Designing a Pronucleotide Stratagem: Lessons from Amino Acid Phosphoramidates of Anticancer and Antiviral Pyrimidines

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Abstract: Phosphoramidate pronucleotides have proven to be an effective strategy for the intracellular delivery of nucleoside 5'-monophosphates. This review will summarize our efforts to understand the in vitro and in vivo behavior of phosphoramidate monoesters of 3'-azido-3'-deoxythymidine (AZT), 3'-fluoro-3'-deoxythymidine (FLT) and 5-fluoro-2'-deoxyuridine (FUdR). Insights drawn from these studies have proved valuable for the future design of phosphoramidate-based pronucleotides.

INTRODUCTION

Research in our lab has focused on the development of nucleoside phosphoramidate monoesters as a potential prodrug strategy in the treatment of HIV and breast cancer. This approach is a modification of the nucleoside amino acid phosphoramidate diesters, which were designed and synthesized by McGuigan and coworkers (**Figure 1**). This approach was initially employed in the development of 3′-azido-3′-deoxythymidine, AZT, pronucleotides.

In this approach, one phosphorus oxygen of AZT monophosphate was replaced by the nitrogen of alanine methyl ester to form a phosphoramidate. The remaining phosphorus oxygen was protected either with an aryl, haloalkyl, or alkyl moiety (**Figure 2**) [1-3].

Phosphoramidate diesters with aryl protecting groups were found to be at least equipotent with haloalkyl phosphoramidates; however, AZT was found to be approximately 10 fold more potent at inhibiting the growth of HIV than either class of prodrug [2,3]. AZT phosphoramidates containing simple alkyl protecting groups were generally inactive [1].

To determine whether these phosphoramidates were delivering AZT or AZT monophosphate, their antiviral activity was determined with HIV-2 infected CEM cells deficient in thymidine kinase (CEM/TK-). Since thymidine kinase catalyzes the intracellular phosphorylation of AZT to AZT-MP, cells which lack this enzyme are resistant to AZT. Therefore, any phosphoramidate that retains activity in CEM/TK- cells is likely to be acting through a mechanism independent of the intracellular conversion of AZT to AZT-MP. AZT alanine phosphoramidate diesters had better activity than AZT in CEM/TK- cells; however, their potency decreased in every case by approximately two orders of magnitude when compared to their potency in HIV-2 infected CEM cells. This result suggested that AZT alanine phosphoramidate diesters were only partially effective in the delivery of AZT-MP [3].

In an effort to circumvent the problems associated with AZT phosphoramidate diesters, amino acid phosphoramidates of 2′,3′-didehydro-2′,3′–dideoxythymidine, d4T, (**Figure 1**) were also synthesized [4]. Since the rate-limiting step in the formation of d4T triphosphate intracellularly appears to be the conversion of d4T to d4T monophosphate, synthesis of d4T pronucleotides seemed reasonable [5]. Alanine phospho-ramidate diesters of d4T containing various substituted aryl esters were tested for anti-HIV activity in CEM and CEM/TK- cell lines. Four of the phosphoramidates tested were approximately ten fold more potent than d4T in MT-4 cells infected either with HIV-1 or HIV-2 strains and were able to retain full activity in CEM/TK- cells infected with HIV-1 strain, suggesting successful delivery of d4T monophosphate [6]. McGuigan and coworkers have also analyzed the role of neutral or hydrophobic amino acids on anti-HIV activity by synthesizing a series of d4T amino acid phosphoramidates. In this study alaninyl phosphoramidates of d4T were approximately 10 to 100 fold more potent than any other amino acid phosphoramidate [7].

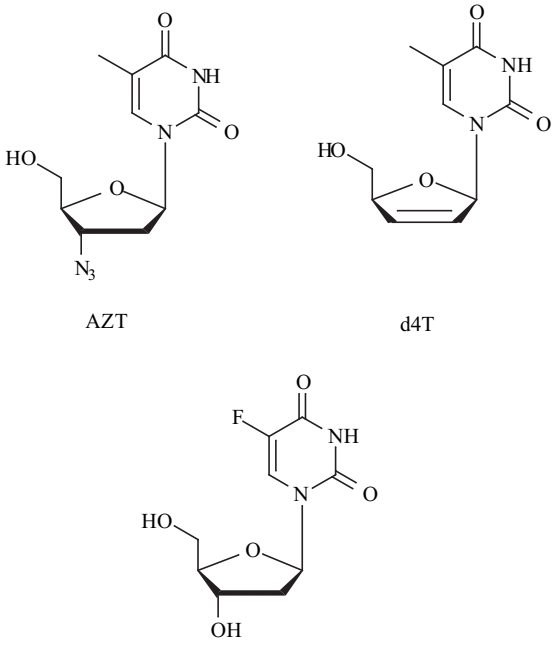

Fig. (1). Three pyrimidine neucleoside analogs currently approved for clinical treatment.

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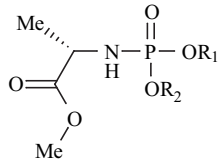
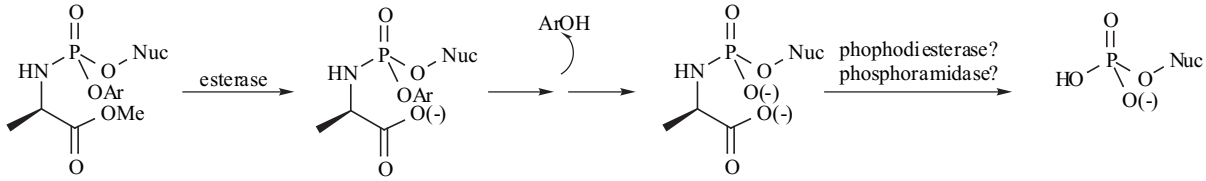


R_1 = AZT or d4T
R_2 = CH_3, CH_2CCl_3, phenyl

Fig. (2). Structure of AZT and d4T alaninyl phosphoramidate diesters. [1-4]

After demonstrating the successful delivery of nucleoside monophosphate, a metabolic pathway for conversion of the phosphoramidate diesters to monophosphates of dideoxy nucleosides was proposed which implicated two uncharacterized enzymes (**Scheme 1**).



Scheme 1. Proposed carboxyesterase-mediated breakdown of aryl phosphodiester amidates. [8,9]

In this metabolic pathway, the alanine methyl ester is hydrolyzed by an esterase, followed by the elimination of phenol and transient formation of a cyclic phosphoramidate. Further analogs studies demonstrated the dependence of this step on the amino acid and phenol structure [8]. Evidence for a phosphoramidate alanine carboxylic acid intermediate was provided when ^{3}H-d4T alaninyl aryl phosphoramidate diester was incubated in CEM cells. HPLC analysis confirmed the presence of a peak which coeluted with an authentic sample of the d4T alanine carboxylic acid phosphoramidate in three different chromatography conditions [9]. The critical conversion, to monophosphate, was hypothesized to result from the action of an uncharacterized phosphodiesterase activity [8].

