Articles

Probing the Mechanism of Action and Decomposition of Amino Acid Phosphomonoester Amidates of Antiviral Nucleoside Prodrugs

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The decomposition pathways in peripheral blood mononuclear cells (PBMCs) and the *in vitro* anti-HIV-1 activity of the structurally similar 3′-azido-3′-deoxythymidine (AZT) phosphoramidates **1**−**6** and 3′-fluoro-3′-deoxythymidine (FLT) phosphoramidates **7**−**10** are reported. The AZT phosphoramidates exhibited no cytotoxicity toward CEM cells at concentrations as high as 100 μM, whereas the FLT phosphoramidates **9** and **10** had CC_{50} values of 95.6 and 35.1 μM, respectively. All 10 compounds exhibited no cytotoxicity toward PBMCs at concentrations as high as 100 μM and were effective at inhibiting viral replication. In particular, the AZT phosphomonoester amidate **4** displayed comparable antiviral acitivity to the parent nucleoside analog AZT. Mechanistic studies on the amino acid carbomethoxy ester phosphomonoester amidates revealed that their decomposition pathway differs from that of amino acid carbomethoxy ester aryl phosphodiester amidates of nucleotide prodrugs. AZT phosphomonoester amidates are internalized by lymphocytes to the same extent as AZT by a nonsaturable process. In lymphocytes, the amino acid carbomethoxy ester phosphomonoester amidates of AZT are not significantly metabolized to either AZT or the mono-, di-, or triphosphate of AZT. The amount of active anabolite, AZT-5′-triphosphate, formed in PBMCs incubated with the AZT phosphomonoester amidates **3** and **4** was 2- and 3-fold less than that observed after treatment with AZT, respectively. In contrast, FLT phosphomonoester amidates are rapidly converted to FLT-5′-monophosphate by a process that is antagonized by the corresponding AZT derivative **4**. These results suggest that the metabolism of aromatic amino acid carbomethoxy ester phosphomonoester amidate nucleotide prodrugs by PBMCs does not require prior conversion to the corresponding carboxylic acid before proceeding to P−N bond cleavage.

Introduction

Chemotherapeutic nucleosides, such as 3′-azido-3′-deoxythymidine (AZT) and 3′-fluoro-3′-deoxythymidine (FLT), once converted to their 5′-triphosphates, function as competitive inhibitors of viral polymerases and chain terminators of growing viral DNA. The bioconversion is mediated by nucleoside and nucleotide kinases. The antiviral activity of these nucleosides, however, can be decreased by either altering or removing the nucleoside kinase responsible for monophosphorylation, as has been recently reported after *in vitro* or *in vivo* long-term exposure to AZT.[1−4] Furthermore, it has recently been demonstrated that the toxicity of AZT is subject to diurnal effects which may be related to monophosphorylation.[5] Consequently, in order to overcome decreased intracellular phosphorylation, a number of systems have been developed for the delivery of monophosphorylated antiviral nucleosides.[6−20]

Of the various prodrug approaches, amino acid phosphoramidate derivatives have shown promise as potent antiviral agents, since in some cases they have exhibited enhanced antiviral activity and reduced cytotoxicity when compared to the parent nucleoside.[17,19,20] In order to define the parameters governing the extracellular and intracellular activation of phosphoramidates, a decomposition pathway for aryl amino acid phosphodiester amidates of chemotherapeutic nucleosides (such as 2′,3′-dideoxy-3′-oxyadenosine, isoddA) in serum and CEM cell extracts has been proposed (Scheme 1).[11] According to the outlined mechanism, amino acid carbomethoxy aryl phosphoramidates of isoddA (**A**) are converted, first, to their corresponding free acids (**B**) by a carboxyesterase followed by nucleophilic attack on the phosphoramidate by the liberated oxygen anion of the carboxylic acid and subsequent release of the aryl alcohol. The unstable cyclic mixed anhydride species (**C**) undergoes rapid hydrolysis to generate the corresponding phosphomonoester amidate (**D**) which can then be processed by endogenous phosphodiesterases and phosphatases to the 5′-monophosphate (**E**) and nucleoside (**F**). The carboxymethyl ester of the phosphomonoester amidate, as an alternative metabolic intermediate, was not observed.

The generality of this decomposition pathway for aryl phosphoramidates was supported by studies with d4T phosphoramidates.[17,20] Lymphocytic cells treated with an alaninyl d4T phosphodiester amidate exhibited increased levels of d4T-5′-monophosphate (d4T-MP). In addition, the free acid of the d4T alaninyl phosphomonoester amidate was shown to be the major product of the prodrug decomposition.[20,21] However, the relation-

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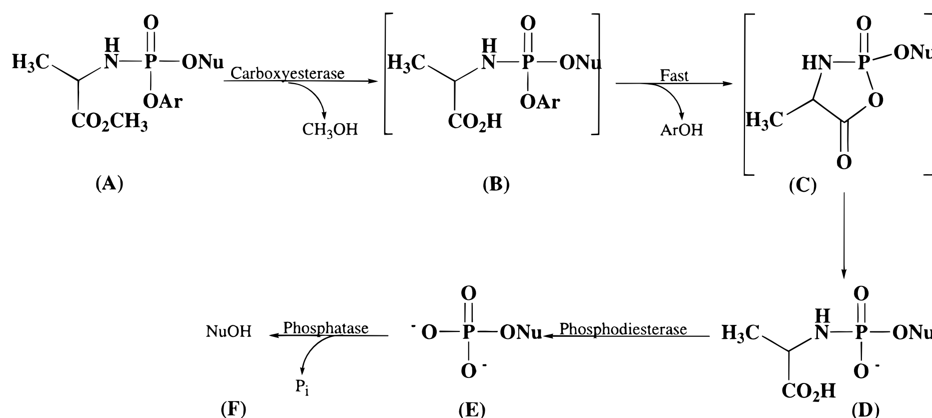
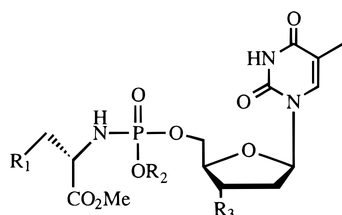
Scheme 1. Proposed Carboxyesterase-Mediated Breakdown of Aryl Phosphodiester Amidates[a]



[a] Nu: either isoddA[11] or d4T.[17] Ar: either C_6H_5 or *p*-$NO_2C_6H_4$.

