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Bioorganic & Medicinal Chemistry 13 (2005) 5408–5423
Bioorganic & Medicinal Chemistry

Effect of change in nucleoside structure on the activation and antiviral activity of phosphoramidate derivatives

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Received 21 January 2005; accepted 25 April 2005
Available online 1 July 2005

Abstract—Changing the nucleoside group of a series of phosphoramidate derivatives affects the enzyme mediated hydrolysis rate of the compounds. d4T and AZT-substituted analogs were activated by enzymes such as lipases, esterases, and proteases. On the other hand, 3dT-substituted derivatives were comparatively less prone to hydrolysis under similar experimental conditions. From the experimental results, we propose that the most preferable nucleoside group for enzyme activation is d4T rather than AZT or 3dT. Additionally, we also observed that depending on the enzymes used the chiral selectivity of the enzymes for the phosphorus center of these phosphoramidate derivatives differed, demonstrating the importance of the nucleoside structure for this class of compounds.

1. Introduction

The HIV/AIDS pandemic continues its spread at a rate of over 15,000 new infections every day. Three categories of antiretroviral agents in clinical use are nucleoside reverse transcriptase inhibitors (NRTI) such as zidovudine and stavudine (d4T),[1,2] protease inhibitors, and non-nucleoside reverse transcriptase inhibitors (NNRTI).[3–6] The 5′-triphosphates of 2′,3′-dideoxynucleoside analogs (ddN), which are generated by nucleoside and nucleotide kinases, are potent inhibitors of human immunodeficiency virus (HIV) reverse transcriptase.[7,8] The rate limiting step for the conversion of 3′-azido-3′-deoxy thymidine (AZT) to its bioactive metabolite, AZT-triphosphate, is proposed to be the conversion of the monophosphate derivative to the diphosphate derivative. By contrast, the rate limiting step for the intracellular generation of the bioactive stavudine metabolite, stavudine-triphosphate, was reported to be the conversion of stavudine to its monophosphate derivative.[9–11] Anti-HIV ddN derivatives primarily rely on nucleoside and nucleotide kinases to convert them into the corresponding 5′-triphosphates as discussed before. However, such compounds were found to act as poor substrates for nucleoside kinases.[9,12–15] Consequently, the development of prodrug strategies has been sought to bypass the initial nucleoside kinase mediated activation step. In an attempt to overcome the dependence of ddN analogs on intracellular nucleoside kinase activation, we and others have prepared a number of aryl phosphate derivatives of zidovudine and stavudine, some of which were found to be potent anti-HIV agents with subnanomolar IC_{50} values.[10,11,16–25]

Our lead anti-HIV compound, stampidine, is a phosphoramidate derivative of stavudine.[23] Stampidine was 100 times more active than stavudine and twice as active as zidovudine against nine clinical HIV-1 isolates of non-B envelope subtypes (A, C, F, and G) originating from South America, Asia, and sub-Saharan Africa.[20] Stampidine was effective against 20 genotypically and phenotypically nucleoside analog reverse transcriptase inhibitor (NRTI)-resistant and 6 non-nucleoside inhibitor (NNRTI)-resistant HIV-1 isolates at subnanomolar to low nanomolar concentrations.[20] Orally or intraperitoneally administered stampidine exhibited significant and dose-dependent in vivo anti-HIV activity against an NRTI-resistant clinical HIV-1 isolate in severe combined immunodeficient (SCID) mice reconstituted with peripheral blood (PBL) mononuclear cells from seronegative human donors.[21] Orally administered stampidine

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doi:10.1016/j.bmc.2005.04.083

showed a dose-dependent antiretroviral effect in chronically FIV-infected cats.[22] Stampidine therapy was not associated with any clinical or laboratory evidence of toxicity at dose levels as high as 500 mg/kg or at cumulative dose levels as high as 8.4 g/kg. Stampidine exhibited favorable pharmacokinetic behavior in mice, rats, dogs, and cats following oral administration.[22,24] The documented in vitro potency of stampidine against primary clinical HIV-1 isolates with genotypic and/or phenotypic NRTI- or NNRTI-resistance as well as non-B envelope subtypes together with its in vivo antiretroviral activity in HIV-infected Hu-PBL SCID mice and FIV-infected cats warrants its further development as a new anti-HIV drug.

The generation of the active metabolites for the phosphoramidate derivatives of nucleosides was originally proposed to require the esterase-mediated hydrolysis of the carbomethoxy group associated with the alanine side chain of stampidine.[10,11,18–20,26–28] We hypothesized that in various tissue microenvironments such compounds may be metabolized through the action of other hydrolytic enzymes in addition to esterase. This was supported recently by a study showing the lipase and protease activations of various phosphoramidate derivatives.[29] The focus of this structure–activity relationship study was the role of the nucleoside group in a series of phosphoramidate derivatives to understand enzyme recognition and activation of this class of compounds.

2. Results and discussion

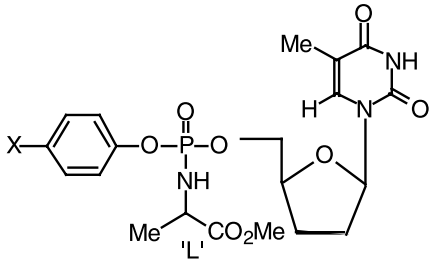
It has been postulated that phosphoramidate derivatives of stavudine are activated by an enzymatic hydrolysis mediated by esterases.[26–28] No other enzymes have been proposed as alternative mediators of the hydrolysis of this promising class of anti-HIV compounds. Since lipases have been shown to be capable of hydrolyzing other chiral compounds with a phosphorous center,[30–34] we sought to determine if the phosphoramidate derivative of d4T could be hydrolyzed to their corresponding active metabolites by a lipase as well. We observed that the lipase-mediated hydrolysis, as well as protease-mediated hydrolysis, of d4T derivatives was more efficient than esterase-mediated hydrolysis.[29]

We extended our investigations to examine the relationship between the nucleoside moiety of these phosphoramidate derivatives and their hydrolysis rate and antiviral activity. For this purpose, a series of compounds were prepared where only the nucleoside group was altered. The synthesis and characterization of the nucleoside-substituted phosphoramidate derivatives have been reported.[18–20] We have focused on three nucleoside analogs for this study, AZT, d4T, and 3dT.

Table 1 shows the first order rate constants for the enzymatic hydrolysis observed for the phosphoramidate derivatives of 3dT with electron donating and withdrawing substituents. Compounds with an electron donating group, such as the methoxy group-substituted analog, showed a 2- to 3-fold slower rate for lipase-mediated hydrolysis. In the case of the lipase-mediated hydrolysis, compounds with an electorn-withdrawing group, such as the bromo-substituted analog, showed a faster rate of hydrolysis. Unexpectedly, the nitro-substituted analog did not show significant hydrolysis, although one would have expected such a trend. This result suggested that lipase activation is not efficient in the case of nitro-substituted analog either due to the highly polar characteristic of the nitro group or to the lack of fit in the enzyme pocket. For the esterase-mediated hydrolysis, the trend was almost reversed. The compounds with electron donating groups on the aryl moiety such as OMe group hydrolyzed much faster than those with electron withdrawing groups.

In the presence of the protease subtilisin Carlsberg, all the substituted derivatives (NO_2, Cl, Br, OMe, and F) underwent hydrolysis at a rate ranging from 1.83 to 1.45 h^{-1} which was faster than the hydrolysis of the unsubstituted phenyl analog of 3dT. Similar results were obtained for all compounds, except for the phenyl-substituted compound in the presence of the protease enzyme, Carica papaya.

Table 1. First order rate constant values for 3dT-substituted phosphoramidate derivatives in the presence of enzymes



Compound (X)	Lipase	Esterase	Subtilisin Carlsberg	Carica papaya
NO_2	0.67 ± 0.2	0.10 ± 0.03	1.31 ± 0.1	1.01 ± 0.03
Br	2.90 ± 0.6	0.9 ± 0.08	1.05 ± 0.1	0.89 ± 0.1
Cl	1.4 ± 0.2	0.8 ± 0.2	1.8 ± 0.1	0.7 ± 0.07
OMe	1.2 ± 0.6	3.06 ± 0.7	1.83 ± 0.05	0.84 ± 0.01
H	1.01 ± 0.1	1.72 ± 0.9	0.23 ± 0.04	0.41 ± 0.06
F	1.25 ± 0.1	1.5 ± 0.7	1.45 ± 0.05	0.41 ± 0.08

Rate constants are expressed per hour.

Table 2 shows the first order rate constants of hydrolysis observed for the phosphoramidate derivatives of d4T using the same enzymes as those used with the 3dT derivatives. As with the 3dT derivatives, the lipase-mediated hydrolysis showed the fastest rates for compounds with electron withdrawing groups. However, the rate of lipase-mediated hydrolysis was faster for the d4T derivatives. For example, stampidine, the bromo-substituted phenyl phosphoramidate derivative showed a rate value of 3.10 h^{-1} in comparison with 3dT (2.9 h^{-1}) for lipase-mediated hydrolysis.

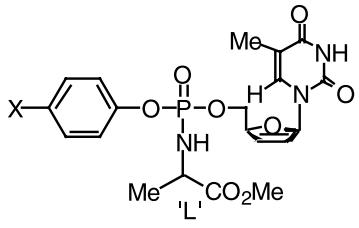
The fluoro-substituted compound underwent faster hydrolysis than the unsubstituted compound in the presence of subtilisin Carlsberg but not Carica papaya. In the presence of the esterase enzyme, the OMe- and Br-substituted compounds underwent faster hydrolysis (rate of hydrolysis values of 3.90 and 3.3 h^{-1}, respectively), than other compounds (rates of hydrolysis: 2.28–3.04 h^{-1}). The most striking results, however, were shown using serine protease. This enzyme gave the highest rate of hydrolysis, especially for the subtilisin Carlsberg. For example, subtilisin Carlsberg showed hydrolysis rate in the range of 4.2–8.5 h^{-1} for all the derivatives. On the other hand, the cysteine protease, Carica papaya, caused slow hydrolysis of the compounds. With the exception of stampidine (1.43 h^{-1}), the hydrolysis rates for all the compounds were in the range of 0.5–0.9 h^{-1}. From the results, it appears that subtilisin Carlsberg protease enzyme recognizes all of the substituted phosphoramidate derivatives of d4T.