Inspired by the studies with phosphoramidate diesters, we decided to investigate the anti-HIV activity of AZT amino acid phosphoramidate monoesters. Specifically, we wanted to explore whether protection of the phosphate with a lipophilic aryl protecting group was necessary for biological activity. Removal of the aryl group in principle would allow for direct conversion of the phosphoramidate to the monophosphate and potentially increase the diversity of possible incorporatable amines. Earlier work in the late 1960s by Shabarova and coworkers had demonstrated that the adenosine phenylalanine methyl ester phosphoramidate monoester was a substrate for a partially purified rabbit liver phosphoramidase [10]. Although further studies of this activity were not pursued, we hypothesized that P-N bond cleavage might be carried out by an intracellular phosphoramidase in human tissues.

In order to draw comparisons with previous phosphoramidate diester studies, we pursued the development of AZT amino acid phosphoramidate monoesters with anti-HIV activity. In addition, because we had shown that AZT is an inhibitor of the growth of MCF-7 cells, a human breast cancer cell line, we have investigated the anti-breast cancer activity of AZT phosphoramidates. Our earlier investigations had demonstrated that AZT is a more potent inhibitor of MCF-7 cell proliferation than CEM cell proliferation. Subsequent experiments revealed that the selectivity that AZT displays for breast cancer can be correlated with a 3 fold increase in levels of phosphorylated metabolites in MCF-7 cells when compared to the levels observed in CEM cells. In particular, AZT triphosphate levels were more than 10 fold higher in MCF-7 cells than in CEM cells. Finally, AZT was shown to be effective in preventing tumor growth in rats with N-methyl nitrosourea induced tumors for at least six weeks [11].

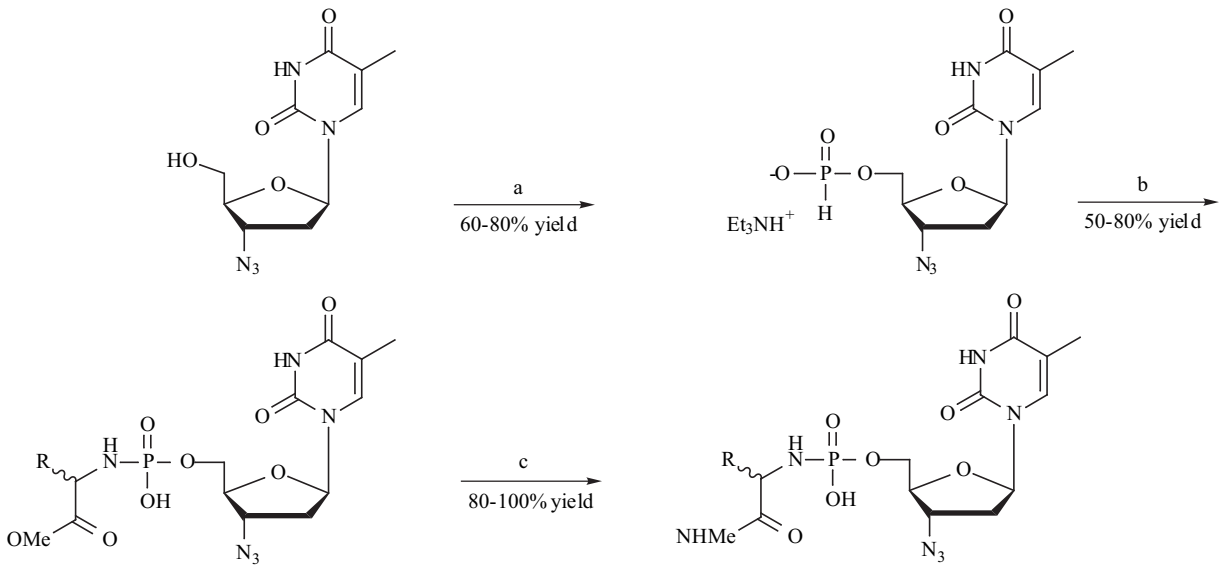
The rationale for studying AZT amino acid phosphoramidate monoesters has been outlined above. To explore this pronucleotide delivery strategy in more detail, a series of amino acid phosphoramidates of AZT have been synthesized and tested for biological activity. Furthermore, pharmacological, metabolic, pharmacokinetic, and cellular uptake studies were carried out in order to determine their mechanism of action and in vivo behavior. We have also performed animal studies to demonstrate their efficacy in the rat mammary tumor model. In addition, studies aimed at characterizing the intracellular nucleoside phosphoramidase activity will also be summarized.

SYNTHESIS AND CHEMICAL PROPERTIES

Nucleoside phosphoramidates can be easily synthesized using H-phosphonate chemistry (**Scheme 2**) [12]. Although previously we have employed other methodologies for synthesizing nucleoside phosphorami-dates, such as phosphate coupling [13] or phosphoramidite chemistry [14], H-phosphonate chemistry has proven to be the most robust and lacked dependence on the nucleoside or amino acid structure.

Briefly, the nucleoside is treated with one equivalent of diphenylphosphite to form the nucleoside-5′-H-phosphonate, which can be oxidized with iodine and treated with an excess of the amino acid methyl ester to form the desired phosphoramidate in 30-60% overall yields. Conversion of the amino acid methyl ester to the methyl-amide is accomplished by reacting the phosphoramidate in methyl-amine saturated methanol [12].

All of the AZT phosphoramidates are highly water soluble with log P values ranging from –0.686 to –1.894 [12]. Determination of the pK_A by titration with HCl using ^{31}P NMR demonstrated a pK_A of approximately 1 for a typical amino acid phosphoramidate monoester, which ensures full ionization under physiological conditions (D. Drontle and C. R. Wagner, unpublished data). In addition, AZT and 5-fluoro-2′-deoxyuridine (FUdR) amino acid phosphoramidate monoesters are indefinitely stable either in serum or cell medium [14,15].



Scheme 2. Synthetic scheme for nucleoside amino acid phosphoramidate monoesters [12].