Table 1. Effect of Nucleoside Derivatives on HIV-1 Replication in PBMCs and Cytotoxicity in PBMCs and CEM Cells[a]



				EC_{50}	CC_{50}	
compd[b]	R_1	R_2	R_3	PBMCs	PBMCs	CEM
1	phenyl	cyanoethyl	N_3	>1	>100	>100
2	3-indolyl	cyanoethyl	N_3	0.4	>100	>100
3	phenyl	H	N_3	0.6	>100	>100
4	3-indolyl	H	N_3	0.01	>100	>100
5	phenyl	methyl	N_3	0.09	>100	>50
6	3-indolyl	methyl	N_3	0.3	>50	>50
7	phenyl	methyl	F	0.21	>100	>100
8	3-indolyl	methyl	F	0.29	>100	>100
9	3-indolyl	cyanoethyl	F	0.2	>100	95.6
10	3-indolyl	H	F	0.9	>100	35.1
AZT				0.08	>100	14.3
FLT				0.001	8.6	0.5

[a] Values are in μM. EC_{50}: effective concentration required to inhibit the replication of HIV by 50%. CC_{50}: cytotoxic concentration required to kill 50% of the cells as compared to control cultures. [b] Prior to biological testing, the purities of the individual compound preparations were shown to be ≥99.3% by HPLC.[19]

ship of this intermediate to the intracellular concentration of d4T mono-, di-, and triphosphates was not determined. Furthermore, as predicted from the proposed mechanism of phosphodiester amidate decomposition, the carboxymethyl ester of the phosphomonoester amidate was not observed as an intermediate.

Our laboratory has recently reported the biological activity of a series of aromatic amino acid carbomethoxy ester phosphomono- and -diester amidates of AZT (Table 1, **1**−**6**).[19] In contrast to amino acid carbomethoxy ester aryl phosphodiester amidates, the phosphomonoester amidates, **3** and **4**, were shown to have significant antiviral activity and reduced cytotoxicity. For example, phosphoramidate **4** was found to be 8-fold more active at inhibiting the replication of HIV-1 in human peripheral blood mononuclear cells (PBMCs) than the parent nucleoside and was at least 10-fold less cytotoxic. The monoester derivatives (**3** and **4**) were shown to be stable in heat-inactivated fetal bovine serum and human serum (data not shown) at pH 7.2, 37 °C, for greater than 6 days. Although **3** and **4** were shown not to be substrates for phosphodiesterase I or alkaline phosphatase, they were shown to be substrates for acid phosphatase. Preliminary mechanistic studies demonstrated that **3** and **4** were internalized by PBMCs to the same extent as AZT.[19] However, in contrast to PBMCs incubated with AZT, little or no free nucleoside and nearly 4-fold more total phosphorylated AZT (i.e., AZT-5′-mono-, di-, -triphosphate and phosphoramidate) were observed in cells incubated with either **3** or **4**.[19] Taken together, these results for the AZT phosphomonoester amidates were unexpected, since the decomposition of amino acid carbomethoxy ester phosphomonoester amidates of isoddA and d4T to their corresponding biologically active nucleoside monophosphates was not observed.

In order to gain insight into the mechanism of action of nucleoside phosphoramidates, we examined the intracellular decomposition of **4** in PBMCs and cell extracts of PBMCs. In addition, we synthesized a structurally similar set of FLT phosphoramidates, **7**−**10**, and compared their biological activity and metabolic fate to the results obtained for AZT phosphomonoester amidates.

Results and Discussion

Chemistry. Phosphoramidates **1**−**10** were synthesized as previously described via a phosphoramidite-based methodology with a few minor modifications (Table 1).[22] Synthesis of the cyanoethyl phosphoramidates, **1**, **2**, and **9**, began by treating AZT or FLT with 2 equiv of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in acetonitrile, yielding the corresponding phosphoramidites. The phosphoramidites were converted to their corresponding methyl phosphites by treatment with tetrazole and methanol. The solvent was removed, and the crude product mixture was redissolved in dry acetonitrile or THF followed by the addition of either phenylalanine methyl ester or tryptophan methyl ester and iodine. The crude product mixture was purified by column chromatography on silica gel, yielding the cyanoethyl phosphoramidates in 30−70%. A similar protocol was used for synthesizing the methyl phosphoramidate diesters, **7** and **8**, where methyl *N,N*-diisopropylchlorophosphoramidite was used in place of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite to afford the desired products in 75−90% yield. The phosphomonoester amidates, **3**, **4**, and **10**, were obtained by treating **1**, **2**, or **9** with ammonia and

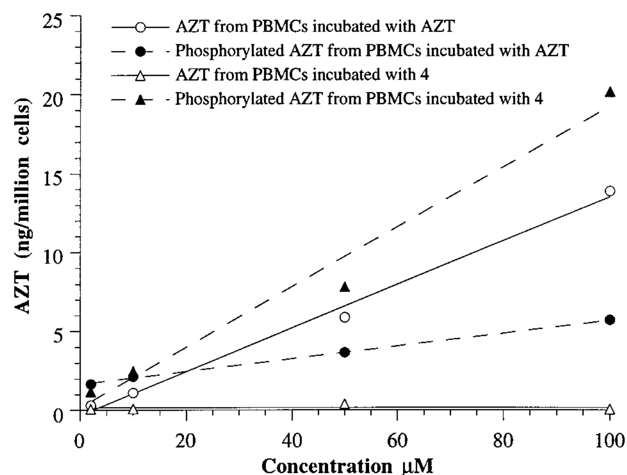


Figure 1. Concentration dependent production of AZT and phosphorylated AZT from PBMCs incubated with AZT or **4**. AZT and **4** were incubated with stimulated PBMCs for 17 h at concentrations of 2, 10, 50, and 100 μM. Quantitation of AZT and total phosphorylated AZT was done as previously described in the Experimental Section.

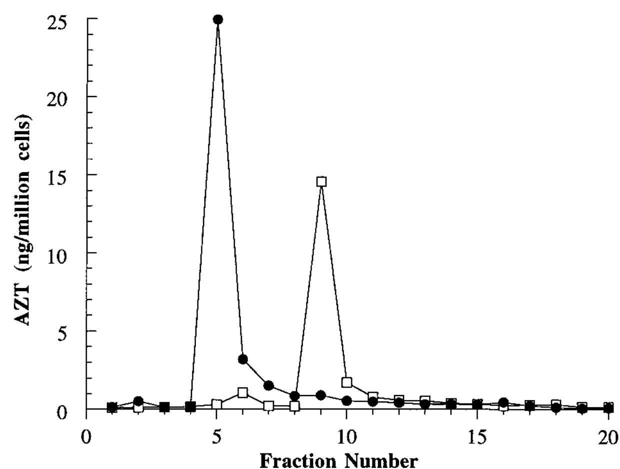


Figure 2. RP-HPLC/RIA analysis of uninfected PBMCs incubated with 100 μM AZT or **4**. Stimulated, uninfected PBMCs were incubated for 17 h in the presence of 100 μM AZT (●) or **4** (□). Fractions were collected and treated as described in the Experimental Section. Retention times for AZT-MP, carboxylic acid of **4**, AZT, and **4** were 1.2, 2.4, 5.0, and 8.9 min, respectively.

methanol followed by cation exchange chromatography yielding the desired products in 86−100%. The methyl phosphoramidate diesters, **5** and **6**, were synthesized from the corresponding phosphoramidate monoesters by conversion of the ammonium salt by ion exchange to the tetra-*n*-butylammonium salt followed by alkylation with iodomethane in acetonitrile to afford the desired products in 45−62% yield.