Table 3 shows the hydrolysis rate constants observed for various phosphoramidate derivatives of AZT in the presence of the enzymes examined. The rate for the lipase-mediated hydrolysis was comparatively slow for these phosphoramidate derivatives. (0.35–0.8 h^{-1}). The data in Table 3 show that the rate of lipase-mediated hydrolysis for unsubstituted compound is minimal (0.02 h^{-1}). Conversely, the esterase-mediated hydrolysis gave a fast rate of hydrolysis. The rate of esterase-mediated hydrolysis was 5.73 and 4.71 h^{-1} for bromo- and OMe-substituted derivatives, respectively. Additionally, the bromo-substituted the derivative displayed the highest rate of hydrolysis in the presence of the protease enzyme subtilisin Carlsberg (3.56 h^{-1}). The rate of hydrolysis in the presence of cysteine protease Carica papaya was nearly the same for most of the substituted derivatives (0.63–0.81 h^{-1}). Compared with the other derivatives the bromo-substituted compound had a higher rate of hydrolysis with this protease.

2.1. Chiral selectivity at the phosphorus center

2.1.1. Nucleoside change. We examined the differences in the chiral selectivity for the various nucleoside-substitut-

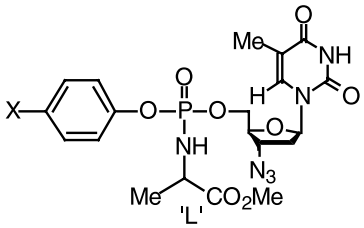
Table 2. First order rate constant values for d4T-substituted phosphoramidate derivatives in the presence of enzymes



Compound (X)	Lipase	Esterase	S. Carlsberg	Carica papaya
Br (stampidine)	3.1 ± 0.5	3.3 ± 0.5	8.55 ± 0.7	1.43 ± 0.4
Cl	3.2 ± 2.28	3.04 ± 0.2	4.21 ± 0.3	0.7 ± 0.1
OMe	1.24 ± 0.8	3.9 ± 1.6	7.07 ± 2.2	0.51 ± 0.05
H	1.81 ± 0.3	2.28 ± 0.9	4.46 ± 2.8	0.83 ± 0.04
F	1.75 ± 0.7	2.78 ± 0.1	4.64 ± 1.8	0.92 ± 0.1

Rate constants are expressed per hour.

Table 3. First order rate constant values for AZT-substituted phosphoramidate derivatives in the presence of enzymes



Compound (X)	Lipase	Esterase	Subtilisin Carlsberg	Carica papaya
NO_2	0.35 ± 0.06	0.22 ± 0.1	0.39 ± 0.04	0.65 ± 0.01
Br	0.83 ± 0.09	5.72 ± 0.2	3.56 ± 1.4	0.81 ± 0.05
OMe	0.46 ± 0.01	4.71 ± 1.2	0.70 ± 0.1	0.63 ± 0.02
H	0.02	—	0.27 ± 0.09	0.08

Rate constants are expressed per hour.

ed phosphoramidate derivatives hydrolyzed by enzymes. Figure 1 shows the HPLC profile comparison for bromo-substituted aryl phosphoramidate derivatives with varying nucleosides treated with lipase. Lipase had the greatest preference for stereoselectivity with the d4T-substituted aryl phosphoramidate derivatives in comparison to the AZT and 3dT derivatives. For esterase-mediated hydrolysis, we observed that AZT-substituted aryl phosphoramidate derivative had the highest chiral selectivity (Fig. 2). This suggests that the esterase discriminates between the diastereoisomers of AZT-substituted arylphosphoramidate derivatives much better than the 3dT and d4T compounds. Both the d4T and 3dT derivatives indicated very little chiral selectivity, among the isomers (Fig. 2). Focusing on the chiral selectivity observed for the protease subtilisin Carlsberg, the highest chiral selectivity was noticed in the case of d4T derivatives, followed by the AZT and 3dT analogs (Fig. 3). Figure 4 illustrates the HPLC profile observed for Carica papaya protease-mediated hydrolysis of the *p*-bromophosphoramidate derivative. For the 3dT- and AZT-substituted compounds, one of the isomers was preferentially hydrolyzed compared to the other. However, the d4T-substituted derivative underwent faster hydrolysis (1.01 for 3dT, 1.43 for d4T, and 0.65 for AZT) with low chiral selectivity. This suggests that Carica papaya recognizes both the 3dT- and AZT-substituted analogs better than the d4T derivatives. The greatest degree of chiral selectivity was observed for the AZT-substituted aryl phosphoramidate derivative followed by 3dT.

Table 4 shows the summary of the trends observed for each of the nucleoside analogs. It shows the groups that were found to yield the fastest rates of hydrolysis for the individual enzymes. In general, the preferred substituent on the phenyl moiety of these phosphoramidate derivatives seems to be a bromo, chloro, or methoxy group. However, for the esterase mediated hydrolysis of 3dT, the methoxy derivative was the only compound that was more readily hydrolyzed, although no chiral selectivity is observed. This is an important observation that may be crucial in deciding which nucleoside analog should be used for antiviral therapy for a particular case.

2.2. Substitution on the phenyl ring and chiral selectivity

We also examined the influence of various para substitutions on the phenyl ring on the chiral selectivity by individual enzymes. Esterase showed very little chiral selectivity irrespective of the substituents present in the phenyl ring demonstrating that these substituents did not affect the recognition of both.

Examining the chiral selectivity of lipase, we found that the chloro and bromo groups on the phenyl ring influenced the chiral selectivity in 3dT and AZT derivatives more than groups such as OMe. This may be attributed to the size and/or polarity of the halo groups. The substituents on the phenyl ring also influenced the chiral selectivity of d4T derivatives. In the case of protease enzymes, chiral selectivity by subtilisin Carlsberg[39] as well as Carcia papaya[37,40] was influenced by some of the substituents on the phenyl ring in all three nucleoside derivatives. As an example, the selectivity indices observed for individual phosphorus isomers of d4T-substituted phosphoramidate analogs treated with lipase and esterase are shown in Table 5.[29,41] The unprecedented selectivity shown by lipase is rationalized due to the tight binding pocket of the lipase compared to esterase.[29] Recently, we also reported the selective indices for protease-mediated hydrolysis of phosphoramidate derivatives of stavudine and found that substituents on the phenyl ring have a profound effect on the selectivity.[41]

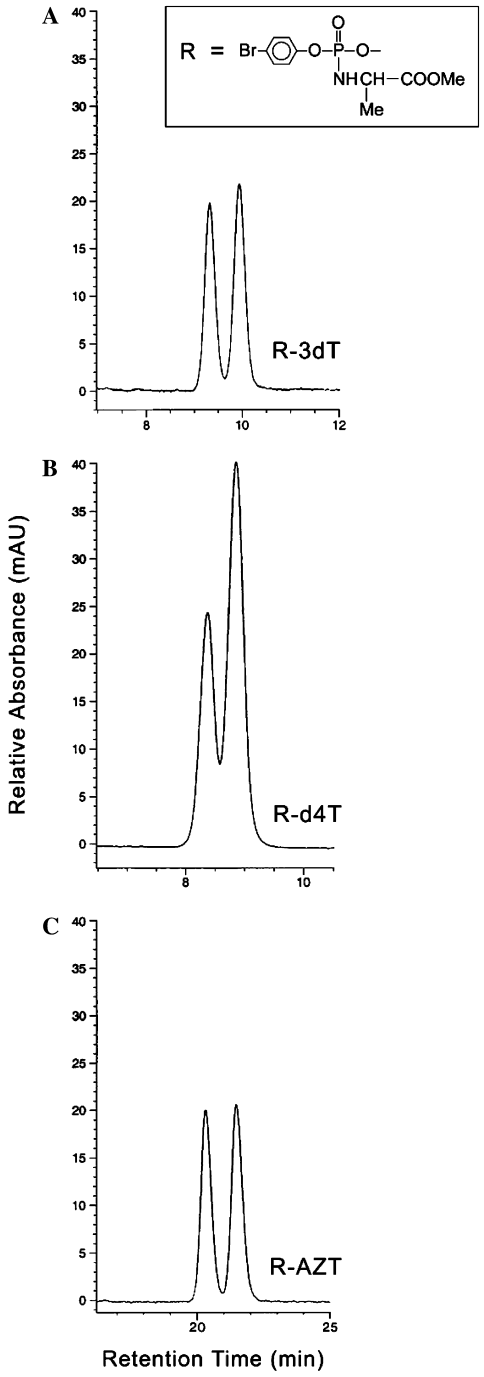


Figure 1. Chiral selectivity of lipase-mediated hydrolysis of the *p*-bromophenyl phosphoramidate derivatives of 3dT (A), d4T (B), and AZT (C) as observed by HPLC at $t = 15$ min.

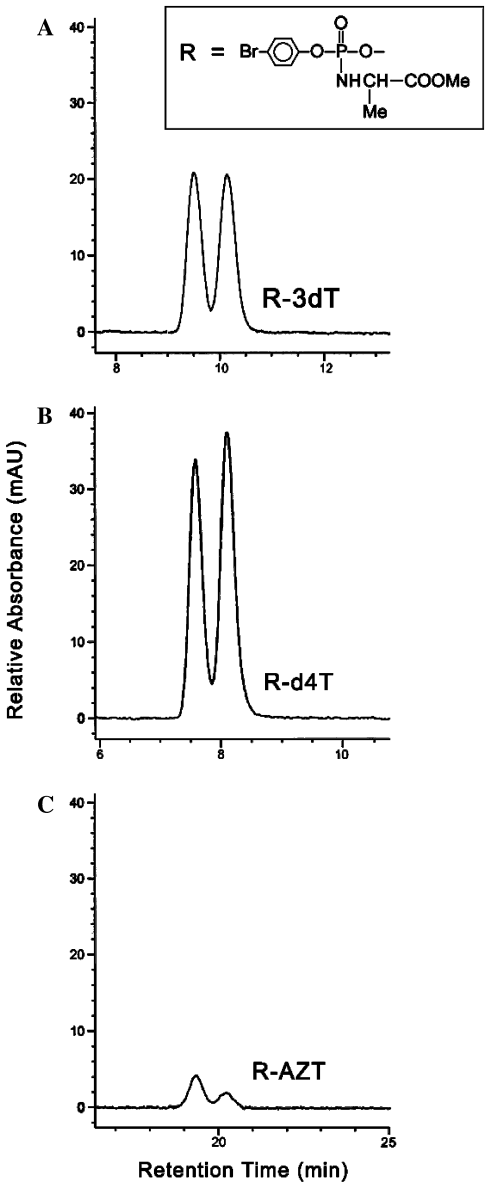


Figure 2. Chiral selectivity of esterase-mediated hydrolysis of the *p*-bromophenyl phosphoramidate derivatives of 3dT (A), d4T (B), and AZT (C) as observed by HPLC at $t = 15$ min.