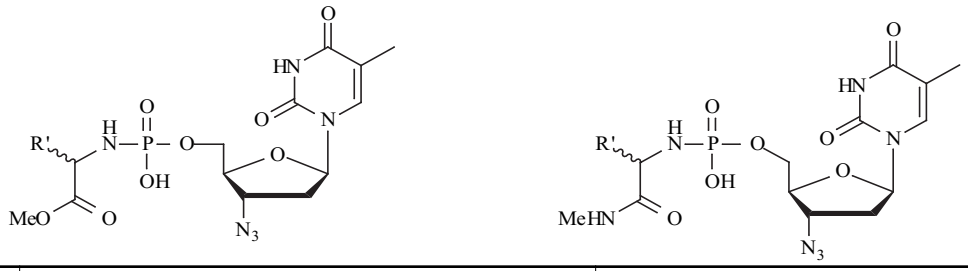
CELL CULTURE STUDIES

AZT amino acid phosphoramidates have been tested for anti-HIV activity with both HIV-1 infected PBMCs and CEM cells and anti-breast cancer activity with MCF-7 cells. However, structure-activity relationship (SAR) studies for the anti-HIV and anti-breast cancer activities are distinctly different.

For anti-breast cancer activity (**Table 1**), two general observations can be discerned from the data. First, amino acids with L stereochemistry were significantly more potent than their D counterparts in MCF-7 cells. Second, side chains with an aromatic ring were found to be more potent than alkyl side chains, as demonstrated by the tryptophan and phenylalanine phosphoramidates (labeled L-ATO and L-APO on table 1).

The effect of substituting a methyl amide for a methyl ester had no interpretable trend. The most potent inhibitor of MCF-7 cell replication in the series was the L-tryptophan methyl amide [12].

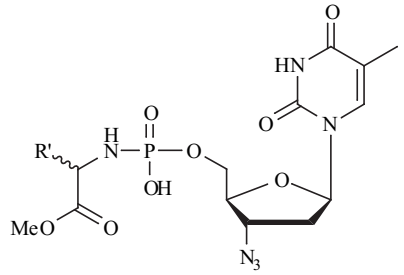
Table 1. ***In vitro*** **Activity of Amino Acid Phosphoramidates Against MCF-7 Cells [12]**

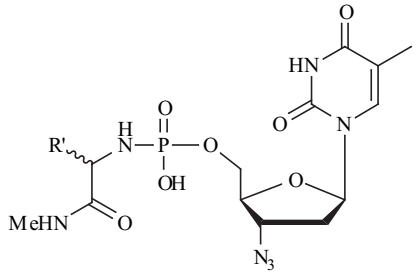


R	Methyl esters			Methyl amides		
	entry	CC_{50} (μM)[a]	Log *P*	entry	CC_{50} (μM)[a]	Log *P*
L-amino acid						
H-		>100	–1.240		5	–1.154
CH_3-		5.8	–1.252		>100	–1.205
$(CH_3)_2CH$-		6	–1.560		2.2	–1.640
$(CH_3)_2CH_2CH$-		>100	–1.498		>100	–1.524
4-HO-$PhCH_2$-		10	–1.592		>100	–1.376
$PhCH_2$-	**L-APO**	0.4	–1.836		1.4	–1.492
3-indolyl-CH_2-	**L-ATO**	0.059	–0.686	**L-ATN**	0.016	–1.382
D-amino acid						
$PhCH_2$-		>100	–1.329		>100	–1.701
3-indolyl-CH_2-		>100	–1.364		>100	–1.894

a) CC_{50} is the required concentration (μM) to reduce MCF-7 cell growth by 50% compared to control samples.

Table 2. The Effect of AZT Phosphoramidate Monoester Derivatives on HIV-1 Replication and Their Cytotoxicity in PBMCs [16][a]





R	Methyl esters EC_{50}[a]		Methyl amides EC_{50}[a]	
	Donor 1	Donor 2	Donor 1	Donor 2
L-amino acid				
CH_3-	0.05	0.03	ND	ND
$(CH_3)_2CH$-	1.3	0.9	ND	ND
$(CH_3)_2CH_2CH$-	1.3	1.0	ND	ND
$PhCH_2$-	30	15	0.18	0.25
3-indolyl-CH_2-	2.0	0.35	0.7	1.8
D-amino acid				
$PhCH_2$-	0.089	0.080	0.90	0.10
3-indolyl-CH_2-	1.0	1.0	0.40	0.18

a) Effective concentration needed to inhibit HIV-1virion production by PBMCs from either Donor 1 or Donor 2 by 50%. ND = Not determined.

For the anti-HIV activity (**Table 2**), no consistent relationship in HIV-1 infected PBMCs between activity and amino acid stereochemistry could be determined. Again, substituting methyl amides for methyl esters had no predictable effect on potency. The most potent anti-HIV agent for infected PBMCs was the phosphoramidate containing L-alanine methyl ester [16]. Although the anti HIV results differ significantly with the results for anti-breast cancer activity determined in MCF-7 cells, these results are consistent with previous studies on HIV-1 infected cells, which implicated the L-alanine methyl ester as a necessary component of highly potent nucleoside phosphoramidate diester [7].

In contrast, the L-phenylalanine methyl ester (L-APO) and L-tryptophan methyl ester (L-ATO) had low nanomolar antiviral activity in CEM cells infected with HIV-1. These results demonstrated that these compounds were potent, and that L-APO was as potent as AZT (IC_{50} of <1 nM) [16].

The lack of a common pattern in the SAR for the anti-HIV and anti-breast cancer potencies of these compounds suggests that their biological potency and activity may be governed by different processes. Whether the observed SAR differences are dependent on cellular differences in either the uptake or processing of these compounds remains to be determined.

MECHANISTIC STUDIES

To gain further understanding of the biological activity of nucleoside phosphoramidates, we have devoted considerable effort to understanding their intracellular pharmacology. These agents were designed to be prodrugs of AZT-MP, which would be subsequently phosphorylated to AZT-TP. Consequently, quantitation of the intracellular concentration of AZT, phosphorylated AZT, and AZT phosphoramidates has expanded our understanding of this pronucleotide approach.

As can be seen from table 3, the level of phosphorylated AZT metabolites was 3.3 fold higher in MCF-7 cells than in CEM cells when incubated with 100μM AZT.

Table 3. Intracellular Levels of AZT and Phosphorylated AZT in PBMC, MCF-7 and CEM Cells [12,17][a]

Compound	AZT phosphate[b]		Phosphoramidate		
	CEM	MCF-7	CEM	MCF-7	PBMC
AZT	810.9	2658.4	-	-	-
L-APO	120.7	221	7.8	295	62.8
L-ATO	115.8	196.5	14.6	213.6	66.2

a) Amounts were determined by RIA-RPLC after incubation with 100uM compound for 17 hr as previously described are expressed a pmoles/million cell.
b) AZT phosphate = total amount of AZT-MP, AZT-DP and AZT-TP.