Analysis of **1**, **2**, **6**, **8**, and **9** by ^{31}P NMR revealed that the cyanoethyl and the tryptophan methyl phosphodiester amidates were composed of two diastereoisomers about the phosphorus atom in a 1:1 ratio, which was also observed by reverse phase HPLC (RP-HPLC) analysis. Only a single broad phosphorus peak was observed by ^{31}P NMR for the phenylalanine methyl phosphodiester amidates (**5** and **7**). As anticipated, the phosphomonoester amidates, **3**, **4**, and **10**, in which the chirality of the phosphate center has been lost, exhibited a single phosphorus peak by ^{31}P NMR.

Comparison of the Internalization of AZT and AZT Phosphoramidates. To determine the relative differences in cellular uptake between the AZT phosphoramidates and AZT, PBMCs were incubated for 17 h with various concentrations of AZT or **4**. As can be seen from Figure 1, the intracellular concentration of AZT increased linearly from 0.3 ng/10^6 cells in the presence of 2 μM added AZT to 14 ng/10^6 cells in the presence of 100 μM AZT. The intracellular amount of total phosphorylated AZT reached 2 ng/10^6 cells in the presence of 2 μM AZT but increased to only 5.7 ng/10^6 cells in the presence of 100 μM AZT. These results are consistent with previous studies demonstrating that the production of total phosphorylated AZT by PBMCs is saturable and that the increase in total intracellular AZT is due to the increase in the intracellular concentration of nonphosphorylated nucleoside.[23]

In contrast, virtually no AZT was observed in cells treated with **4**. Furthermore, the intracellular amount of total phosphorylated AZT increased linearly from 1.3 to 20 ng/10^6 cells over the entire concentration range. When the results are compared, the total amount of intracellular AZT was shown not to be substantially different for PBMCs treated with either AZT or **4**.

Analysis of Metabolism of AZT Phosphomonoester Amidates. Previously, we developed a sensitive radioimmunoassay (RIA) in order to quantitate the intracellular amounts of AZT and total phosphorylated AZT after exposure of PBMCs to **3** or **4**.[19] The RIA assay, used to determine the amounts of AZT and total phosphorylated AZT, could not distinguish between phosphorylated AZT species and AZT phosphoramidates, since both **3** and **4**, upon treatment with potato acid phosphatase, are converted to AZT. Consequently, a coupled RP-HPLC/RIA assay was developed, which allowed mono-, di-, and triphosphorylated AZT to be separated from AZT, the carboxylic acid of **3** or **4**, and **3** or **4**. PBMCs were treated with 100 μM of either **3** or **4** for 14 h and the intracellular metabolites analyzed by RP-HPLC/RIA. As can be seen from Figure 2, little or no intracellular mono-, di-, or triphosphate AZT was detected in PBMCs incubated with either AZT or **4**. In addition, no free carboxylic acid and only negligible amounts of AZT were observed. Unmetabolized AZT phosphoramidate was the only significant species observed.

The decomposition of AZT phosphomonoester amidates was further evaluated by examining the metabolism of radiolabeled [*methyl*-^{3}H]-**4** by cell free extracts of activated PBMCs. After incubation for 2 h at 37 °C, the reaction mixture was analyzed by anion exchange and reverse phase HPLC and the radioactivity of selected fractions determined. As was observed by analysis of the intracellular metabolites from PBMCs treated with **4**, only unmetabolized **4** was detected.

Typically, the amount of AZT-5′-triphosphate (AZT-TP) produced by PBMCs was considerably lower than that produced by continuous cell lines, such as CEM or MT-4, which are often used as host cells to examine the antiviral activity and metabolism of nucleosides. To further examine the intracellular production of AZT-TP by PBMCs, we chose to treat PBMCs with either AZT or **4** over a concentration range of 2−100 μM and measure the intracellular amounts of AZT-TP with a radioactive reverse transcriptase assay. The intracellular amounts of AZT-TP increased nearly 7-fold, from 327 to 2220 fmol/10^6 cells when the AZT concentration

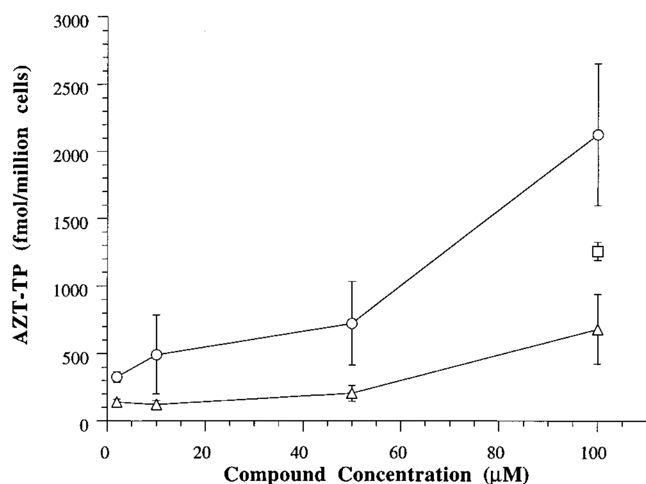


Figure 3. Concentration dependent production of AZT-TP from PBMCs incubated with AZT, **4**, or **3**: (○) amount of intracellular AZT-TP measured in cells incubated with AZT, (△) AZT-TP levels found in cells incubated with **4**, and (□) AZT-TP levels found in cells incubated with **3**. AZT and **4** were incubated in stimulated PBMCs for 17 h at concentrations of 2, 10, 50, and 100 μM. Compound **3** was incubated at a concentration of 100 μM. Quantitation of AZT-TP was done was previously described in the Experimental Section.

was increased from 2 to 100 μM (see Figure 3). In contrast, a constant level of about 155 fmol/10^6 cells of AZT-TP was observed for PBMCs treated with 2–50 μM **4**. The intracellular amounts of AZT-TP could be increased 4-fold upon incubation of the cells with 100 μM **4**. Nevertheless, across the concentration range of added AZT and **4**, the amounts of AZT-TP generated from **4** were between 2- and 4-fold less than that observed after treatment with AZT. Thus, very little of the AZT phosphoramidate **4** was metabolized to AZT-TP in PBMCs. These results are surprising since **4** was found to be at least as potent as AZT, suggesting that the activity of **4** is not entirely dependent upon rapid AZT-TP generation.