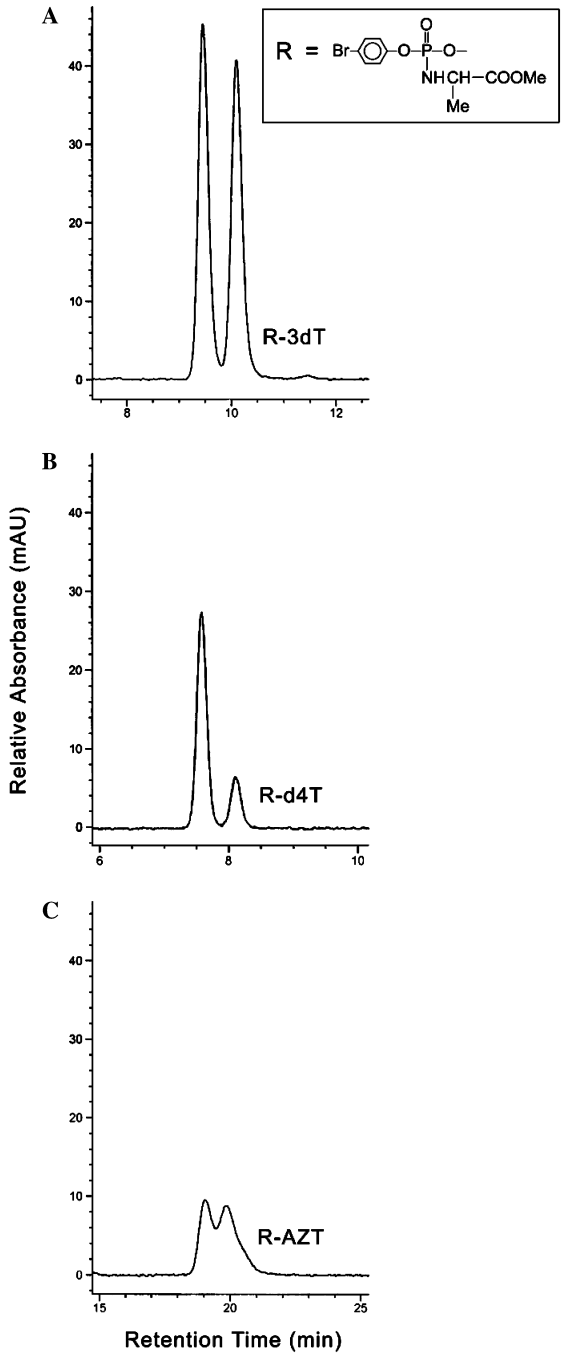


Figure 3. Chiral selectivity of subtilisin Carlsberg mediated hydrolysis of the *p*-bromophenyl phosphoramidate derivatives of 3dT (A), d4T (B), and AZT (C) as observed by HPLC at $t = 15$ min.

Based on our earlier studies we tentatively assigned the stereochemistry of the isomers that under go hydrolysis in the presence of enzymes for d4T derivatives.[29] Lipase preferably hydrolyzes the (*S*) isomers of all the phosphoramidate derivatives faster than the (*R*) isomers. Esterase showed no isomeric preference. The protease subtilisin Carlsberg preferably hydrolyzes (*R*) isomers and the cysteine protease prefers to hydrolyze the (*S*) isomers. This point is further illustrated by examining a mixture of phosphoramidate derivatives treated with various enzymes. Figure 5 shows a series of representative HPLC chromatograms of a mixture of phosphoramidate treated with lipase over time. The peaks at 6.0 and 6.5 min represent the phosphorus diastereoisomer [R_p or S_p] of 2-bromophenyl phosphoramidate of stavudine (DDE605). The peaks at 4.3 and 4.59 correspond to the phosphorus diastereoisomers [R_p or S_p] of 4-methoxy phenyl alaninyl phosphoramidate of stavudine (DDE598). At 18 min of the reaction, additional peaks are visible in the HPLC chromatogram. The peaks at 1.98 min represent stavudine (d4T), followed by the peak at 3.1 min of *p*-methoxyphenol. The peak at 11.3 min correspond to that of 2-bromophenol. The isomers are hydrolyzed preferentially, demonstrating the chiral selectivity of lipase. Figure 6 shows the selectivity indices for the various phosphoramidate derivatives of stavudine treated with serine and cysteine proteases. Cysteine protease shows the highest degree of

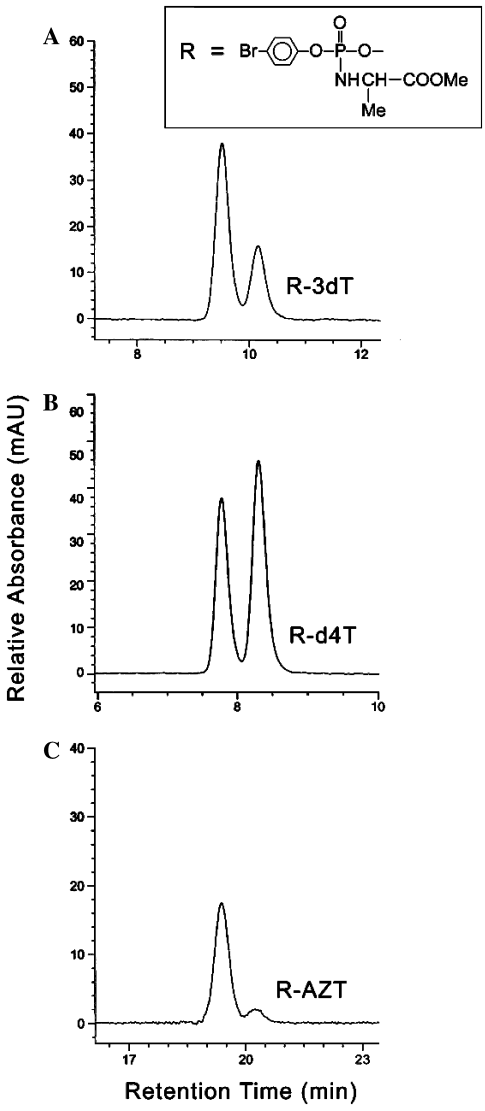


Figure 4. Chiral selectivity of Carica papaya mediated hydrolysis of the *p*-bromophenyl phosphoramidate derivatives of 3dT (A), d4T (B), and AZT (C) as observed by HPLC at $t = 15$ min.

Table 4. Fast rate of hydrolysis of the best substituents present on the phenyl ring of phosphoramidate derivatives of nucleosides

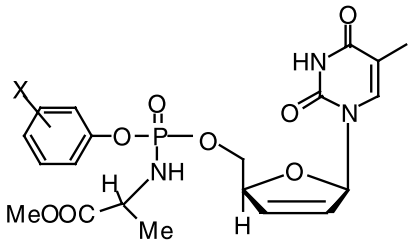
Nucleoside	Esterase	Lipase	Carlsberg	Carica papaya
d4T	OMe, Br, Cl	Br, Cl	Br	Br
AZT	OMe, Br	Br	Br	Br
3dT	OMe	Br	Cl	Br

chiral selectivity among the substituents as compared to the other protease.

2.3. Antiviral activity

We focused our attention on the antiviral activity of the aryl-substituted phosphoramidate derivatives belonging to one of the three classes of nucleoside analogs: AZT, d4T, and 3dT. Table 6 shows the antiviral activity shown by various analogs. In the case of the AZT derivatives, the bromo-substituted compound was the most potent with

Table 5. Selectivity index of various arylphosphoramidate derivatives of d4T[29,41]



X	SI			
	Lipase	Esterase	Carlsberg	Carica papaya
H	0.7	1.0	0.1	6.1
4Br	4.0	1.5	0.4	1.4
4Cl	5.4	1.3	0.2	2.0
4F	1.9	1.3	0.4	5.2
4OMe	2.7	0.9	0.5	0.8
2,6-OMe	1.0	1.3	—	—

SI denotes the selectivity index is the ratio of rate of hydrolysis observed for each of the isomers.

an IC_{50} value of 0.006 μM. The other derivatives showed only marginal to low antiviral activity (0.048–0.057 μM). Among the groups of nucleoside analogs studied, we found that the d4T derivatives were 5–40 times more potent than the AZT and 3dT compounds. On the other hand, most d4T phosphoramidate derivatives showed considerable potency (0.001–0.004 μM). However, all 3dT derivaties were shown to possess micromolar activity, indicating that saturation of the double bond in the structure of the sugar ring is not beneficial.

In the previous discussion, we have indicated that most of the enzymes hydrolyzed d4T derivatives more efficiently compared to the corresponding AZT and 3dT analogs. Relating the antiviral activity to the rate of enzymatic hydrolysis, we conclude that in order for a drug to be effective we need to have a structural framework that can be recognized by a variety of enzymes. Also it shows that hydrolysis may be one of the key factors for the biological activity shown by these nucleoside analogs. In addition, we also point out that the metabolite resulting from the hydrolysis of the nucleoside analogs needs to be further recognized by kinases to form the active compounds.

