultures were set up in 24-well tissue culture plates, with 1 mL/well. The drugs, prepared in doubly distilled $H_2O$ ($ddH_2O$), were then added to duplicate or triplicate cultures. Uninfected and untreated cultures as well as infected untreated cultures were grown at equivalent cell density as controls. The cultures were maintained at 37 °C for 6 days. Samples were then collected from CEM cultures for supernatant p24 detection using a p24 ELISA (Cellular Product, NY).

**Cytotoxicity Assay.** The compounds were evaluated for their potential toxic effects on uninfected, PHA-stimulated human PBMCs and CEM cells. After cell density and viability determination (trypan blue dye exclusion method), cells were distributed at a density of $2.5 \times 10^5$ cells/well into 96-well tissue culture plates to which diluted drug solutions and medium had been added. The plates were then incubated for 6 days at 37 °C in $H_2O$-saturated air with 10% $CO_2$. After incubation, 50 $\mu$L of XTT/PMS solution (XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide sodium salt; PMS, phenazine methosulfate; Sigma, MI) was added to each well. The XTT/PMS solution was made by first dissolving 0.15 g XTT in 144 mL of RPMI 1640 medium (without phenol red). Then, 1% (w/v) of PMS solution was prepared in PBS (phosphate buffer saline). 6 mL of PMS solution was then added to the XTT solution and mixed well before being applied to each well. The plates were incubated for 4 h at 37 °C to allow for the PMS-coupled XTT reduction. Cell viability was quantified by measuring the absorbance at 450 nm using 630 nm as a reference wavelength.

**Radioactive RT Assay.** In vitro RT assays were performed as described previously with purified recombinant HIV-1 RT (#NEI-490, NEN Life Science Products, Inc., MA) and poly(rA)-oligo$(dT)_{12-18}$ or poly(rC)-oligo$(dG)_{12-18}$ as the primer templates.[19] The extent of HIV-1 RT inhibition was determined from a set of six drug concentrations and expressed as 50% effective molar concentration at which 50% of HIV-1 RT activity was inhibited ($IC_{50}$).[28]

**Antiviral Activity against AZT-Resistant HIV-1 Strain.** The antiviral activity assays against an AZT-resistant HIV-1 strain were performed according to the previously described antiviral activity assay procedures.[10] The AZT-resistant HIV-1 strain (NIH #1082) was used instead of LAI/HIV-1. This AZT-resistant HIV-1 strain is an AZT intermediate isolate, with an $IC_{50}$ of 0.1 $\mu$M for AZT.[15]

**Antiviral Activity Assays in Cultures Pretreated with Compounds.** Uninfected, PHA-stimulated PBMCs were distributed into 24-well tissue culture plates at a density of 2.5 $\times$ $10^5$ cells/mL. Each well contained 1 mL of culture. The compounds, dissolved in $ddH_2O$, were added to the wells in triplicate and the cultures were incubated in a $CO_2$/air incubator at 37 °C for 2 h. At the end of the incubation, LAI/HIV-1 (5000 DPM/$10^6$cells) was added to each well and the plates were further incubated at 37 °C for 6 days. Parallel cultures, where PBMCs were first infected with HIV-1 for 3 h then treated with compounds, were also set up for comparison. At the end of 6 days, the culture supernatants were assayed for viral RT activity.

**Time of Addition Studies.** Time of addition studies for AZT, **3a** and **4a** were performed according to the method previously reported by Pauwels et al. with PHA-stimulated PBMCs.[16] The drug concentrations used were: AZT, 1 $\mu$M; compound **3a**, 10 $\mu$M; compound **4a**, 30 $\mu$M. Viral $p24^{gag}$ production was determined 29 h postinfection by a sandwich ELISA (Cellular Culture Product, Inc., NY) as described above.

**Inhibitory Effect on Chronic HIV-1 Infection in U1 Cells.** U1 cells were obtained from National Institute of Health, AIDS Research and Reference Reagent Program (#165). Cells were culture at 37 °C in a 5% $CO_2$−95% air environment in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FBS. Assays were performed in 24-well tissue culture plates at a cell density of 1.5 $\times$ $10^5$ cells/well. Each well contained 2 mL of RPMI 1640 medium supplemented with 10% FBS and 10 ng/mL TNF-α. Compound **3a** or **4a**, dissolved in $ddH_2O$, was added to duplicate cultures. Parallel controls included: no compound, TNF-α alone, no TNF-α. The cultures were maintained in a humidified 5% $CO_2$−95% air incubator at 37 °C for 48 h. At the end of the incubation, 100 $\mu$L of culture supernatant was removed and assayed for viral $p24^{gag}$ antigen using the ELISA. 1 mL of culture from each well was withdrawn and discarded, and the remaining culture was used for the XTT cytotoxicity assay.