Inhibition of Viral Enzymes. As reported previously, neither phosphoramidate **3** nor **4** were found to significantly inhibit HIV-RT at concentrations > 100 μM.[19] In accord with these results, **9** and **10** were found not to significantly inhibit the activity of HIV-RT. Compound **4** (IC_{50} > 100 μM) was also found neither to be an inhibitor nor function as a substrate for HIV protease (data not shown).

Recently, monophosphorylated AZT in micromolar to millimolar concentrations has been shown to inhibit the activity of HIV integrase and RNase H.[24–27] Consequently, the phosphoramidates were tested for their ability to inhibit these viral enzymes. Neither AZT phosphoramidate **3** nor **4** were found to inhibit HIV integrase or HIV RNase H activity at concentrations as high as 500 μM.

Biological Activity of AZT and FLT Phosphoramidates. In order to probe the role of the 3′-azido group on the antiviral activity of the AZT phosphoramidates, the antiviral activity and cytotoxicity of FLT phosphoramidates, **7** and **10**, were evaluated and compared to those of compounds **1**–**10**[19,28] (Table 1).

As previously reported, compounds **1**–**3**, **5**, and **6** were 12.5-, 5-, 7.5-, 1.1-, and 3.8-fold less active than AZT, respectively, while only the tryptophan derivative **4** was 8-fold more active than AZT against HIV-1.[19] In contrast, the FLT derivatives **7**–**10** were 200–900-fold less active than the parent nucleoside FLT. In addition, **7**–**10** exhibited no cytotoxicity toward PBMCs at concentrations as high as 100 μM and were 68–200-fold less cytotoxic toward CEM cells than the parent nucleoside, respectively.[29,30]

Comparison of the EC_{50} values for the AZT and FLT phosphomono- and -diesters revealed unique structure–activity requirements. First, despite the substantial difference in lipophilicity due to the cyanoethyl moiety, a modest 1.7-fold enhancement of the activity of the AZT phenylalanine monoester, **3**, relative to the cyanoethyl diester, **1**, was observed. Nevertheless, the methyl diester, **5**, was nearly 6-fold more active than the monoester, **3**. In contrast to the phenylalanine derivatives, a 40-fold enhancement of the activity of the AZT tryptophan monoester, **4**, was observed relative to the cyanoethyl diester, **2**, and a 30-fold enhancement was observed relative to the methyl diester, **6**. Only the FLT tryptophan monoester, **10**, showed a 4.5-fold decrease in activity as compared with the corresponding cyanoethyl diester, **9**, and a 3.1-fold decrease relative to the methyl diester, **8**. When the influence of the tryptophan and the phenylalanine moieties was directly compared, a modest 2.5-fold increase in activity was demonstrated for the tryptophan cyanoethyl AZT diester, **2**, over the phenylalanine derivative, **1**. In contrast, the phenylalanine methyl diester, **5**, was 3-fold more active than the tryptophan derivative, **6**. The phenylalanine methyl diester, **7**, and the tryptophan methyl diester, **8**, were roughly equipotent, with **7** being 1.4-fold more active than **8**. When the influence of the 3′-fluoro group was examined, a modest 2.3-fold decrease in activity for the phenylalanine methyl diester of FLT, **7**, over the AZT derivative, **5**, was observed, with the tryptophan methyl diesters, **6** and **8**, being equipotent. In contrast, the tryptophan cyanoethyl diester of FLT, **9**, was 2-fold more active than the corresponding AZT derivative, **2**. When the phenylalanine and tryptophan monoesters were compared, a 60-fold increase in the activity of the tryptophan AZT monoester, **4**, over the phenylalanine monoester, **3**, was observed, despite their similar lipophilicities (data not shown). Interestingly, the tryptophan monoester of AZT, **4**, was also found to be 90-fold more potent than the corresponding tryptophan monoester of FLT, **10**. Consequently, the antiviral activity of **4** appears to be dependent on the combination of the 3′-azido, 3-indolylmethyl, and phosphomonoester amidate hydroxyl moieties.

Time Dependence of AZT and FLT Phosphomonoester Amidate Internalization. To assess the effect of the substitution of the 3′-azido group with a fluorine atom on the internalization of nucleoside phosphoramidates by lymphocytes, stimulated PBMCs were incubated with 100 μM of either compound **4** or **10** and the amount of intracellular intact phosphoramidate was measured at various time points over 14 h via a RP-HPLC assay (Figure 4). As was anticipated from the above studies of the concentration dependence on internalization of **4**, the intercellular levels of **4** steadily increased with time in a nonsaturable manner. In contrast, the intracellular amounts of **10** reached a plateau by the end of the first hour of incubation, resulting in 5.5-fold less internalized **10** relative to **4** at the end of a 14 h incubation. As can be seen from Figure

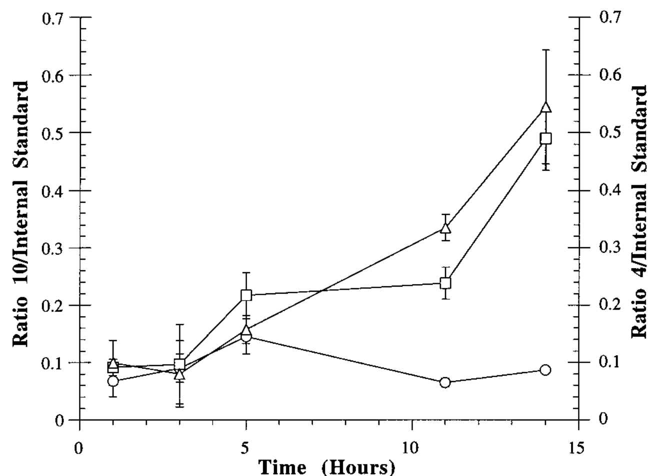


Figure 4. Time dependent uptake and metabolism of **10** in the presence and absence of **4** in PBMCs: (○) ratio of **10**/internal standard in PBMCs incubated with 100 μM **10** only, (□) ratio of **10**/internal standard in PBMCs incubated with both 100 μM **4** and **10** together, and (△) ratio of **4**/internal standard in PBMCs incubated with both 100 μM **4** and **10** together.