2.4. Role of the amino acid

To evaluate the influence of the amino acid of these phosphoramidate derivatives on their anti-HIV activity we prepared AZT analogs with different amino acids. We chose the 4-bromo substitutions primarily because the bromo derivatives were found to be more active as well as more easily hydrolyzed. Table 7 shows the rate of hydrolysis observed for the compounds in the presence of the various enzymes examined. Among the amino acids chosen alanine was found to be the best amino acid with the highest rate of hydrolysis for all the enzymes. However, phenyl alanine-substituted aryl phosphoramidate derivative of AZT showed a faster rate for both esterase and subtilisin Carlsberg-mediated hydrolysis. The proline, tryptophan, leucine, and phenyl

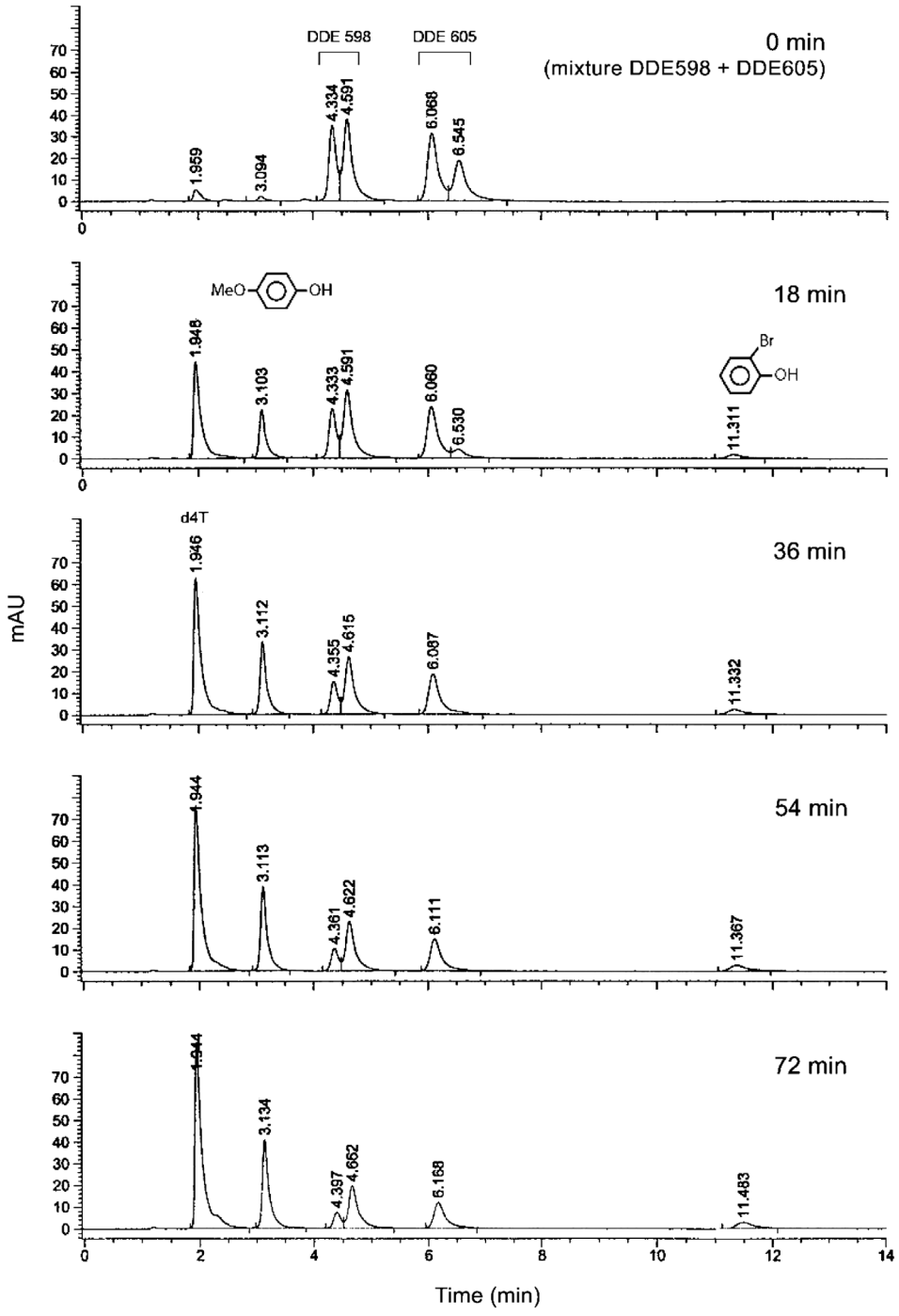


Figure 5. HPLC profiles of a mixture of the 4-methoxy- (DDE598) and the 2-bromophenyl phosphoramidate (DDE605) derivatives of d4T treated with lipase at various intervals of time.

alanine substituted phosphoramidate derivatives all had a slower rate of hydrolysis with lipase. The antiviral potency shown by the alanine-substituted phosphoramidate derivative was 10-fold higher compared to other amino acid derivatives.[35]

2.5. Mechanism of enzyme mediated hydrolysis of phosphoramidate derivatives of nucleosides

It has been proposed that the rate limiting step for the conversion of AZT to its bioactive metabolite, AZT-triphosphate, is the conversion of the monophosphate derivative to the diphosphate derivative. By contrast, the rate limiting step for the intracellular generation of the bioactive stavudine metabolite, stavudine-triphosphate, was reported to be the conversion of the nucleoside to its monophosphate derivative.[9–11] The difference in the rate limiting steps for these two nucleosides indirectly points out that the kinases differentiate between the structural differences of the two compounds. In other words, the change of the nucleoside may impart a change in the binding characteristics with kinases. We

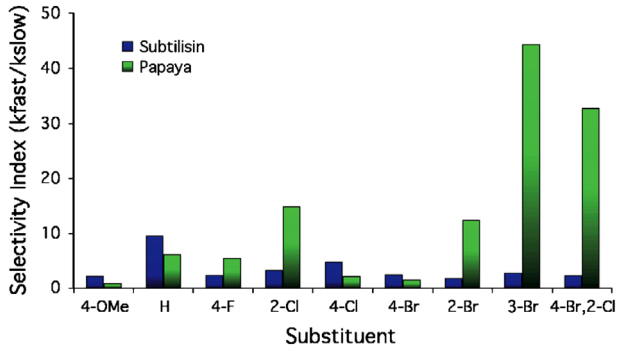
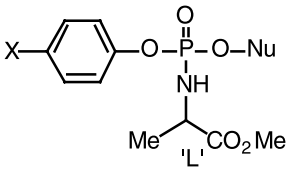


Figure 6. Selectivity index for two proteases for varying substituents on the phenyl ring of phosphoramidate derivatives of stavudine. Left to right increasing value of Hamett sigma.

Table 6. Antiviral activity of various phosphoramidate derivatives of nucleoside analogs



Substituents	AZT	d4T[18]	3dT[18]
H	0.055[17]	0.002	2.1
OMe	0.057[17], 0.057[18]	0.004	1.3
F	0.048[17], 0.029[18]	0.001	3.1
Cl	—	0.001	2.1
Br	0.006	0.001 (stampidine)	1.2

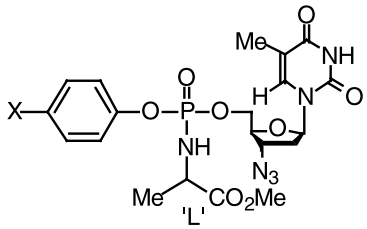
Antiviral activity expressed in μM.
The viral strain used for the above studies was HTLVIII$_B$. HIV-1 strain. The applied p24 enzyme immunoassay (EIA) was unmodified kinetic assay commercially available from Coulter Corporation/ Immunotech, Inc. (Westbrooke, ME), which utilizes a murine mAb to HIV core protein coated on to microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells (i.e., virus controls).

speculate that a similar trend occurs for the 3dT derivatives, although detailed studies have not been initiated due to the low activity profile of this compound. This analogy is supported by how bacterial enzymes differentiate between these three nucleosides as observed from their hydrolysis profile. It is essential that the phosphoramidate derivatives be hydrolyzed first to remove the phenoxy moiety in order to be activated by kinases. As such, the proposed hydrolysis pathway may be playing the key role for initiating the metabolism of these compounds. For this to happen the necessary enzymes in the cells need to recognize the substrate structure. To illustrate this, we propose the following pathway for hydrolytic profile of phosphoramidate derivatives of nucleosides (Scheme 1).

We chose only d4T and AZT derivatives as these phosphoramidate derivatives showed the most promising antiviral activity. In the first case, we observed that the rate of hydrolysis by the bacterial enzyme serine protease, subtilisin Carlsberg, yielded the fastest rate of hydrolysis for all the stavudine derivatives examined. Both lipase and esterase showed similar trends, however at a lower rate of hydrolysis. We also point out that cysteine protease Carcia papaya showed a 3- to 5-fold lower hydrolysis rate, implying that it is not being recognized as well as the d4T phosphoramidates.

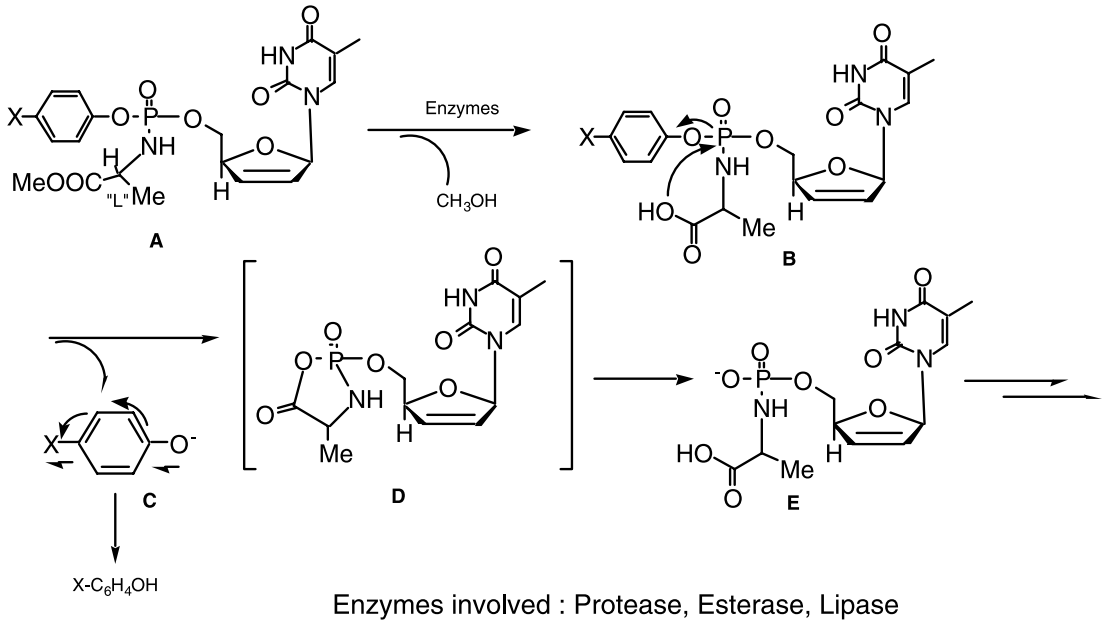
In the case of AZT derivatives, esterase showed considerable activation as compared to lipase and Carica papaya. However, in the case of subtilisin Carlsberg, all four derivatives (leucine, tryptophan, phenyl alanine, and alanine) showed moderate rate of hydrolysis. Based on the result, we propose that bromo- and methoxy-substituted AZT derivatives of phosphoramidate derivatives are well recognized by esterase and subtilisin Carlsberg as compared to other enzymes. Lipase showed very low rate of hydrolysis in the case of AZT derivatives as compared to both d4T and 3dT analogs. This is understandable, given that the binding pocket of lipase is very small [29] and the azido group in AZT present in the sugar moiety is not able to enter the binding pocket. In the case of 3dT presence of two hydrogen atoms in the sugar moiety may affect the binding although to a lesser extent than the azido group. On the other hand, esterase has a larger area for binding and we observe a faster rate of hydrolysis in each of the nucleoside analogs. A similar trend was observed with subtilisin Carlsberg. In summary, we hypothesize that each structural modification of the phosphoramidate derivatives of nucleosides has a significant role toward activation by different enzymes.