**Inhibitory Effect on HIV-1 Attachment.** Viral attachment assay were carried out for AZT, **3a** and **4a** with CEM-SS cells as described by Rice et al.[29] The final concentrations for these three compounds were 0.001, 0.01, 0.1, 1, 10, and 100 $\mu$M. Dextran sulfate was used as a positive control at 100, 10, 1, and 0.1 $\mu$g/mL.

**Measurement of Intracellular Concentration of Total AZT Phosphates.** Intracellular concentrations of AZT phosphates were determined as previously reported by RIA−RP-HPLC.[10] PBMCs or CEM cells were incubated with either 10 $\mu$M AZT, **3a**, or **4a** for 18 h at 37 °C and the concentration of total AZT phosphates was determined. Cells were then washed with 1 mL of Hank's balanced salt solution three times. Cell pellets were then resuspended in 1 mL of 60% methanol and stored at −20 °C overnight. On the next day, cell debris and supernatant medium were separated by centrifugation (12 000 rpm, 10 min, 4 °C). The supernatant was transferred to a new tube and dried. The dried residue was reconstituted in 20 $\mu$L of $ddH_2O$ and injected onto a RP-HPLC system to separate the phosphorylated species. The column used was a 4.6- $\times$ 250-mm 5-mm Spherisorb reverse-phase C8 column (Chrom Tech, MN). The HPLC system consisted of a Spectra-Physics SP8800 ternary HPLC pump and SP4600 integrator; a Kratos Spectraflow 757 absorbance detector and a Rheodyne manual injector. The metabolites were eluted at 1.5 mL/min by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) and monitored at 260 nm. The gradient changed linearly from 85% A to 75% A over the first 10 min. From 10 to 15 min, there was a decrease in the gradient from 75% A to 72% A. From 15 to 20 min, the gradient changed linearly back to 85% A. Fractions were collected at 1-min intervals and dried. The fractions were then dephosphorylated and the amount of AZT released was determined by ZDV-Trac RIA (DiaSorin, MN). Dephosphorylation was performed by treating samples (reconstituted in 200 $\mu$L of 0.1 M potassium hydrogen phthalate buffer, pH 4.8) with acid phosphatase type XA (25 $\mu$L, 45 mg/mL) (Sigma, MI) at 37 °C for 4 h. Samples were dried and stored at −20 °C until assayed. Retention times for AZT-MP, free acid of **4a**, free acid of **3a**, AZT, **4a**, and **3a** were 1.85, 3.10, 3.40, 5.80, 10.11, and 10.39 min, respectively.

**Measurement of Intracellular Concentration of AZT-TP.** PHA-stimulated PBMC and CEM cultures were set up in 24-well tissue culture plates. The cell density was $10^6$ cells/mL and each well contained 1 mL of culture. The concentrations of test compounds, **3a** or **4a**, were 10 $\mu$M and the cultures were set up in triplicate. The cultures were incubated in a $CO_2$/air incubator at 37 °C for 18 h. At the end of incubation, cells were harvested and washed three times with 1 mL of Hank's balanced salt solution. Cells were then resuspended in 1 mL 60% methanol and were stored at −20 °C overnight. On the next day, cell debris and the supernatant medium were separated by centrifugation (12 000 rpm, 10 min, 4 °C). The supernatant was transferred to a new tube and dried. The dried samples were then reconstituted in 20 $\mu$L of $ddH_2O$ and used for RT assays. To quantitate the amount of AZT-TP, a modified radioactive reverse transcriptase assay was utilized as described by Robbins and co-workers.[19] RT assays were performed with purified recombinant HIV-1 RT (#NEI-490, NEN Life Science Products, Inc., MA) and poly(rA)$(dT)_{12-18}$ as the primer template. The reactions were carried out in a total volume of 80 $\mu$L in 96-well microtiter plates. To each well, 20 $\mu$L of reconstituted AZT-TP standard (Moravek, CA) or cell extracts from PBMCs or CEM cells were added along with 40 $\mu$L of the master mix. All samples were done in triplicate. The master mix consisted of 0.2 mL of lysis buffer, 0.2 mL of 0.5 M Tris, pH 8.0/5 mM EDTA, 20 $\mu$L of 0.5 M $MgCl_2$, 24 $\mu$L of $^3$H-TTP (specific activity 80 Ci/mmol), 0.2 mL of primer template (250 $\mu$g/mL stock), and 0.32 mL of $ddH_2O$. The reaction was initiated by adding 20 $\mu$L of diluted HIV-1 RT (1 U/mL in 10 mM CHAPS (Sigma, MI) solution in $ddH_2O$) into each well and incubating at 37 °C for 2 h. To stop the reaction, 100 $\mu$L of 10% TCA/0.45 mM $Na_4P_2O_7$ was added to each well. The plates were then chilled at −20 °C for 10 min before the labeled DNA was harvested and counted. The amount of intracellular AZT-TP was then determined based on the inhibition of HIV RT, relative to the AZT-TP standards.