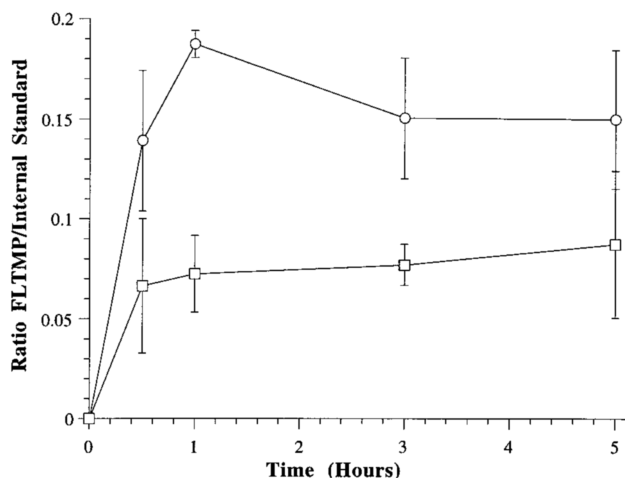


Figure 5. Time dependent production of FLTMP from PBMC lysates incubated with **10** in the presence and absence of **4**: (○) relative amount of FLTMP detected in PBMC lysates incubated with 1 mM **10** only and (□) relative amount of FLTMP detected in PBMC lysates incubated with 1 mM **10** and mM **4**.

4, when stimulated PBMCs were incubated in the presence of 100 μM **4** and **10**, the intracellular accumulation of **10** was increased 5-fold, to nearly identical levels observed for **4**. Consequently, the internal metabolic processes that govern the concentration of **4** maybe modulated by **10**.

Metabolism of FLT Phosphoramidates by Cell Extracts. Recently, we have shown that the tryptophan 5′-phosphoramidate of 5-fluoro-2′-deoxyuridine (FUdR) is converted by CEM cell extracts to the corresponding FUdR-5′-monophosphate followed by rapid conversion to FUdR.[31] Consequently, we examined the metabolism of **10** by PBMC extracts for the generation of FLT-MP.

Typically, cell lysates of PBMCs were incubated with 1 mM **10** at pH 7.5, 37 °C, for a specified period of time and analyzed by anion exchange HPLC. As can be seen in Figure 5, within the first hour, a rapid increase of FLT-MP was readily observed followed by a time dependent decrease, which was probably due to the slow conversion of FLT-MP to FLT by a phosphatase, such as 5′-nucleotidase. Moreover, when cell lysates of PBMCs were incubated with 1 mM of both **4** and **10**, the level of FLT-MP dropped over 3-fold and the initial rate of FLT-MP formation was reduced 2-fold, which is consistent with the increase in intracellular accumulation of **10** observed in cells incubated with both **4** and **10**. These results demonstrate that, unlike **4**, the FLT phosphoramidate, **10**, is readily converted in cell extracts to FLT-MP, while **4** may serve as an inhibitor of this activity.

Mechanistic Conclusions

The mechanism for decomposition of amino acid carbomethoxy ester aryl phosphodiester amidate nucleoside prodrugs, as recently proposed, requires sequential hydrolysis to the carboxylic acid, nucleophilic attack at the phosphorus center by the carboxylic acid group, dissociation of the aryl alcohol, and hydrolysis of the P−N bond, as depicted in Scheme 1. Two observations offered support for this pathway. First, significant amounts of amino acid phosphomonoester amidates of d4T and isoddA and not the carbomethoxy esters were generated by cells and cell extracts incubated with the phosphodiester amidates.[11,17,20] Second, cell extracts incubated with the corresponding carbomethoxy ester phosphomonoester amidate did not produce detectable amounts of the carboxylic acid or monophosphate.

Evaluation of the biological activity and metabolism of phosphomonoester amidates by lymphocytes found that (1) AZT phosphomonoester amidates are not significantly metabolized to either the mono-, di-, or triphosphate of AZT, (2) AZT phosphomonoester amidates are internalized to the same extent as AZT by a nonsaturable process, (3) FLT phosphoramidates are not as potent as their AZT counterparts, and (4) FLT phosphomonoester amidates are rapidly converted to FLT-MP by a process that is antagonized by the corresponding AZT derivative.

These results demonstrate that aromatic amino acid phosphomonoester amidates of AZT and FLT are able to be transported across the cellular membrane. Moreover, in contrast to previously reported phosphodiester amidates, hydrolysis of the carbomethoxy ester moiety is not a prerequisite for antiviral activity.[11,20] The conversion of FLT phosphomonoester amidates to FLT-MP suggests an alternative phosphoramidate decomposition pathway involving P−N bond cleavage by an unidentified phosphoramidate hydrolase, which maybe similar to the enzyme proposed for the conversion of phosphoramidates of FUdR and acyclic unsaturated nucleoside analogs.[31−33] This activity is dependent, however, on the choice of amino acid and nucleoside, since little if any of the AZT phosphomonoester amidates was found to be converted to AZT-MP. The reduced toxicity of the AZT phosphoramidates, relative to that of AZT, is in accord with the lack of detectable monophosphate production, since recent data have suggested that high levels of AZT-MP significantly contribute to the toxicity of AZT.[34−37] In addition, because reduced amounts of AZT-TP were observed for PBMCs treated with AZT phosphoramidates as compared to cells treated with comparable amounts of AZT, the antiviral activity of these compounds does not

depend on the generation of similar or increased amounts of phosphorylated AZT species. Consequently, phosphomonoester amidates of AZT warrant further investigation as potential nontoxic antiviral agents.

Experimental Section

Materials and Methods. Histopaque 1077 and template-primer, poly(rA)·poly(dT)$_{12-18}$, were purchased form Pharmacia Biotechnology Inc., Piscataway, NJ. AZT, phytohemagglutinin, penicillin G, and acid phosphatase type XA were purchased form Sigma Chemical Co., St. Louis, MO. The ZDV-Trac RIA kit was obtained from Incstar, Stillwater, MN. RPMI 1640 medium and gentamicin were obtained from GIBCO, Grand Island, NY. Fetal bovine serum was purchased from Summit Biotechnology, Ft. Collins, CO. Recombinant HIV-1 reverse transcriptase and [*methyl*-^{3}H]thymidine-5′-triphosphate were purchased from DuPont NEN Research Products, Boston, MA. All other chemicals were of highest grade possible.