After incubating MCF-7 cells with 100μM L-ATO or L-APO, the level of phosphorylated AZT metabolites was at least 10 fold lower than the level detected after incubation with AZT. For CEM cells, the amount of intracellular phosphorylated AZT derived from AZT was seven fold greater than from either phosphoramidate. The increased concentrations of phosphorylated AZT metabolites from

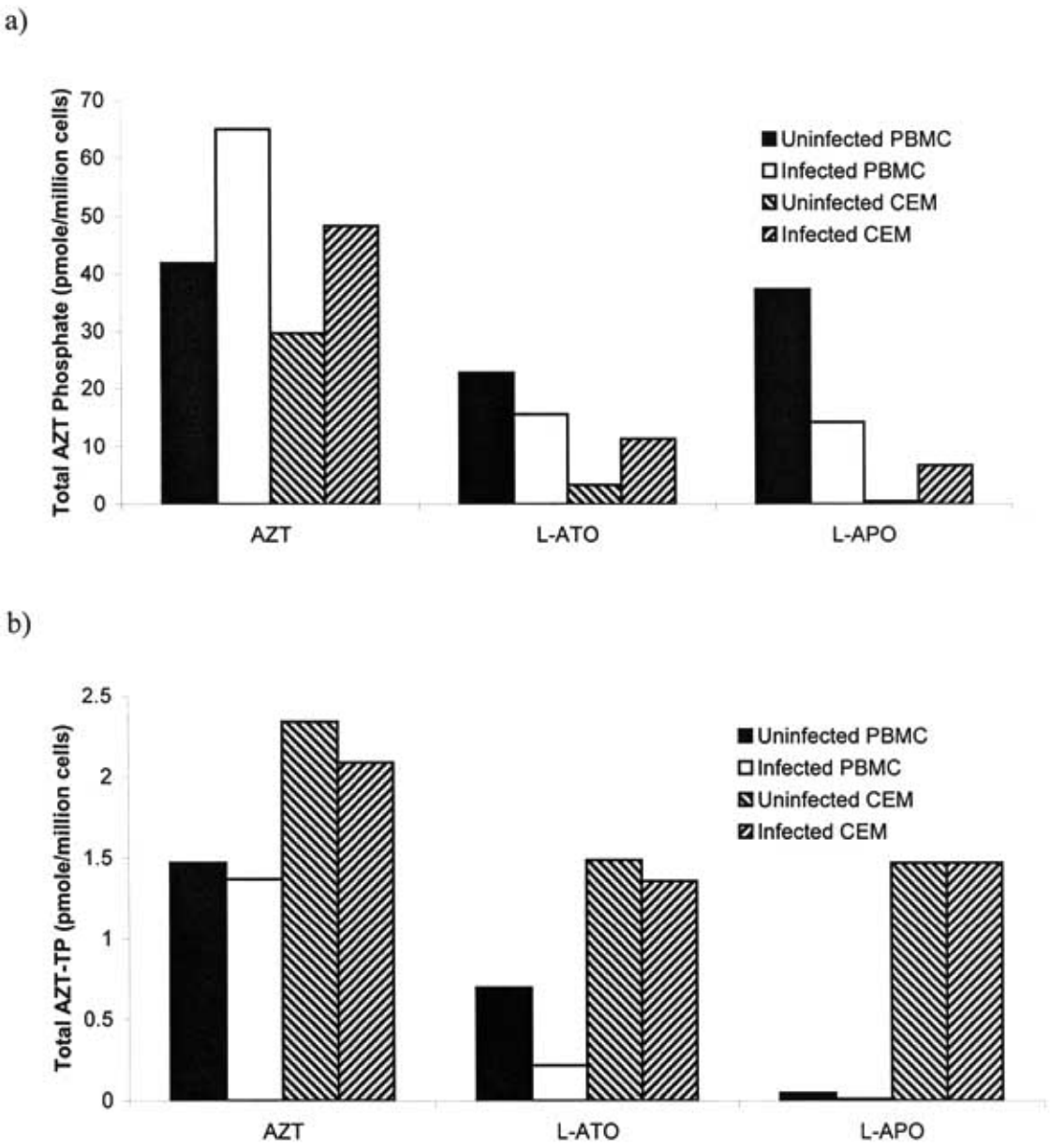


Fig. (3). Intracellular concentrations of (a) total AZT phosphates and (b) total AZT triphosphate in infected and uninfected PBMC and CEM cells treated with 10 μM AZT, L-ATO or L-APO for 18 hours at 37 °C [16].

AZT, L-ATO, and L-APO that are found in MCF-7 cells when compared to CEM cells are consistent with the increased toxicity of AZT toward MCF-7 cells [11,12].

The levels of total phosphorylated AZT metabolites (AZT-MP, DP, TP) generated from AZT, L-ATO and L-APO in PBMCs and CEM cells were also determined. To gain further insight into the effect that infection had on intracellular metabolism, these experiments were also conducted with HIV-1 infected cells. As can be seen from figure **3a**, AZT generally produced higher levels of total phosphorylated AZT metabolites than L-ATO or L-APO either in CEM cells or PBMCs. Furthermore, L-APO and L-ATO generated higher levels of total phosphorylated AZT metabolites in PBMCs than in CEM cells [16].

The results from measurements of total phosphorylated AZT metabolites were inconsistent with the results of the cell culture assays that suggested that L-ATO and L-APO had greater anti-HIV potency in CEM cells than in PBMCs. To clarify these results, AZT triphosphate levels were also measured in CEM cells and in PBMCs (Fig. **3b**). AZT generated high levels of AZT triphosphate both in CEM cells and in PBMCs. L-ATO and L-APO generated substantially higher levels of AZT triphosphate in CEM cells than in PBMCs. Furthermore, the levels of AZT triphosphate generated from L-ATO and L-APO in CEM cells were comparable with the levels of AZT triphosphate generated from AZT in CEM cells. These results correlate well with the anti-HIV potency of AZT, L-ATO and L-APO in CEM cells and PBMCs, suggesting that the anti-HIV

potency is dependent upon conversion to AZT triphosphate [16].

Since AZT competes with thymidine intracellularly to exert its activity, addition of thymidine to cell cultures that are incubated with AZT will decrease its efficacy. This will also be true for a prodrug either of AZT or AZT-MP. Thymidine decreased the potency of L-ATO and L-APO and this effect correlated with decreased levels of AZT-TP [16,17].

Time of addition studies are a useful pharmacological tool that indicates the stage of the viral life cycle an antiviral agent inhibits [18]. Antiviral agents that are added after the stage in the viral replicative cycle that they inhibit will be inactive. HIV reverse transcriptase inhibitors, such as AZT, must be added to the cell culture within the first six hours after incubation with virus to retain activity. Consistent with its inhibition of HIV reverse transcriptase, L-ATO was shown to require a time of addition no later than six hours after infection to retain activity [16]. In addition, previous studies have demonstrated that AZT-MP may act as a moderate inhibitor of HIV integrase and RNase H [19-22]. Therefore, studies were undertaken to test whether or not AZT phosphoramidates function as direct inhibitors of these enzymes. Neither L-ATO nor L-APO inhibited HIV reverse transcriptase, integrase, or RNase H. Furthermore, L-ATO and L-APO were found to be neither substrates for, nor inhibitors of, HIV protease [15,23].