Table 7. Rate of hydrolysis of phosphoramidate derivatives of AZT with enzymes



Amino Acid (L) (R_1)	Lipase	Subtilisin Carlsberg	Esterase	Carica papaya
Leucine	0.03 ± 0.02	2.8 ± 2.5	5.06 ± 0.9	0.17 ± 0.1
Tryptophan	0.05 ± 0.04	2.8 ± 0.08	5.65 ± 0.5	0.23 ± 0.2
Phenyl alanine	0.31 ± 0.2	8.6 ± 4.6	11.23 ± 0.8	0.17 ± 0.1
Alanine	0.83 ± 0.09	3.6 ± 1.4	5.72 ± 0.2	0.81 ± 0.05

Rate constants are expressed per hour.



Scheme 1. Enzymatic hydrolysis pathway of phosphoramidate derivatives of stavudine.

2.6. Tentative pathway for the activation of phosphoramidate derivatives of stavudine with various enzymes

Based on the results obtained from the enzymes examined we propose that there are two steps involved in the conversion of these phosphoramidate derivatives to their active metabolites (Fig. 7). We selected stampidine, a 4-bromophenylmethoxylalaninyl phosphoramidate of stavudine, as the example compound represented in the pathway. In the drug activation step, either one or all the three enzymes could act on the compound to convert it into the active metabolite following the mechanism depicted in Scheme 1. Based on the rate of reaction we hypothesize that first protease may be involved (due to the highest rate of reaction), followed by other enzymes. Under these circumstances, the drug is a competitive substrate for hydrolysis between these three broad classes of enzymes. Once the active metabolite is formed, a subsequent step involves the conversion of the above by thymidine kinase in an ATP-dependent mechanism to inhibit the viral reverse transcriptase (viral inhibition step, Fig. 7).

After having established that proteases and other bacterial enzymes could be involved in conversion of these phosphoramidate derivatives of stavudine to the active metabolite, the next question was to determine whether cellular or viral proteases could also result in drug activation. To address this, we conducted experiments that involved the treatment of stampidine with commercially available HIV-1 protease, recombinant, expressed in *Escherichia coli* suitable for cleaving the HIV substrate. We examined the reaction between this protease and stampidine by HPLC and monitored the reaction over time. We found that there was no reaction between the para-bromo-substituted phosphoramidate derivative of stavudine and this protease under our experimental conditions. This could possibly be explained due to the specificity of the HIV-1 protease. These proteases do not accept a broad spectrum of compounds. However, our results demonstrate that the HIV-1 protease is not directly involved in the activation step depicted in our flowchart. Based on this result we propose that cellular proteases may be involved instead of HIV-1 protease in the activation step illustrated in Scheme 1. Further work is in progress to evaluate the role of intra/extracellular proteases with these phosphoramidate derivatives.

2.7. Biological relevance of bacterial enzymes used in the present study

To compare our results with the bacterial enzymes[39,40] to biologically relevant systems, we have conducted a series of experiments using a variety of cells: we used five different cell lines to examine the intracellular metabolism of stampidine, namely Jurkat (T-cell line), Cos-7, (monkey kidney cell line), RAW 264 (macrophage-like cell line), CEM (T-leukemia cell line), and LL2 (mouse lung cancer cell line). Cells in phosphate buffer were treated with stampidine and aliquots of the supernatant were collected at various time intervals and analyzed by HPLC. A guard column was used in order to avoid any protein or particulate from entering the column. In 15 min, treatmentof RAW 264 cells with stampidine resulted in the formation of d4T and *p*-bromophenol. In 30 min, we observed an increase in the amount of d4T and *p*-bromophenol; however, one of the isomers was still present in intact form (Fig. 8). Similar results were observed with Cos-7 cells. Furthermore, one of the isomers of stampidine was found to get hydrolyzed preferentially, implying that similar enzymes in RAW 264 and Cos-7 cell lines may be responsible for the chiral selectivity of hydrolysis. (Table 8).

For the next set of experiments, we used Jurkat cells. Treatment of Jurkat cells with stampidine resulted in

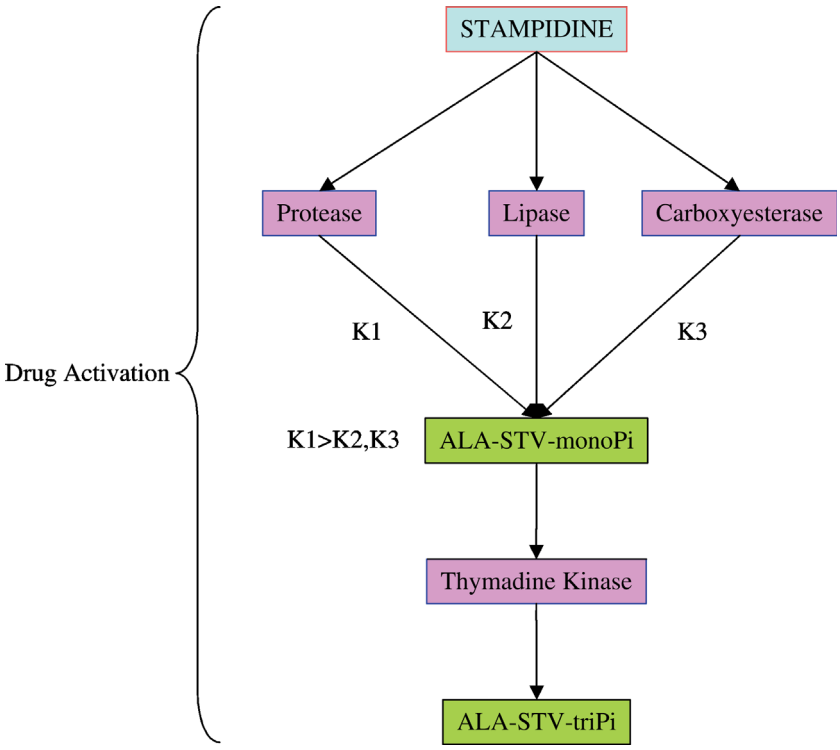


Figure 7. Conversion of stampidine into active metabolites. Stampidine is thought to be a competitive substrate for hydrolysis by three broad classes of enzymes: proteases, lipases, and carboxyesterases. The product of this drug activation step is ala-stv-monophosphate. The in vitro studies suggest that proteases are the most active in hydrolyzing stampidine (K1 > K2, K3). Thymidine kinase then converts ala-stv-monophosphate to ala-stv-triphosphate in an ATP dependent mechanism to inhibit the viral reverse transcriptase (viral inhibition step). Stampidine was 100-fold more potent than stavudine and 2-fold more potent than zidovudine against primary clinical HIV-1 isolates of non-B envelope subtype originating from South America, Asia, and sub-Saharan Africa.[20,21] It is well known that both in stavudine and zidovudine, the active component is primarily due to the formation of triphosphate. Our experimental results suggest that the potency of stampidine against these clinical isolates indirectly points out that alanine stavudine triphosphate could be formed instead of stavudine triphosphate. Additionally, we have shown in our pharmacokinetic studies indeed alanine stavudine monophosphate is formed in vivo studies.[37,38]

the intracellular hydrolysis of stampidine within 30 min. One isomer underwent hydrolysis faster than the other (Fig. 9). Additionally, it was evident that this isomer was different from the isomer undergoing hydrolysis in RAW 264 and Cos-7 cells. Thus, two different types of enzymes each preferentially recognizing one of the isomers may be operative in different cell types. The hydrolysis of stampidine in CEM Cells and LL2 mouse lung cells (Figs. 10 and 11) mimicked its hydrolysis in Jurkat cells.

2.8. Comparison of the rate of hydrolysis

The fastest rate of hydrolysis for stampidine was observed in RAW 264 cells and the slowest rate of hydrolysis was observed with CEM cells (Fig. 12). The rate of hydrolysis of stampidine in RAW 264 cells was 3-fold faster than its rate of hydrolysis in Cos-7 cells and 7-fold faster than its rate of hydrolysis in Jurkat cells. Similarly the rate of hydrolysis in RAW 264 cells was 5-fold faster than in LLL2 cells and ≈15 times faster than in CEM cells.

Depending on the cell line utilized we observed varying chiral selectivity in the hydrolysis of stampidine isomers. This observation indicates that different enzymes may be operative in the hydrolysis of stampidine in different cell types. We also propose that various other enzymes are capable of hydrolyzing stampidine apart from esterase. Overall, the results demonstrated that the hydrolysis occurs immediately after incubating the compound with cells. In addition, enzyme inhibitors were found to slow down the rate of hydrolysis. We used both protease and esterase inhibitors to demonstrate the inhibition. In the future, we plan to extend our studies to other enzymes that are potentially involved in the metabolism of these phosphoramidate derivatives by examining the hydrolysis profile in enzyme deficient cells.

3. Conclusion

In summary, the data support the proposal that a change in the nucleoside structure in these phosphoramidate derivatives causes significant alteration in the chiral selectivity, hydrolysis rate, and the antiviral activity.