NMR (^{1}H and ^{31}P) spectra were recorded on Varian VXR-300 and GE Omega-300 spectrometers. An external standard of 85% H_3PO_4 was used for all ^{31}P NMR spectra. An asterisk refers to doubling of peaks in the NMR due to the presence of diastereomers. FAB mass spectra were obtained on a VG 7070E-HF mass spectrometer. Analytical TLC was performed on Analtech silica gel GHLF (0.25 mm) or Machery-Nagel polygram sil G/UV$_{254}$ (0.2 mm) plates. Column chromatography was performed using grade 62, 60−200 mesh silica gel from Aldrich Chemical Co. Flash chromatography was performed using grade 60, 230−400 mesh Merck silica gel from Aldrich. Methyl *N,N*-diisopropylchlorophosphoramidite was purchased from Aldrich. THF was distilled, under nitrogen, from sodium-benzophenone ketal just prior to use. Methanol was distilled from magnesium methoxide and stored over 3 Å molecular sieves. Dichloromethane and acetonitrile were distilled from P_2O_5 and stored over 3 Å molecular sieves. All other solvents were reagent grade and used as received. Concentration under reduced pressure refers to solvent removal on a Buchi rotary evaporator. High vacuum refers to $<10^{-2}$ psi attained with a DuoSeal mechanical pump.

Chemistry. 3′-Azido-3′-deoxythymidine Methyl *N*-(1-Carbomethoxy-2-phenylethyl)phosphoramidate (5). The corresponding phosphomonoester **3** (100 mg, 0.19 mmol) was dissolved in water, applied to a Dowex 50W (Bu_4N^+) column (1.5 cm × 10 cm), and eluted with water. The 5 mL fractions containing the tetra-*n*-butylammonium salt of **3** were combined and lyophilized. The resulting powder was dissolved in dry acetonitrile (5 mL) under nitrogen. Iodomethane (0.018 mL, 0.31 mmol) was added via syringe, and the reaction was heated to reflux for 6 h. The reaction mixture was then cooled to room temperature and concentrated to dryness followed by silica gel flash column chromatography (chloroform−methanol gradient). The resulting methyl phosphoramidate diester **5** was isolated as a viscous oil (41 mg, 45% yield). ^{1}H NMR ($CDCl_3$): δ 9.4* (1H, br s, H3(NH)), 7.4−7.1 (6H, m, H6, Ph), 6.2* (1H, m, H1′), 4.3* (1H, m, H3′), 4.2−3.8 (5H, m, H4′, H5′, C*H*CO$_2$Me, NHP(O)), 3.7 (3H, s, CO_2CH_3), 3.55 (3H, dd, P(O)OCH$_3$), 3.17 (1H, m, PhC*H*$_2$), 2.90 (1H, m, PhC*H*$_2$), 2.4 (1H, m, H2′), 2.2 (1H, m, H2′), 1.95 (3H, s, 5-CH$_3$). ^{31}P NMR: δ 9.84. FABMS: *m*/*e* [M + H]$^+$ 523.[45]

3′-Azido-3′-deoxythymidine Methyl *N*-(1-Carbomethoxy-2-indolylethyl)phosphoramidate (6). The same procedure was followed as for the preparation of the phenylalanine derivative **5**. The product was obtained as a colorless solid (47 mg, 62% yield). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.47 (1H, s, H6), 7.30−6.98 (5H, m, indole aromatic Hs), 6.0 (1H, t, H1′), 4.2−3.7 (6H, m, H3′, H4′, H5′, C*H*CO$_2$Me, NHP(O)), 3.66 (3H, s, CO_2CH_3), 3.45 (3H, dd, P(O)OCH$_3$), 3.21 (1H, m, TrpC*H*$_2$), 3.05 (1H, m, TrpC*H*$_2$), 2.2 (1H, m, H2′), 2.0 (1H, m, H2′), 1.95 (3H, s, 5-CH$_3$). ^{31}P NMR: δ 10.23 and 10.06 (diastereoisomers). FABMS: *m*/*e* [M + H]$^+$ 561. Anal. ($C_{23}H_{28}N_7O_8P\cdot1.4H_2O$) C, H, N.

3′-Fluoro-3′-deoxythymidine Methyl *N*-(1-Carbomethoxy-2-phenylethyl)phosphoramidate (7). FLT (100 mg, 0.41 mmol) was dissolved in a mixture of dry dichloromethane (5 mL) and dry acetonitrile (5 mL) under nitrogen and cooled to 0 °C in an ice bath. Diisopropylamine (0.14 mL, 0.78 mmol) was added to the solution by syringe followed by methyl *N,N*-diisopropylchlorophosphoramidite (0.103 mL, 0.53 mmol). After stirring at 0 °C for 30 min, little remaining FLT could be detected by TLC (hexane−ethyl acetate, 1/1) in the reaction mixture. Tetrazole (115 mg, 1.64 mmol) was added, and after 5 min dry methanol (0.033 mL, 0.82 mmol) was added. While stirring, the reaction mixture was allowed to warm to room temperature over the course of 1 h.

Phenylalanine methyl ester free amine (600 mg, 3.35 mmol) was prepared by dissolving the hydrochloride salt in water (15 mL), basifying with K_2CO_3 (926 mg, 6.7 mmol), extracting with dichloromethane (4 × 10 mL), and then concentrating to dryness under reduced pressure. The phenylalanine methyl ester free amine (600 mg, 3.35 mmol) was then dissolved in dry THF (10 mL) and added to the reaction mixture followed by the addition of iodine (156 mg, 0.62 mmol). The reaction mixture was stirred at room temperature under nitrogen for 2 h, after which the excess iodine was quenched by the addition of ∼1 mL of a saturated $NaHSO_3$ solution. Water (20 mL) was added and the resulting mixture extracted with dichloromethane (4 × 15 mL). The organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. After flash column chromatography (chloroform−methanol gradient) and gravity column chromatography (chloroform−methanol gradient), the product was isolated as a colorless, crystalline solid (184 mg, 90% yield). ^{1}H NMR ($CDCl_3$): δ 9.4* (1H, br s, H3(NH)), 7.5−7.2 (6H, m, H6, Ph), 6.3* (1H, m, H1′), 5.2* (1H, m, H3′), 4.3* (1H, m, H4′), 4.1−3.8 (4H, m, H5′, C*H*CO$_2$Me, NHP(O)), 3.7 (3H, s, CO_2CH_3), 3.55 (3H, dd, P(O)-OCH$_3$), 3.15 (1H, m, PhC*H*$_2$), 2.95 (1H, m, PhC*H*$_2$), 2.6 (1H, m, H2′), 2.1 (1H, m, H2′), 1.95 (3H, s, 5-CH$_3$). ^{31}P NMR: δ 9.93. FABMS: *m*/*e* [M + H]$^+$ 500.37. Anal. ($C_{21}H_{27}N_3O_8PF$) C, H, N.