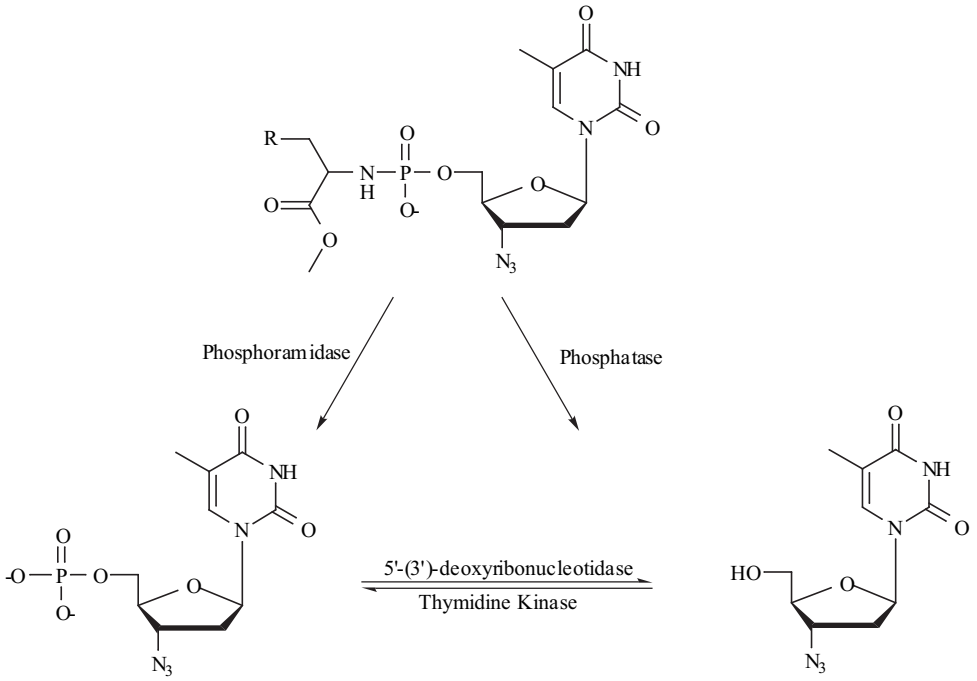
CHEMICAL EVIDENCE FOR PHOSPHORAMIDASE ACTIVITY

Based on our results, the initial hypothesis that AZT phosphoramidates act through a mechanism similar to AZT seemed plausible and implied that phosphoramidates are hydrolyzed intracellularly. Two mechanistic questions then became important. First, does the hydrolysis of AZT phosphoramidates result from uncatalyzed hydrolysis, or is this process enzyme mediated? Second, do nucleoside phosphoramidates undergo primarily P-O bond or P-N bond hydrolysis? Both whole cell and cell extract based metabolism studies have been performed to address these issues (**Scheme 3**).

One piece of evidence that cellular enzymatic activity may be responsible for the activation of nucleoside phosphoramidate monoesters was carried out with AZT and a thymidine analog, 3′-fluoro-3′-deoxythymidine (FLT). Incubation of L-tryptophan methyl ester of FLT phosphoramidate in PBMC lysates generated FLT-MP. This process was shown to be antagonized by coincubation with L-ATO [15]. This result suggested that the FLT phosphoramidate was metabolized enzymatically and that this process could be inhibited by L-ATO. When combined with the stability studies described earlier, these results provided reasonable evidence that the hydrolysis of nucleoside phosphoramidates was enzymatically catalyzed.

The simplest method for demonstrating P-N bond cleavage is through the use of kinase deficient cells since P-N bond hydrolysis releases the nucleoside monophosphate, whereas P-O bond hydrolysis releases the free nucleoside. McGuigan and coworkers demonstrated the successful delivery of d4T monophosphate through the use of CEM/TK- cells [6]. This evidence suggests that their compounds are metabolized initially to d4T-MP, which results from P-N bond cleavage.

Previously, we had found evidence that a cellular phosphoramidase is likely to be responsible for the activation of fluorodeoxyuridine (FUdR) phosphoramidates in permeabilized lymphocytic cells [24]. Examination of the anticancer activity of FUdR L-tryptophan and L-phenylalanine phosphoramidates activity both in CEM/TK- and L929/TK- cell lines demonstrated that the



Scheme 3. Proposed decomposition pathway for AZT Amino Acid Phosphoramidates.

phosphoramidates were modestly active when compared to FUdR. To gain insight into the intracellular pharmacology of FUdR phosphoramidate monoesters, the ability of both phosphoramidates to directly inhibit thymidylate synthetase was determined in intact and in permeabilized L929/TK- cells (**Table 4**).

Table 4. Inhibition of Cellular Thymidylate Synthetase Activity in Intact and Permeabilized L929 TK- Cells [24]

	IC_{50}[a], μM		
Compound	**Intact**	**Permeabilized**	**I/P**
FUdR	7.3 ± 3.6	11 ± 4.9	0.66
FUdR-MP	>10	0.008 ±0.004	>1250
L-Phe-FuDR	>1000	1.1 ± 0.74	>909
L-Trp-FuDR	>1000	0.67 ± 0.55	>1492

a) IC_{50} is defined as the concentration of compound, expressed in micromolar, necessary to inhibit thymidylate synthetase activity by 50%.

In this experiment, the activity of FUdR decreased slightly, whereas the activity of FUdR monophosphate increased by greater than 1250 fold. This result was expected because FUdR can readily cross the intact cell membrane. However, in the case of FUdR monophosphate, which is dephosphorylated before crossing the cell membrane, permeabilizing the cell membrane greatly increased potency. Permeabilizing the cell membranes also increased the activity of both phosphoramidates to approximately the same extent as FUdR monophosphate. This data suggested that the most significant barrier to activity in the TK- cell line was not the lack of thymidine kinase, but rather that the phosphoramidates were either unable to cross the cell membrane or were rapidly hydrolyzed after crossing the cell membrane [24].

Additional evidence in support of direct P-N bond cleavage was found when the rates of formation of FUdR and FUdR monophosphate were determined in CEM cell extracts (Fig. **4**). FUdR monophosphate was detected within 30 minutes after incubation with 6-^{3}H-labeled FUdR L-tryptophan phosphoramidate and increased steadily for the first 90 minutes post incubation. FUdR was undetectable until 90 minutes after incubation. The simplest interpretation of this result is that FUdR L-tryptophan phosphoramidate monoester is first converted to FUdR monophosphate and then subsequently converted to FUdR [24].