**Antiviral Activity in HIV-1-Infected Resting PBMCs.** Ficoll-purified PBMCs from healthy individuals were divided into two groups. One group was cultured in medium (RPMI 1640 + 10% FBS, 100 U/mL penicillin G, 100 $\mu$g/mL streptomycin) and incubated in a humidified $CO_2$/air incubator at 37 °C for 2 days to allow macrophages and monocytes to adhere to the plastic surface of the flask and to ensure cells were in a resting stage. The other group was cultured in parallel in the same medium with the addition of 10 $\mu$g/mL PHA. At the end of the incubation, nonadherent PBMCs from both groups were counted and infected with LAI/HIV-1 (5000 DPM/$10^6$ cells) at 37 °C for 3 h. After 3 h for virus adsorption, cells were washed three times with 15 mL of Hank's balance salt solution. Cells were resuspended in medium at a density of 2.5 $\times$ $10^5$ cells/mL and distributed to 24-well tissue culture plates. For PHA-stimulated cultures, cells were resuspended in medium containing 10 U/mL IL-2. No IL-2 was added into the medium of the resting cell populations. Compounds, prepared in $ddH_2O$, were then added to each well in triplicate, and the cultures were incubated at 37 °C for 6 days. At the end of 6 days, cells

were pelleted (1500 rpm, 10 min, room temperature). The supernatant wasassayed for RT activity and the cell pellets were used for proviral DNA PCR. To prepare the template for proviral DNA PCR, cells were washed with 1 mL of PBS then boiled for 10 min in 55 $\mu$L of $ddH_2O$. Proteinase K digestion was then carried out for each sample at 55 °C for 1 h (2 $\mu$g/sample). Proteinase K was then inactivated by boiling the samples for 6 min. The resulting cell lysates were then used directly for proviral DNA PCR. The total volume of PCR reaction was 50 $\mu$L including $ddH_2O$, 2.5 $\mu$M of the four deoxyribonucleotides (dTTP, dCTP, dATP, and dGTP) (Perkin-Elmer Cetus, CA), 1× PCR buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 0.001% gelatin, and 50 mM KCl), 1.6 $\mu$M 5′- and 3′-primers (5′-GTCAGCCAAAATTACCCTATAGTGCAGAAC-3′, 5′-ACATAGTCTCTAAAGGGTTCCTTTGGTCCT-3′), and 2.5 units of Ampli Taq DNA polymerase (Perkin-Elmer Cetus, CA). The amplification reactions were initiated by denaturation at 94 °C for 5 min, followed by cooling to 65 °C for 7 min (Taq was added at 65 °C) and then cycling (94 °C, 45 s; 60 °C, 45 s; 72 °C, 1 min) for 25 cycles. To normalize the amount of template in each proviral DNA PCR reaction, human interferon-$\beta$1 (IFN-$\beta$1) DNA was amplified using an IFN-$\beta$1 amplimer set (upstream primer, 5′-ATGACCAACAAGTGTCTCCTCCAAA-3′; downstream primer, 5′-GTTTCGGAGGTAACCTGTAAGTCTG-3′) (Clontech, CA). The concentration for both 5′- and 3′-primers was 0.6 $\mu$M and the same PCR conditions were used as for the proviral DNA PCR. After amplification, 15 $\mu$L of the PCR products were analyzed by electrophoresis on a 1% agarose gel.