4. Experimental

4.1. Compounds

All the compounds in the present study were prepared using known literature procedures and were characterized using analytical techniques, as reported in our

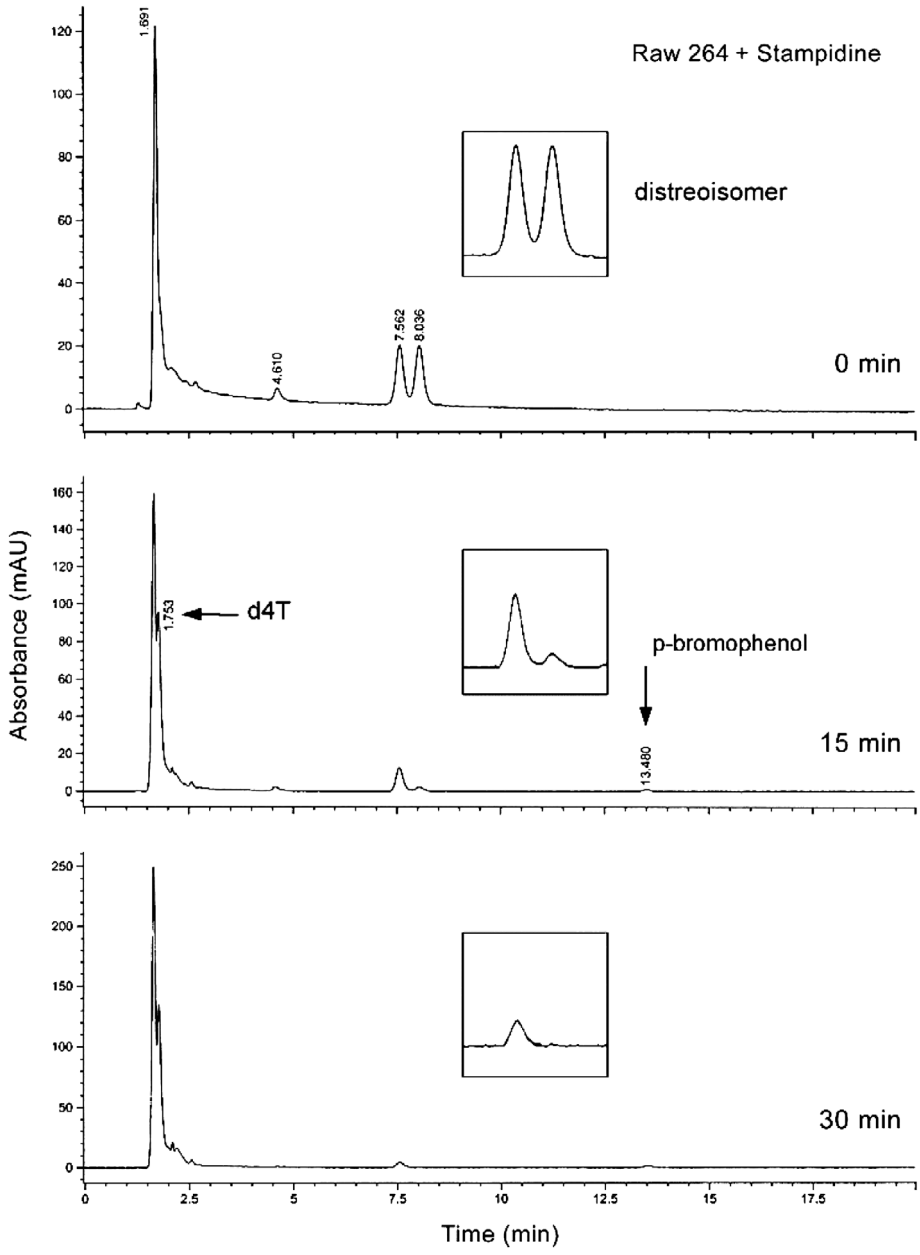


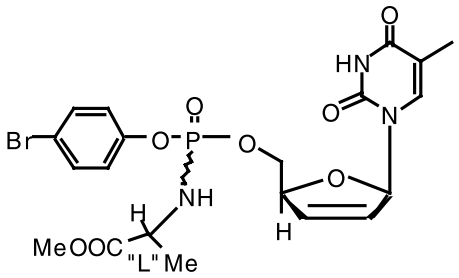
Figure 8. HPLC profile of extracts from RAW 264 cells treated with stampidine.

earlier publications.[18–20] Lipase (Candida Antartica-B, cat #ICR-110), protease (subtilisin Carlsberg, cat #ICR–119), as well as protease (Carica papaya, cat #ICR-124) were obtained from Biocatalytics Inc., Pasadena, CA 91106. The solid enzymes were used as such without further purification.

4.2. Experimental conditions for lipase study

For the kinetic study, a known amount of a phosphoramidate derivative was carefully weighed (5–7 mg) using an analytical balance and transferred into a 20 mL scintillation glass vial. Using a pipette man, 3 mL of methanol was added and the contents were vortexed for 2 min until a homogeneous solution resulted. Using another pipet man, 100 μL of that stock solution was transferred into another scintillation vial along with 900 μL of water and the contents were vortexed. Separately the enzyme stock solution was prepared using 5 mg of solid lipase powder and was transferred to a volumetric flask. To this was added 8 mL of water and the contents were shaken to dissolve the enzyme. The reaction mixture was prepared as follows for the kinetic study. From the stock solution of the compound as mentioned above, 500 μL of the methanolic solution of the phosphoramidate derivative was pipetted out into another glass vial and to this 500 μL of enzyme solution was added. The contents were shaken to form a homogeneous solution. From this reaction mixture, 50 μL was used for HPLC analysis. The column used was a Lichrospher RP-18 analytical column (5 μm, 4×250 mm). The eluent used for HPLC was water (0.1% TFA/TEA) and acetonitrile in the ratio of 65:35. The column was maintained at room temperature. The flow rate was maintained at 1 mL/min, the detection

Table 8. Chiral selectivity of phosphorus stereoisomers of phosphoramidate derivative stampidine with different cell treatment



Cells used	Fastest hydrolyzing isomer	Rate per hour
RAW 264 (macrophages)	'*R*' isomer	4.03
Cos-7 (monkey kidney cells)	'*R*' isomer	1.41
Jurkat cells	'*S*' isomer	0.69
CEM cells	'*S*' isomer	0.28
LL2-lung cells	'*S*' isomer	0.75

Tentative stereochemistry assignment based on previous publication.[29]

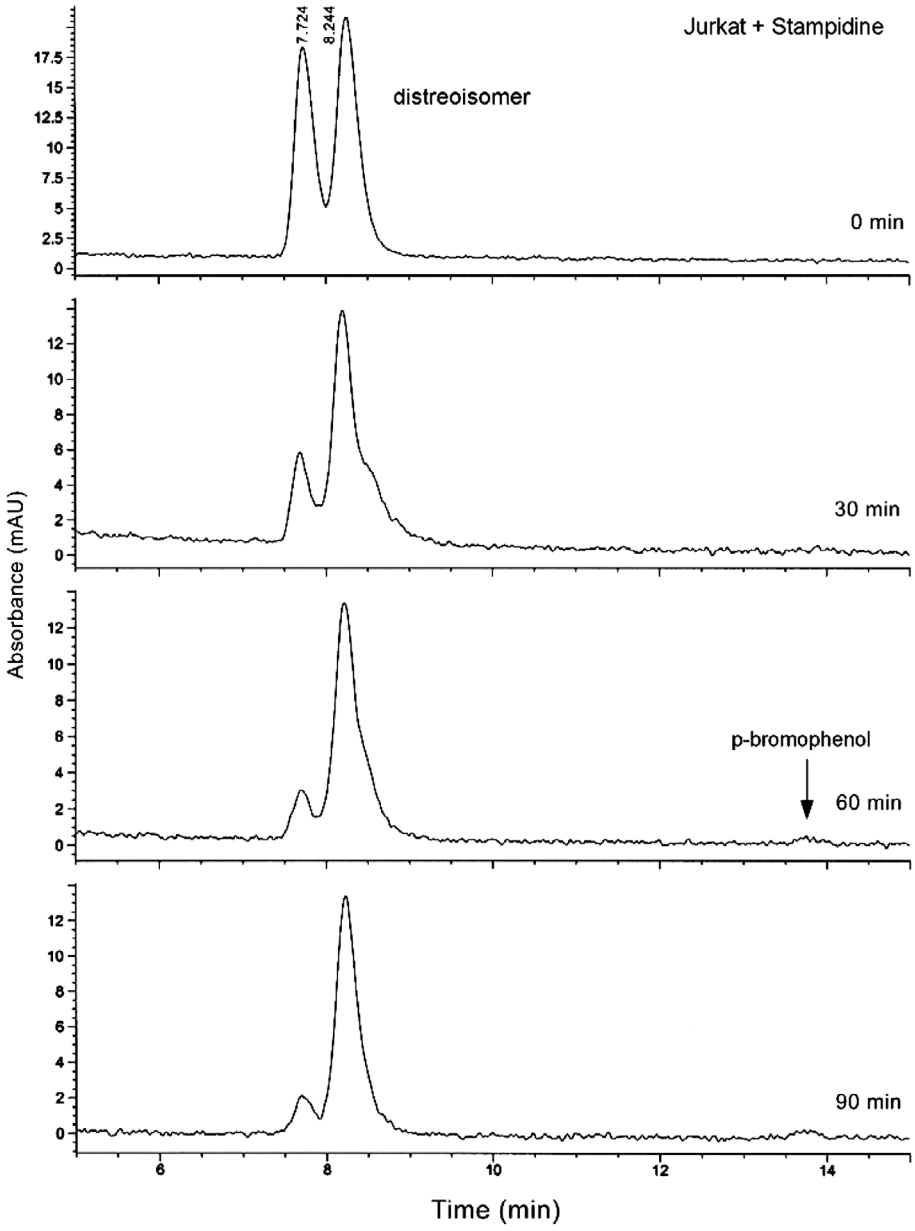


Figure 9. HPLC profile of extracts from Jurkat cells treated with stampidine.