Further support for the existence of nucleoside phosphoramidase activity was found in CEM cell extracts incubated either with AZT, L-ATO or L-APO. The cell extracts were unable to directly convert AZT to AZT-MP since ATP was not added. No AZT monophosphate was detected in these cell extracts when incubated with 1 mM AZT. When lysates were incubated at 37°C with 1mM either of L-ATO or L-APO, 6.39 and 6.30 nmoles of AZT-MP/mg protein/mL were formed in 45 minutes, respectively. Furthermore, this activity was potently inhibited by carbamate analogs, in which the phosphate has been replaced by a carbonyl. This result suggests that CEM cell extracts are capable of hydrolyzing AZT phosphoramidates to AZT-MP [16].

We have also tested the ability of AZT amino acid phosphoramidates to inhibit replication in a CEM/TK- cell line. However, all phosphoramidates tested in these cells were essentially inactive, which suggests that they are efficiently converted to AZT either by P-O bond cleavage or P-N bond cleavage followed by rapid dephosphorylation of AZT-MP (S.-L. Chang and C.R. Wagner, unpublished data). In support of the later possibility, Balzarini and coworkers have shown that AZT-MP is an excellent substrate for 5′-(3′)-deoxynucleotidase, a phosphatase, which rapidly hydrolyzes AZT-MP [25]. Furthermore, since AZT-MP is not readily converted to AZT-DP, the hydrolysis of AZT-MP by 5′-(3′)-deoxynucleotidase is a significant metabolic pathway [26]. Fridland and coworkers have also found that the CEM TK- cell line is able to export nucleoside monophosphates, such as AZT-MP, via a

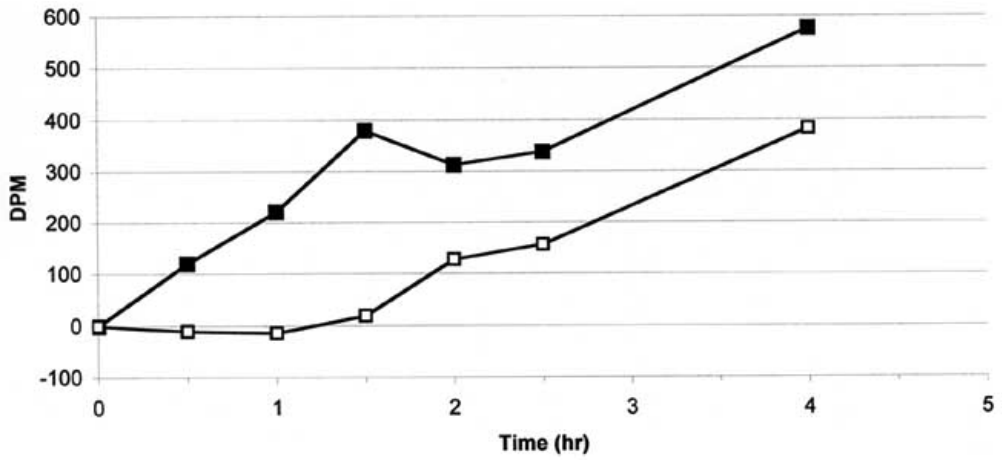


Fig. (4). Production of FUdR 5-monophosphate (■) and FUdR (❑) from cell free extracts of CEM cells with radiolabeled FUdR L-Tryptophan Methyl Ester Phosphoramidate [24].

transporter mediated mechanism [27]. Therefore, the lack of activity of AZT phosphoramidates in CEM/TK- cells may reflect rapid conversion of AZT-MP to AZT or export of AZT-MP and the parent phosphoramidate by these cells.

BIOLOGICAL EVIDENCE FOR PHOSPHORAMIDASE ACTIVITY

Given our evidence for the intracellular metabolism of nucleoside phosphoramidates, the isolation and characterization of putative phosphoramidases would greatly facilitate our understanding of their native biological formation and our ability to design phosphoramidate pronucleotides.

The first reported identification of phosphoramidase activity was from bovine spleen in the 1950s. This enzyme was capable of hydrolyzing p-chloroaniline phosphoramidate, N-phosphorylglycine, and α-N-D/L-phosphoryltryptophan [28]. Shortly after this discovery, several other phosphoramidases were isolated that hydrolyzed phosphoramidates containing a terminal phosphate [29-31]. These phosphoramidases, isolated from both mammalian tissues and bacteria, were not only capable of hydrolyzing phosphoramidates, but also hydrolyzed phosphate esters such as phosphotyrosine, pyrophosphate, and glucose 6-phosphate [30,32]. Although these discoveries are certainly relevant to the question of whether a cellular phosphoramidase activity exists, they are unlikely to be responsible for the hydrolysis of nucleoside amino acid phosphoramidates since their substrates were phosphoramidates containing a terminal phosphate, rather than a nucleoside monophosphate.

As mentioned earlier, Shabarova and coworkers partially purified a phosphoramidase from rabbit liver that hydrolyzed ribonucleoside phosphoramidates preferentially. It was purified 20- to 25- fold using ammonium sulfate fractionation and Sephadex G-100 chromatography. The optimum pH for this enzyme was found to be 5.0 and this enzyme was shown to be specific for phosphoramidates versus phosphate esters [10].

To further probe the mechanism of the rabbit liver phosphoramidase, a series of uridine 5′– phosphoramidates was synthesized in which the basicity of the amine component of the phosphoramidate was varied. These compounds were then subjected both to acidic and enzymatic hydrolysis and the rates for each process were determined. As can be seen from figure 5, the maximum rate of acidic hydrolysis occurred when the amine had a pK_A of 9, and phosphoramidates containing amines with a higher pK_A were found to have a lower rate of hydrolysis.

On the other hand, the phosphoramidase was shown to maintain its maximal rate of hydrolysis for amines with a pK_A of 9 or greater. This result suggested that the reaction took place with two separate steps: first, protonation of the phosphoramidate nitrogen and nucleophilic attack by the enzyme to form a uridylated enzyme intermediate, followed by hydrolysis of this covalent enzyme intermediate to form UMP [33,34].

To evaluate the in vivo validity of this result, Imbach and coworkers recently designed and synthesized a series of AZT phosphoramidates with differing basicities at the phosphoramidate nitrogen [35]. They then evaluated their anti-HIV potency in CEM-SS TK- cells and measured their stability in CEM-SS cell extracts. These phosphoramidates were found to be more potent anti-HIV agents than AZT in CEM-SS TK-. In addition, these compounds possessed long half-lives, ranging from 72 to 696 hours. Consistent with the work by Shabarova, a pK_A of 9 or higher for the amine component of the phosphoramidate resulted in the shortest half-lives in CEM-SS cell extracts. While correlation between stability of the phosphoramidate and activity was not perfect, phosphoramidates with long half-lives tended to be less active, and the phosphoramidate with the shortest half-life exhibited the greatest potency [35].