**Thymidine Competition Studies.** To determine the $EC_{50}$ value of compounds in the presence of thymidine, antiviral activity assays were performed with PHA-stimulated PBMCs as previously described. Test compounds, **3a** and **4a**, and thymidine were all prepared in $ddH_2O$. Six concentrations: 0.001, 0.01, 0.1, 1, 10, and 100 $\mu$M, were used for test compounds, and 50 $\mu$M thymidine was used throughout these assays. Parallel cultures with only the test compounds were also included for comparison. The incubations were carried out for 6 days and the culture supernatants were assayed for RT activity. The results were then expressed as disintegrations/min/0.25 mL of original culture. $EC_{50}$ values are defined as the 50% effective concentration to inhibit the replication of HIV-1 by 50%.

The effect of thymidine on intracellular AZT-TP concentrations in PHA-stimulated, drug-treated HIV-1-infected PBMCs (donor 2) cultures was also evaluated. The concentration of the test compounds was 10 $\mu$M, and the thymidine concentration was 50 $\mu$M. The procedures were the same as described previously in the measurement of the intracellular concentrations of AZT-TP.

**Phosphoramidase Activity in CEM Cell Lysates.** To determine the phosphoramidase activity in CEM cells, $1.5 \times 10^8$ CEM cells were lysed in 1 mL of lysis buffer (20 mM Tris-HCl, 500 mM NaCl pH 7.5) with sonication. Sonication was done on ice 4 × 4 s using the Virsonic 300 cell disrupter (model 175893, VirTis Co., NY). The activity assay was done in a 1.5-mL microcentrifuge tube which contained 80 $\mu$L of the cell lysate, 10 $\mu$L of lysis buffer, and 10 $\mu$L of compound **3a** or **4a** from 10 mM stock. A control with no compound was also included in parallel. The reaction was incubated at 37 °C for 45 min and stopped by the addition of 150 $\mu$L of 100% methanol, followed by freezing on dry ice. The reaction mixture was then stored at −20 °C overnight. On the next day, the reaction mixture was centrifuged at 12 000 rpm at 4 °C for 10 min. The supernatant was transferred to a new tube and dried using a Centrivap Concentrator (Labconco). Dried pellets were then reconstituted in 100 $\mu$L of $ddH_2O$ and 30 $\mu$L of the reconstituted sample was then injected onto a RP-HPLC system to separate AZT-MP from phosphoramidates. The separation conditions used were identical to those described above, for the measurement of intracellular concentrations of total AZT phosphates.

**Carbamate Inhibition Studies in CEM Cell Lysates.** Similar to the phosphoramidate activity assay, $1.5 \times 10^8$ CEM cells were lysed in 1 mL of lysis buffer (20 mM Tris-HCl, 500 mM NaCl pH 7.5) with sonication (4 × 4 s on ice). The inhibition studies were carried out with 80 $\mu$L of cell lysate, 10 $\mu$L of compound **3a** or **4a** from 10 mM stock, and 10 $\mu$L of **8** or **9** from 10 mM stock. Compounds **3a** and **4a** were prepared in $ddH_2O$; compounds **8** and **9** were prepared in 100% DMSO. Control reactions that contained only lysates, and lysates and either **3a** or **4a** were also included for comparison. The reactions were incubated at 37 °C for 45 min. At the end of the incubation, 150 $\mu$L of methanol was added to stop the reactions. The reactions were frozen on dry ice immediately and stored at −20 °C overnight. On the next day, protein and cell debris were separated from the supernatant by centrifugation (12 000 rpm, 10 min, 4 °C). The supernatant was then freeze-dried and reconstituted into 100 $\mu$L of $ddH_2O$. 30 $\mu$L of the reconstituted sample was then injected onto a RP-HPLC. The amount of AZT-MP generated in each reaction was then determined with the previously described RP-HPLC−RIA.[10]

**Protein Quantification.** The amount of protein in a given sample was determined by the Coomassie protein assay (Pierce, IL). In this assay, 20, 15, 10 and 5 $\mu$g/mL BSA (bovine serum albumin; Sigma, MO) standards were prepared in $ddH_2O$. Protein samples, if necessary, were also diluted in $ddH_2O$. 250 $\mu$L of diluted protein samples, or standards, was mixed with an equal volume of assay reagent and the absorbance was measured at 595 nm. The protein concentration of each sample was then calculated according to the standard curve.

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