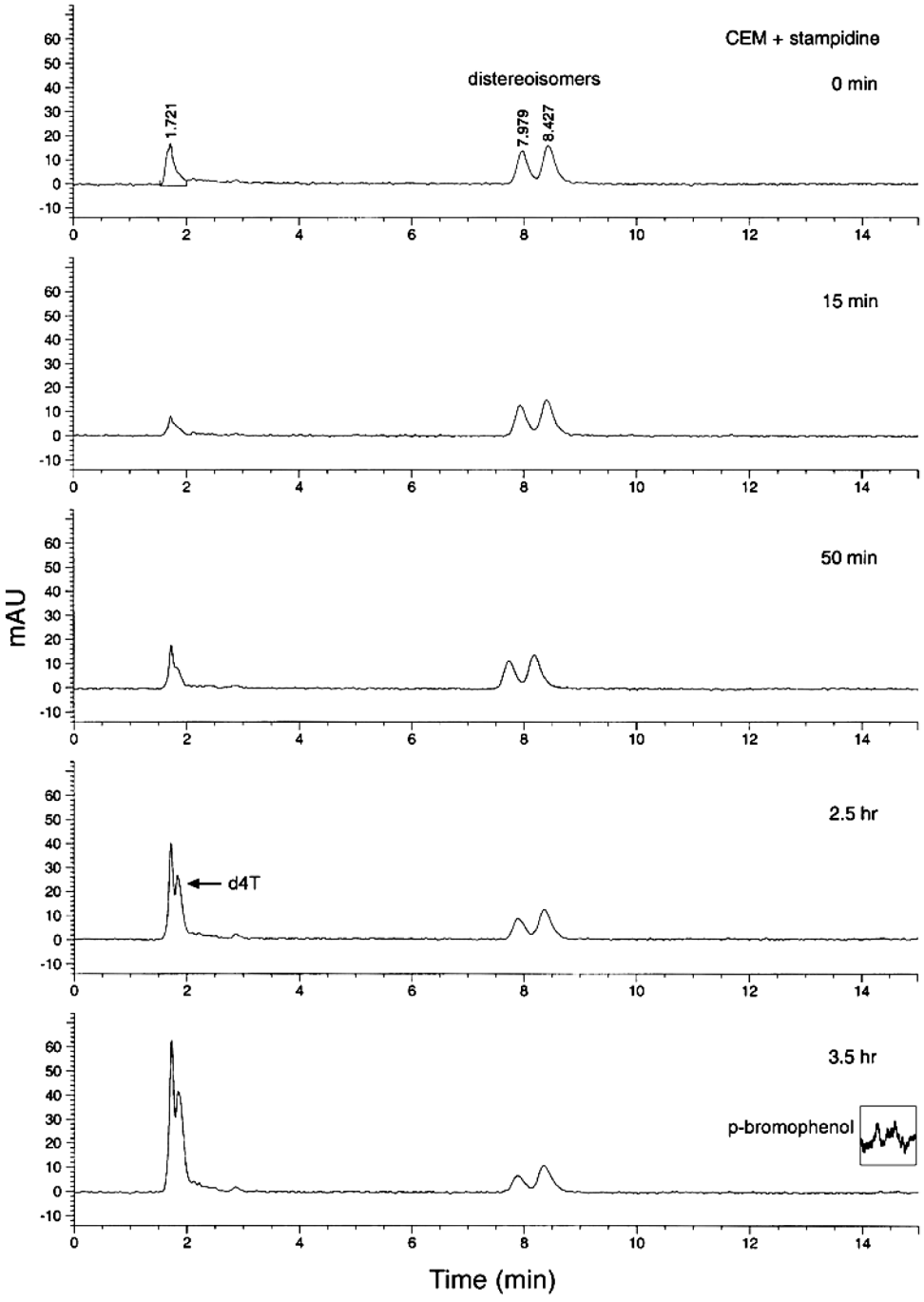


Figure 10. HPLC profile of extracts from CEM cells treated with stampidine.

wavelength was adjusted to 265 nm, and the reference wavelength was kept at 400 nm. Aliquot of the sample was drawn at various intervals of time from the reaction vial and analyzed. For fast reactions, two HPLC instruments were used simultaneously to obtain the rates. Hydrolysis rates were determined by fitting single exponential decay equations to the disappearance of each isomer substrate in the presence of enzyme.

4.3. Experimental conditions for protease study

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (3 mg) using an analytical balance and transferred into a scintillation glass vial. Using a pipette man, 3 mL of methanol was added and the contents were vortexed for 2 min until a homogeneous solution resulted. Using another pipetman, 100 μL of the compound stock solution was transferred into another scintillation vial and to this was added 900 μL of water and the contents were vortexed. Separately the enzyme stock solution was prepared using 5.8 mg of solid protease powder and transferred to a volumetric flask, diluted with 8 mL of water, and the contents were shaken to dissolve the enzyme. The reaction mixture was prepared as follows for the kinetic study. From the stock solution of the compound 150 μL

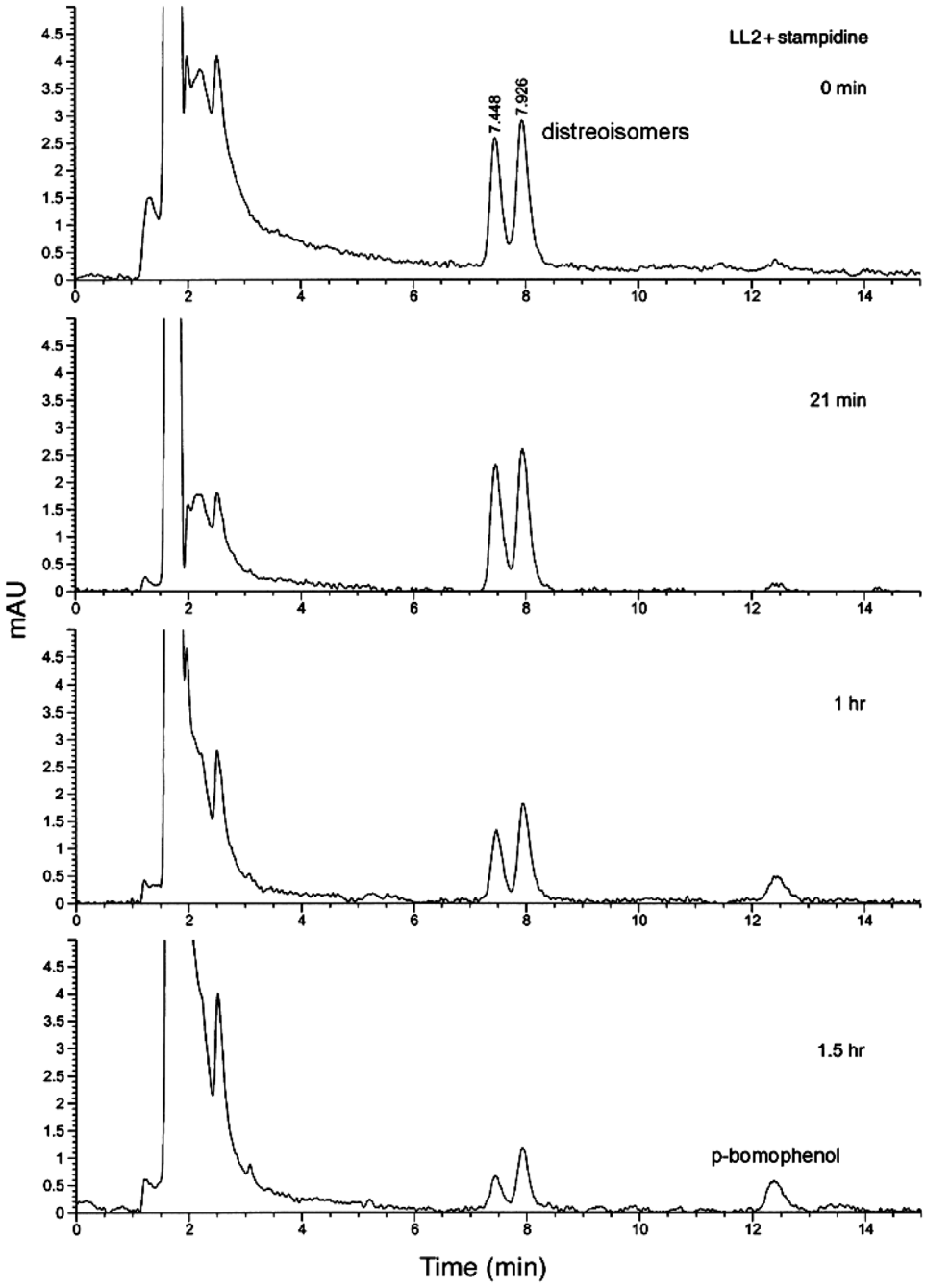


Figure 11. HPLC profile of extracts from LL2 mouse lung cells treated with stampidine.

was pipetted into another glass vial. To this, 150 μL of protease stock solution was added and the contents were shaken to form a homogeneous solution. From this reaction mixture, 20 μL was used for HPLC analysis.

4.4. Experimental conditions for esterase study

For the esterase studies, 10 μL of esterase solution was transferred into a vial and 40 μL of water was added and the contents were vortexed for 2 min. The sample was prepared using a similar procedure as reported in other studies (3 mg/3 mL methanol, 100 μL taken, and diluted with 900 μL of water). For the kinetic study, 40 μL of the diluted compound was transferred into a vial. To this, was added 40 μL of water followed by 10 μL of esterase enzyme solution.

4.5. Estimation of products

The amount of products observed during the reactions was estimated from the area obtained from the HPLC profiles. In addition, authentic samples of the products when possible were run to identify the peaks observed during the reaction. The rate of reaction was computed by using first order rate constants and an average of eight to nine time points were used for this estimate. The rate constants reported refer to rate per hour, since some of the reactions were too slow to obtain meaningful results.

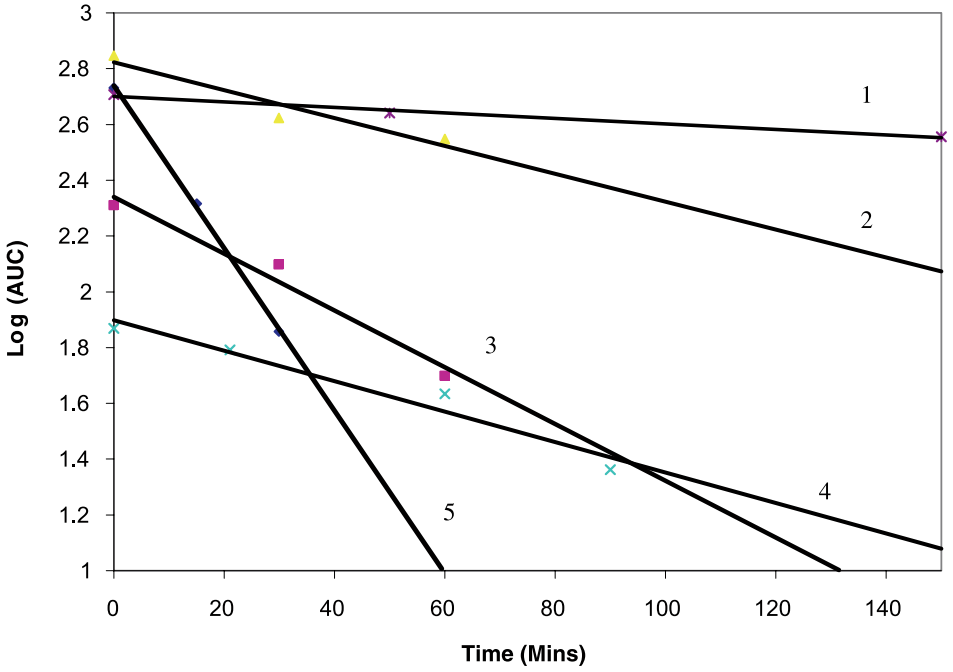


Figure 12. Rate of hydrolysis of stampidine treated with various cells: 1, CEM; 2, Jurkat; 3, Cos-7; 4, LL2 Lung; 5, RAW 264.