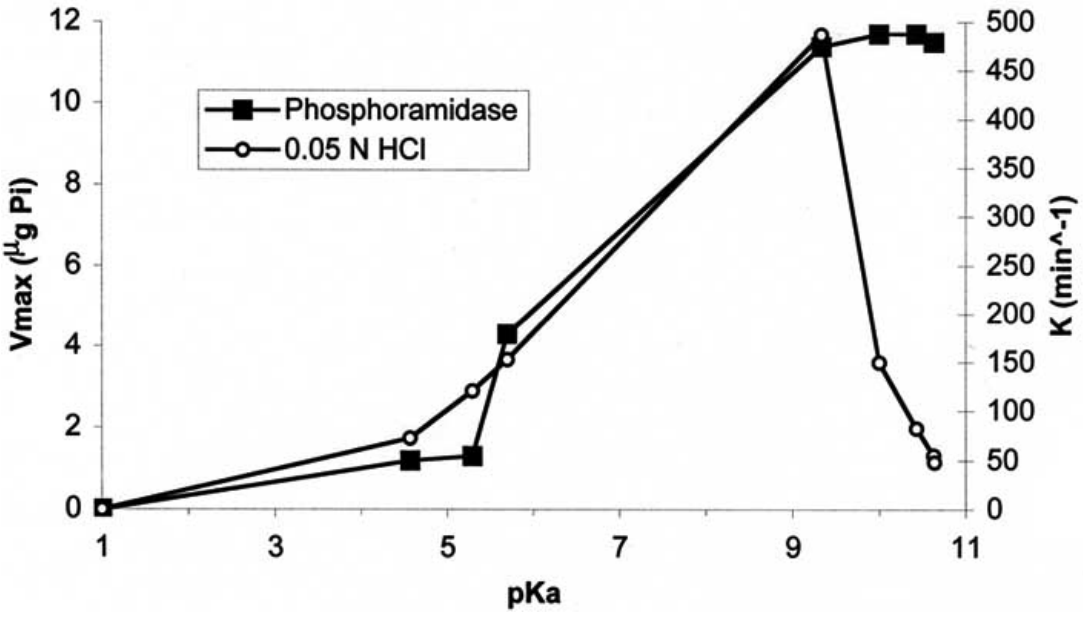


Fig. (5). Effect of variation of amine pKa on the rate of acidic and enzymatic hydrolysis [33].

Recently, a protein, previously identified as Protein Kinase C inhibitor (PKCI) and originally purified from rabbit heart tissue, has been shown not to have a regulatory role on PKC, but to hydrolyze adenosine phosphoramidates [36,37]. This enzyme, which belongs to the histidine triad (HIT) superfamily, is histidine triad nucleotide binding protein 1 (Hint1). Related sequences to Hint1 have been found in all known genomes, suggesting that they may have an essential cellular function [38,39]. The hallmark of this superfamily is the presence of the C-terminal consensus sequence HXHXHXX, where X is a hydrophobic amino acid [38]. The most well-studied member of this family is galactose-1-phosphate uridylyltransferase (GalT), which is responsible for the conversion of UDP glucose to UDP galactose [40]. Fragile histidine triad nucleotide binding protein (FHIT) has also received attention because of its putative role as a tumor suppressor and ability to carry out diadenosine polyphosphate (AP_4A) hydrolysis [41,42].

The Hint isoform that was isolated from rabbit heart was initially purified by affinity chromatography with adenosine agarose beads [36]. The protein had a molecular weight of approximately 14 kD by SDS-Page; however, size exclusion chromatography of the purified protein showed that it had a molecular weight of 28 kD. This result suggested that Hint existed as a homodimer, which was confirmed when the X-ray crystal structure was obtained [39]. A crystal structure of the human counterpart, hHint1, has also been determined [43].

In order to determine the natural substrate for Hint, its ability to hydrolyze a series of natural and nonnatural nucleotides was determined. The only substrates hydrolyzed to any significant extent were AMP phosphoramidates, the best substrate being adenosine-5′-phosphoramidate, $AMPNH_2$. The K_M and k_{cat} for $AMPNH_2$ for rabbit Hint were found to be 68 nM and 0.20 s^{-1}, respectively [37].

Mechanistically, rabbit Hint is thought to be similar to other members of the HIT superfamily that have been identified. The mechanism proposed involves nucleophilic attack of an active site histidine on the 5′-phosphate of the nucleotide substrate to form a nucleotide histidine intermediate, which is subsequently cleaved to form product [44].

Since characterization of the enzyme necessary for the biological activity we have observed would greatly enhance the future design of phosphoramidate pronucleotides, we have attempted to isolate the protein responsible for hydrolysis of AZT amino acid phosphoramidates. After construction of a cDNA library from PBMCs in T7 phage, we employed the phage display technique to attempt to isolate the enzyme by affinity chromatography [45]. After screening the phage with an affinity column constructed from the AZT L-tryptophan carbamate, DNA was extracted from several phage plaques selected by the column, and PCR was performed using primers specific to hHint1. Amplification of the sequence for hHint1 occurred in many of these plaques, suggesting that hHint1 bound to the affinity column (J. Cheng, T. F. Chou, and C. R. Wagner, unpublished data). The enzyme has also been cloned and overexpressed in bacteria (T. F. Chou and C. R. Wagner, unpublished data). Whether this enzyme is responsible for the hydrolysis of AZT amino acid phosphoramidates in human tissues is currently under investigation.

CELLULAR UPTAKE OF NUCLEOSIDE PHOSPHORAMIDATES

Previous research in our lab has determined the intracellular levels of L-ATO and L-APO in PBMCs, MCF-7 and CEM cell lines [12,17]. The data, summarized in table 3, indicate that, when incubated either with 100μM L-ATO or L-APO, phosphoramidate concentrations in MCF-7 cells were found to be 5 to nearly 30 fold higher than the amounts observed for PBMCs and CEM cells, respectively [12,17]. Recently, we have carried out cellular uptake and metabolism studies on AZT phosphoramidates in CEM cells and PBMCs with ^{31}P NMR and capillary LC-MS. The intracellular concentration either of L-ATO or L-APO in CEM cells did not plateau, even at extracellular concentrations as high as 2.5mM. (J. Kim and C. R. Wagner, unpublished data) The intracellular amounts of AZT-MP also rose as the extracellular concentrations of either phosphoramidate increased. Based on these studies, L-ATO and L-APO enter CEM cells either through a passive diffusion mechanism or a process that is difficult to saturate. Nevertheless, there appears to be significant variation in the ability of different tissues to accumulate AZT phosphoramidate monoesters. Further studies of the molecular basis and generality of this phenomenon should clarify the mechanism of cellular uptake.