3′-Fluoro-3′-deoxythymidine Methyl *N*-(1-Carbomethoxy-2-indolylethyl)phosphoramidate (8). The same procedure was followed as for the preparation of the phenylalanine deivative **7**. The product was obtained as a colorless, crystalline solid (180 mg, 75% yield). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.6 (1H, s, H6), 7.5−7.1 (5H, indole aromatic Hs), 6.3* (1H, m, H1′), 5.05* (1H, dd, H3′), 4.2 (2H, m, H4′, H5′), 4.0−3.7 (3H, m, H5′, C*H*CO$_2$Me, NHP(O)), 3.8 (3H, s, CO_2CH_3), 3.55 (3H, dd, P(O)OCH$_3$), 3.31 (1H, m, TrpC*H*$_2$), 3.15 (1H, m, TrpC*H*$_2$), 2.4 (2H, m, H2′), 1.89 (3H, s, 5-CH$_3$). ^{31}P NMR: δ 10.50 and 10.17 (diastereoisomers). FABMS: *m*/*e* [M + H]$^+$ 539. Anal. ($C_{23}H_{28}N_4O_8PF\cdot1.0H_2O$) C, H, N.

Isolation of Human PBMCs. PBMCs were obtained from whole blood of healthy HIV-1, syphilis, and hepatitis B virus-seronegative volunteers and collected by single-step sterile Ficoll-Paque discontinuous gradient centrifugation. In brief, whole blood samples were placed in ACCUSPIN tubes (Sigma) with Histopaque 1077 (Ficoll) and centrifuged (800*g*) at room temperature for 20 min to separate the PBMCs from other cells. The cloudy interface containing mainly PBMCs was washed with PBS and centrifuged (600*g*, 10 min) twice to remove the Ficoll solution. Cells were resuspended in media and counted via the trypan blue dye exclusion method.[38]

Antiviral Assays. The procedures for the antiviral assays in human PBMCs have been published previously.[39] Briefly, uninfected phytohemagglutinin-stimulated human PBMCs were infected with HIV-1 (strain LAV-1) (MOI of 0.001) and cultured in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1.5 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 4 mM sodium bicarbonate. The compounds, dissolved in DMSO, were then added to triplicate cultures. Uninfected and untreated PBMCs were grown in parallel at equivalent cell concentrations as controls. The cultures were maintained in a humidified 5% CO_2−95% air incubator at 37 °C for 6 days after infection, at which point all cultures were sampled for supernatant RT activity. The supernatant from each culture was clarified from cells at 300*g* for 10 min., and the viral particles were pelleted at 40 000 rpm for 30 min by using a Beckman 70.1 Ti rotor and suspended in virus-disrupting buffer (50 mM Tris chloride, pH 7.8, 800 mM NaCl, 20% glycerol, 0.5 mM phenylmethane-

sulfonyl fluoride, and 0.5% Triton X-100). The RT assay was performed by a modification of the method of Spira et al. in 96-well microdilution plates with a poly(rA)·poly$(dT)_{12-18}$ as the template-primer.[40] The RT results were then expressed in disintegrations per minute per mL of originally clarified supernatant. Antiviral activity was then determined from a set of six drug concentrations and expressed as the 50% effective concentration to inhibit the replication of HIV by 50% (EC_{50}).

Cytotoxicity Assays in Lymphocytes. The compounds were evaluated for their potential toxic effects on uninfected phytohemagglutinin-stimulated human PBMCs and also in CEM cells. PBMCs were isolated as described above. CEM (CCRF-CEM) cells are a T-lymphoblastoid cell line that was obtained from the American Type Culture Collection, Rockville, MD. The CEM cells were maintained in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 μg/mL). The PBMCs and CEM cells were cultured with and without drug for 6 days, at which time portions were counted for cell proliferation and viability by the trypan blue dye exclusion method.[38]

RT Inhibition Assays. The compounds were evaluated for their potential inhibition of HIV-1-RT. The standard reaction mixture (100 μL) for HIV-1 assays contained 100 mM Tris hydrochloride (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.05 U of poly(rA)·poly$(dT)_{12-18}$/mL (equivalent to 3.1 μg/mL), and 1 mM $[^3H]$dTTP (specific activity 82.3 Ci/mmol). Reactions were initiated by the addition of 10 μL of recombinant HIV-1-RT. The reactions were then incubated at 37 °C for 60 min. Samples of 40 μL were spotted onto ion exchange paper disks (Munktell no. 5, Grycksbo Pappersbulk, Grycksbo, Sweden) and washed in 5% trichloroacetic acid, 0.02 M sodium pyrophosphate, and then ethanol as described previously.[41] The extent of HIV-1-RT inhibition was then determined from a set of six drug concentrations and expressed as 50% inhibitor concentration to inhibit HIV-1-RT by 50% (IC_{50}) as previously described.[42]

Culture of Human PBMCs and Cell Lysis. Human PBMCs were cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, penicillin G (160 U/mL), and gentamicin (50 μg/mL). Cultures were supplemented with phytohemagglutinin (10 μg/mL) and 5% human interleukin-2 (IL-2) where noted. Incubations were done at 37 °C, in a 5% CO_2−95% air environment, at a concentration of 1×10^6 cells/mL. Cells were incubated for indicated lengths of time, then the media were removed, and the cells were washed three times with PBS. Cells were then lysed by the addition of 60% methanol in water, overnight at −20 °C. Cellular debris was then pelleted by centrifugation, and the cellular extracts were then removed by pipet, dried, and stored at −20 °C until assayed.

Dephosphorylation of Phosphorylated AZT by Acid Phosphatase. Dephosphorylation of cell extracts (dehydrated residue) was performed by treating samples (reconstituted in 200 μL of 0.1 M potassium hydrogen phthalate buffer, pH 4.8) with acid phosphatase type XA (200 μL, 14 μg/mL). Dephosphorylation was carried out for 3 h at 37 °C. Samples were then dehydrated and stored at −20 °C until assayed.

Measurement of Phosphorylated AZT by ZDV-Trac RIA. Cell extracts were split into two equal fractions, one of which was dephosphorylated according to the described acid phosphatase procedure, and the second was untreated. These fractions were then reconstituted in kit assay buffer matrix (1.0 mL). The identical matrix was used for standards. These fractions were then assayed for AZT content according to the kit instructions. In brief, each fraction (200 μL in duplicate) was treated with a solution of ^{125}I-labeled AZT (100 μL) followed by the reconstituted anti-AZT antibody solution (100 μL). The mixture was vortexed and incubated at room temperature for 2 h. A double-antibody-precipitating agent (500 μL) was then added, and the mixture was vortexed and allowed to stand for an additional 30 min. Samples were then centrifuged for 20 min at 2500*g*, and the resulting pellet was separated from nonprecipitating antibody and tracer by aspiration of the remaining supernatant. Samples were then counted on a Beckman Gamma 4000 counting system. Counts were compared to a standard curve generated by concurrently assayed kit standards, also done in duplicate, ranging from 0.4 to 256 ng/mL. Counts were inversely proportional to AZT concentration, and sample concentrations were determined from a log−logit plot of the standard curve. The concentration of phosphorylated AZT was determined as previously described[19,43] by subtracting the mean value of the amount of AZT found for the samples that were not treated with acid phosphatase from the amount of AZT found for the acid phosphatase-treated samples ($AZT_{total} - AZT = AZT_{phosphorylated}$).