4.6. Lymphocyte experimental protocol

The cells were propagated in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90% and fetal bovine serum, 10%, at 37 °C in a humidified atmosphere containing 5% CO_2. The cell line was obtained from the Cell Biology Laboratory of the Parker Hughes Cancer Center. To obtain these cells, 30 million frozen cells were plated in a T-150 flask with 20 mL of medium and incubated at 37 °C for 48 h. The cells were collected in a 50 mL conical tube and centrifuged at 1000 rpm for 5 min, the supernatant decanted off and the cells were re-suspended in 90 mL of medium and grown further in three T-150 flasks with 30 mL each at 37 °C for another 48 h to give 120 million cells. The cells were collected by centrifugation at 1000 rpm for 5 min, subsequently washed thrice in 1× PBS and then resuspended in 1 mL of 1× PBS.

For the HPLC measurements, 0.5 mL of the suspended cell mixture in PBS buffer was taken, treated with stampidine, and incubated at 37 °C. At various time intervals, an aliquot of the supernatant was drawn and assayed using HPLC. Authentic samples were used to identify the products during cell mediated hydrolysis of stampidine. A lichrospher-RP-18 column (5 μm, 4 × 250 mm) was used for this purpose and the eluent was a mixture consisting of 65% water containing 0.1% TEA and TFA, and 35% of acetonitrile. The flow rate was maintained at 1 mL per minute and the column was maintained at room temperature throughout the analysis. The rate of reaction was calculated using a first order kinetic equation.

4.7. In vitro assays of anti-HIV activity

Normal human peripheral blood mononuclear cells (PBMNC) from HIV-negative donors (HTLV III_B strain) were cultured 72 h in RPMI 1640 supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS), 3% interleukin-2, 2 mM L-glutamine, 25 mM HEPES, 2 g/L $NaHCO_3$, 50 mg/mL gentamicin, and 4 mg/mL phytohemagglutinin prior to exposure to HIV-1 at a multiplicity of infection (MOI) of 0.1 during a 1 h adsorption period at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, cells were cultured in 96-well microtiter plates (100 mL/well; 2×10^6 cells/mL) in the presence of various concentrations of phosphoramidates and aliquots of culture supernatants were removed from the wells on the seventh day after infection for p24 antigen assays, as previously described.[36] The applied p24 enzyme immunoassay (EIA) was unmodified kinetic assay commercially available from Coulter Corporation/Immunotech., Inc. (Westbrooke, ME), which utilizes a murine mAb to HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells (i.e., virus controls).

References and notes

1. Mansuri, M. M.; Starrett, J. E., Jr.; Ghazzouli, I.; Hitchcock, M. J. M.; Sterzycki, R. Z.; Brankovan, V.; Lin, T. S.; August, E. M.; Prussoff, W. H.; Sommadossi, J. P.; Martin, J. C. *J. Med. Chem.* **1989**, *32*, 462.
2. Horwitz, J. P.; Chua, J.; DaRooge, M. A.; Noel, M.; Klindt, I. L. *J. Org. Chem.* **1996**, *31*, 205.

3. Bell, F. W.; Cantrell, A. S.; Hogberg, M.; Jaskunas, S. R.; Johansson, N. G.; Jordan, C. L.; Kinnick, M. D.; Lind, P.; Morin, J. M., Jr.; Noreen, R.; Oberg, B.; Palkowitz, J. A.; Parrish, C. A.; Pranc, P.; Sahlberg, C.; Ternansky, R. T.; Vasileff, R. T.; Vrang, L.; West, S. J.; Zhang, H.; Zhou, X. X. *J. Med. Chem.* **1995**, *38*, 4929.
4. Cantrell, A. S.; Engelhardt, P.; Hogberg, M.; Jaskunas, S. R.; Johansson, N. G.; Jordan, C. L.; Kangasmetsa, J.; Kinnick, M. D.; Lind, P.; Morin, J. M., Jr.; Muesing, M. A.; Noreen, R.; Oberg, B.; Pranc, P.; Sahlberg, C.; Ternansky, R. J.; Vasileff, R. T.; Vrang, L.; West, S. J.; Zhang, H. *J. Med. Chem.* **1996**, *39*, 4261.
5. Vig, R.; Mao, C.; Venkatachalam, T. K.; Tuel-Ahlgren, L.; Sudbeck, E. A.; Uckun, F. M. *Bioorg. Med. Chem.* **1998**, *6*, 1789.
6. Mao, C.; Vig, R.; Venkatachalam, T. K.; Sudbeck, E. A.; Uckun, F. M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2213.
7. Furman, P. A.; Fyfe, J. A.; St. Clair, M. H.; Weinhold, K.; Rideout, J. L.; Freeman, G. A.; Nusinoff, L. S.; Bolognesi, D. P.; Broder, S.; Mitsuya, H.; Barry, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8333.
8. Hao, Z.; Cooney, D. A.; Hartman, N. R.; Perno, C. F.; Fridland, A.; De Vico, A. L.; Sarngadharan, M. G.; Border, S.; Johns, D. G. *Mol. Pharmacol.* **1988**, *34*, 431.
9. Balzarini, J.; Cooney, D. A.; Dalal, M.; Kang, G. J.; Cupp, J. E.; De Clercq, E.; Broder, S.; Johns, D. G. *Mol. Pharm.* **1987**, *32*, 798.
10. McGuigan, C.; Cahard, D.; Sheeka, H. M.; De Clercq, E.; Balzarini, J. *J. Med. Chem.* **1996**, *39*, 1748.
11. McGuigan, C.; Cahard, D.; Sheeka, H. M.; De Clercq, E.; Balzarini, J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1183.
12. Starnes, M. G.; Gheng, Y. C. *J. Biol. Chem.* **1987**, *32*, 798.
13. Hao, Z.; Cooney, D. A.; Farquhar, D.; Perno, C. F.; Zhang, K.; Masood, R.; Wilson, Y.; Hartman, N. R.; Balzarini, J.; Johns, D. G. *Mol. Pharm.* **1990**, *37*, 157.
14. Johnson, M. A.; Ahluwalia, G.; Connelly, M. C.; Cooney, D. A.; Broder, S.; Johns, D. G.; Fridland, A. *J. Biol. Chem.* **1988**, *263*, 15354.
15. Johnson, M. A.; Fridlan, A. *Mol. Pharm.* **1989**, *36*, 291.
16. McGuigan, C.; Pathirana, R. N.; Mahmood, N.; Devine, K. G.; Hay, A. J. *Antivir. Res.* **1992**, *17*, 311.
17. McGuigan, C.; Pathirana, R. N.; Balzarini, J.; De Clercq, E. *J. Med. Chem.* **1993**, *36*, 1048.
18. Vig, R.; Venkatachalam, T. K.; Uckun, F. M. *Antiviral Chem. Chemother.* **1998**, *9*, 445.
19. Venkatachalam, T. K.; Tai, T. L.; Vig, R.; Chen, C. L.; Jan, S. T.; Uckun, F. M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3121.
20. Uckun, F. M.; Pendergrass, S.; Venkatachalam, T. K.; Qazi, S.; Richman, D. *Antimicrob. Agents Chemother.* **2002**, *46*, 3613.
21. Uckun, F. M.; Qazi, S.; Pendergrass, S.; Lisowaski, E.; Waurzyniak, B.; Chen, C. L.; Venkatachalam, T. K. *Antimicrob. Agents Chemother.* **2002**, *46*, 3428.
22. Uckun, F. M.; Chen, C. L.; Samuel, P.; Pendergrass, S.; Venkatachalam, T. K.; Waurzyniak, B.; Qazi, S. *Antimicrob. Agents Chemother.* **2003**, *47*, 1233.
23. Uckun, F.M.; Vig, R.; U.S. patent 6,030,957. 2000.
24. Uckun, F. M.; Chen, C. L.; Lisowski, E.; Mitcheltree, G.; Venkatachalam, T. K.; Eerbeck, D.; Chen, H.; Waurzyniak, B. *Arzneim.-Forsch.* **2003**, *53*, 357.
25. Uckun, F. M.; Samuel, P.; Qazi, S.; Chen, C. L.; Pendergrass, S.; Venkatachalam, T. K. *Antiviral Chem. Chemother.* **2002**, *13*, 73.
26. Siddiqui, A. Q.; McGuican, C.; Ballatore, C.; Wedgwood, O.; De Clercq, E.; Balzarini, J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2555.
27. Siddiqui, A. Q.; Ballatore, C.; McGuigan, C.; De Clercq, E.; Balzarini, J. *J. Med. Chem.* **1999**, *42*, 393.
28. Wagner, C. R.; McIntee, E. J.; Schinazi, R. F.; Abraham, T. W. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1819.
29. Venkatachalam, T. K.; Samuel, P.; Li, P.; Mao, C.; Qazi, S.; Uckun, F. M. *Bioorg. Med. Chem.*, **2004**, *12*, 3371.
30. Cygler, M.; Grochulski, P.; Kazlauskas, R.; Schrag, J. D.; Bouthillier, F.; Rubn, B.; Serreqi, A. N.; Gupta, A. K. *J. Am. Chem. Soc.* **1989**, *116*, 3180.
31. Tsi, M. D.; Chang, T. T. *J. Am. Chem. Soc.* **1980**, *102*, 5416.
32. Bruzik, K.; Tsi, M. D. *J. Am. Chem. Soc.* **1982**, *104*, 863.
33. Bruzik, K.; Jiang, R. T.; Tsi, M. D. *J. Am. Chem. Soc.* **1983**, *105*, 5455.
34. Jiang, R. T.; Shyy, Y. J.; Tsi, M. D. *Biochemistry* **1984**, *23*, 1661.
35. Venkatachalam, T. K.; D'Cruz, O.; Uckun, F. M. *Antiviral Chem. Chemother.* **2000**, *11*, 31.
36. Uckun, F. M.; Chelstrom, L.; Tuel-Ahlgren, L.; Dibirdik, I.; Irvin, J. D.; Langlie, M. C.; Myers, D. E. *Antimicrob. Agents Chemother.* **1998**, *42*, 383.
37. Chen, C. L.; Yu, G.; Venkatachalam, T. K.; Uckun, F. M. *Drug Metab. Dispos.* **2002**, *30*, 1523.
38. Chen, C. L.; Venkatachalam, T. K.; Zhu, Z. H.; Uckun, F. M. *Drug Metab. Dispos.* **2001**, *29*, 1035.
39. Groves, M. R.; Taylor, M. A.; Scott, M.; Cummings, N. J.; Pickersgill, R. W.; Jenkins, J. A. *Structure* **1996**, *4*, 1193.
40. Chen, S. C.; Bahar, I. *Bioinformatics* **2004**, *20*(Suppl. 1), I77–I85.
41. Venkatachalam, T. K.; Samuel, P.; Qazi, S.; Uckun, F. M.; Europ. *J. Med. Chem.*, **2005**, *40*, 452.