PHARMACOKINETICS AND ANIMAL STUDIES

To demonstrate the potential therapeutic utility of AZT amino acid phosphoramidates, their metabolism and disposition in rats was investigated [46,47]. Eight AZT phosphoramidates were initially evaluated: both the D and L tryptophan and phenylalanine methyl esters and methyl amides (**Table 5**).

These modifications allowed us to investigate the importance of stereochemistry and serum esterase activity on the uptake and metabolism of these compounds. Pharmacokinetic studies performed by intravenous (i.v.) dosing female Sprague-Dawley rats demonstrated that AZT phosphoramidates have a significantly increased half-life over the parent nucleoside. AZT phosphoramidates were shown to have an increased volume of distribution compared to AZT; however, this factor was partially offset by the higher clearance rate for AZT phosphoramidates compared to AZT [46].

Determination of the metabolic products in rat urine indicated that approximately 16% of the total dose for the methyl-esters was excreted unmetabolized. An additional 16-19% was excreted in urine as AZT. In contrast, rats excreted 16 to 26% of the unmetabolized methyl amides, and AZT levels were approximately 12% for all methyl amides [46].

Since these amounts only account for approximately 35% of the total dose, biliary excretion was also studied. After intravenous dosing of L-ATN (see table 1), the biliary excretion was measured and determined to be 54.3% ± 4.9% four hours after dosing. This result suggests that after IV

Table 5. Summary of Estimated Pharmacokinetic Parameters of AZT Amino Acid Phosphoramidates after Single i.v. bolus doses of 190 μmole/kg[c] in rats [46]

Compound	CL[d] (L/hr/kg)	Clr[e] (L/hr/kg)	Vss[f] (L/kg)	$T_{1/2}$[g] (hrs)	Fe[h] %	Fm[i] % (AZT)
L-Trp-AZT- OMe	5.80 ± 1.58	0.80 ± 0.38	3.50 ± 1.97	4.39 ± 0.65	3.50 ± 1.97	19.09 ± 9.59
D-Trp-AZT- OMe	3.07 ± 0.78	0.48 ± 0.07	0.59 ± 0.09	6.67 ± 2.08	16.02 ± 4.42	16.30 ± 3.27
L-Phe-AZT- OMe	ND[a]	0.53 ± 0.40	ND[a]	6.93 ± 1.74[b]	11.8 ± 4.6	22.17 ± 4.97
L-Phe-AZT- NHMe	ND[a]	0.57 ± 0.15	ND[a]	6.59 ± 0.71[b]	22.4 ± 5.4	10.2 ± 2.7
L-Trp-AZT- NHMe	5.74 ± 1.44	0.84 ± 0.15	15.6 ± 6.0	11.65 ± 0.82	16.4 ± 5.6	12.1 ± 5.4
D-Trp-AZT- NHMe	3.24 ± 0.62	0.93 ± 0.27	3.12 ± 1.94	4.67 ± 1.98	26.5 ± 5.3	13.2 ± 9.3
AZT	1.35 ± 0.24	0.57 ± 0.23	0.87 ± 0.10	0.46 ± 0.11	41.3 ± 11.0	-------

a) ND- Not Determined
b) Half-life determined from urinary excretion rate plot.
c) 38 μmole/kg was used for AZT instead of 190 μmole/kg
d) Total body clearance
e) Renal clearance
f) Volume of distribution at steady state
g) Elimination half-life
h) Fraction excreted
i) Fraction metabolized

dosing biliary excretion is the major route of elimination [47].

Not surprisingly, the methyl amides have increased metabolic stability over the methyl esters of L-ATO and L-APO since rat plasma has relatively high esterase activity [48]. Furthermore, the methyl amides were shown to have lower protein binding than the corresponding methyl esters [46].

In this study, we determined the oral bioavailability of the L-tryptophan methyl amide of AZT phosphoramidate and its metabolism in the stomach and intestinal tract. Since phosphoramidates undergo acid hydrolysis to release the corresponding phosphate, L-tryptophan methyl amide of AZT phosphoramidate was partially hydrolyzed in gastric fluid. The rate of hydrolysis was found to be pH dependent, with the rate increasing as the pH decreased. In tissue homogenates or intestinal contents at neutral pH, however, the prodrug was shown to be indefinitely stable [47].

When AZT amino acid phosphoramidates were initially designed, it was envisioned that the amino acid might facilitate oral bioavailability by utilizing intestinal membrane amino acid or peptide transporters. Unfortunately, little or no oral bioavailability was observed for L-ATN when administered to rats [47]. To further address the question of intestinal transport, intestinal perfusion studies were performed. In these studies, rat intestine was cannulated at either end and a solution containing prodrug was delivered via the cannula to the intestines. Since unabsorbed L-ATN exited the intestines via cannula and could be recovered, the concentration of L-ATN absorbed could be determined. These studies demonstrated that intestinal absorption of the prodrug is highly unfavorable, thus at least tryptophan AZT phosphoramidates appear to be unable to utilize specific transporters to increase oral bioavailability.

AZT was detected in plasma and urine samples after oral administration of L-ATN, however, suggesting that the prodrug was being hydrolyzed to AZT, which crossed the intestinal membrane. The AZT derived from the prodrug was found to have a half-life in plasma of 2.97 ± 0.43 hrs, which is significantly longer than the half-life of AZT alone [49]. In addition, a greater amount of the prodrug was converted to AZT than observed after i.v. dosing [46,47].

Given the favorable in vivo properties of AZT phosphoramidates and our earlier anti-tumor studies in rats with AZT, we have begun to evaluate the anti-tumor activity of AZT phosphoramidates [11]. In these studies, female Sprague-Dawley rats have had mammary tumors induced with N-methyl nitrosourea. These rats were then dosed with L-ATN. The results suggest that L-ATN is at least as effective at inhibiting mammary tumor growth as AZT (A. Akanni, Y. Abul-Hajj, and C. R. Wagner, unpublished data).

SUMMARY AND FUTURE DIRECTIONS

In conclusion, our results with nucleoside amino acid phosphoramidates suggest that pronucleotides based on P-N bond cleavage may be a fruitful and generalizable strategy for the delivery of nucleoside monophosphates. Efforts to delineate the substrate specificity and catalytic activity of hHint1, as well as other related nucleoside phosphoramidate hydrolyzing proteins, should greatly enhance the future design of phosphoramidate pronucleotides.

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