Identification of Individual Phosphorylated Species by RP-HPLC/RIA Coupled Assay. PBMCs were cultured with either 100 μM **3** or **4** for 14 h, lysed, and dried as described above. The dried residue was reconstituted in 20 μL of a PBS buffer. Samples were then vortexed and centrifuged (5000*g*, 10 s, 4 °C). Separation of phosphorylated species was performed on a 4.6 × 250 mm 5-μm Spherisorb reverse phase C8 column. 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 by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) and monitored at 260 nm. The gradient ran at 1.5 mL/min and changed linearly from 85% A to 75% A over the first 10 min. From 10 to 15 min there was an increase in the gradient from 75% A to 72% A. From 15 to 20 min there was a linear gradient change 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 RIA as described above. Retention times for AZT-MP, free acid of **4**, AZT, **3**, and **4** were 1.2, 2.4, 5.0, 8.6, and 8.9 min, respectively.

Measurement of AZT-TP by Radioactive RT Assay. In order to quantitate the amount of AZT-TP, a modified radioactive reverse transcriptase assay was utilized as described by Robbins *et al.*[44] RT assays were performed with purified recombinant HIV-1-RT and poly(rA)·p$(dT)_{12-18}$ as the template-primer. The reactions were carried out in a total volume of 80 μL. To each reaction vial containing 20 μL of reconstituted AZT-TP standard or unknown in the PBMC extract was added 40 μL of master mix. Master mix consists of 10 μL of disruption buffer, 10 μL of 0.5 M tris buffer with 5 mM EGTA, 1 μL of 0.5 M $MgCl_2$, 3 μL of $[^3H]$TTP (1 mCi/mL), 10 μL of template-primer, and 16 μL of distilled water. The reaction vials were incubated at 37 °C for 2 h; 60 μL of the reaction mixture was placed on a DE-81 paper (0.5 in. × 2.25 in. per sample). The ion exchange paper was air-dried and then washed four times, 10 min each, with 5% NaH_2PO_4, then once quickly with distilled water, and finally once quickly with 95% ethanol. The paper was air-dried, placed in 5 mL of scintillation cocktail, and then counted on a Beckman LS-3801 liquid scintillation counter. The inhibition of the HIV-RT by AZT-TP was determined with known amounts of AZT-TP (0.2069−206.9 pmol/mL) done in triplicate. All unknowns were also done in triplicate.

Monitoring Cellular Uptake of Phosphoramidates by RP-HPLC. PBMCs were cultured with 100 μM **6** in the presence or absence of 100 μM **4** for various periods of time, lysed and dried as described above. The dried residue was reconstituted in 20 μL of a methanolic PBS solution containing 2′,3′-dideoxy-2′,3′-didehydrothymidine 2-cyanoethyl-*N*-(1-carboxymethyl-2-indolylethyl)phosphoramidate (50 μM). Samples were vortexed and centrifuged (5000*g*, 10 s, 4 °C). Separation and relative quantitation of remaining phosphoramidates present in the PBMCs were performed on a 4.6 × 250 mm 5-μm Spherisorb reverse phase C8 column. The HPLC system was identical to the one described above. The compounds were eluted by using a gradient of 50 mM ammonium acetate (solvent A) and acetonitrile (solvent B) and monitored at 255 nm. The gradient ran at 1.5 mL/min and was identical to the one described above. Retention time for the internal standard, 2′,3′-dideoxy-2′,3′-didehydrothymidine 2-cyanoethyl-*N*-(1-carboxymethyl-2-indolylethyl)phosphoramidate, was 18.5 and 19.0 min (diastereomers). Retention times for compounds **10**

and **4** were 7.6 min and 8.9 min, respectively. Relative amounts of remaining phosphoramidates were expressed as a ratio of the peak area of the compound to the peak area of the internal standard.

Monitoring FLT-MP Production via Strong Anion Exchange (SAX)-HPLC. PBMCs were separated from their culture medium by centrifugation (430*g*, 5 min, room temperature). The residue (about 100 μL) was resuspended in 4 mL of buffer (Tris·HCl 20 mM, NaCl 500 mM, pH 7.5) and sonicated with a VirSonic 300 cell disrupter with microtip adapter (4 × 4 s bursts on ice). Lysate from $\sim 8 \times 10^6$ cells was incubated with **10** (1 mM) in the presence and absence of **4** (1 mM) for indicated lengths of time at 37 °C in triplicate. Enzymatic reactions were halted by quickly freezing the samples in dry ice and storing at −20 °C until assayed. Methanol (180 μL) was added to precipitate proteins and to ensure termination of enzymatic reactions; 20 μL of 0.5 mM 5′-amino-5′-deoxythymidine was added as an internal standard. Samples were then centrifuged (13200*g*, 15 min, 4 °C), and a 20 μL aliquot from the methanolic lysate was subjected to HPLC analysis. Separation and quantitation of the nucleotide FLT-MP was performed on a 4.6 × 150 mm 5-μm Spherisorb SAX column. The HPLC system was identical to the one described above. Metabolites were eluted by using a gradient of 0.01 M KH_2PO_4 (pH 4.5) (solvent A) and 0.5 M KH_2PO_4 (pH 4.5) (solvent B) and monitored at 260 nm. The gradient ran at 0.5 mL/min isocratically at 100% A for 3 min and then changed linearly from 100% A to 90% A over the next 6 min. From 9 to 18 min the gradient was held constant at 90% A. From 18 to 25 min there was a linear gradient change back to 100% A. Metabolite identification was based on the retention time of a coinjected synthesized standard of FLT-MP. The corresponding retention times for FLT-MP and 5′-amino-5′-deoxythymidine when monitored at 260 nm were 15.8 and 3.45 min, respectively. The relative amount of FLT-MP was expressed as a ratio of peak area of FLT-MP compared to the peak area for 5′-amino-5′-deoxythymidine.

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(45) Although the purity of **5** was verified by NMR, HPLC, and MS analyses, the hydroscopic nature prevented the obtaining of satisfactory (<0.5% of theoretical) elemental analyses. The compound was, therefore, judged pure by observation of a single peak using two different RP-HPLC methods.

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