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Bioorganic & Medicinal Chemistry 13 (2005) 1763–1773
Bioorganic & Medicinal Chemistry

Stereochemical influence on lipase-mediated hydrolysis and biological activity of stampidine and other stavudine phosphoramidates

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Received 30 April 2004; accepted 1 December 2004
Available online 21 January 2005

Abstract—Stampidine and other halogen substituted stavudine phosphoramidates can be activated by lipase-mediated hydrolysis. The target site for the lipase appears to be the methyl ester group of the L-alanine side chain. Accordingly, the D-amino acid substituted isomers {Rp or Sp}are resistant to lipase-mediated hydrolysis and exhibit substantially less anti-HIV activity. Molecular modeling results indicate that the L-amino acid configured isomers {Rp or Sp} are preferred in the lipase binding pocket.

1. Introduction

Stampidine is a novel phosphoramidate derivative of stavudine that is being developed as a potential anti-HIV agent.[1–5] 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.[1] Stampidine was effective against twenty genotypically and phenotypically nucleoside analog reverse transcriptase inhibitor (NRTI)-resistant and six non-nucleoside inhibitor (NNRTI)-resistant HIV-1 isolates at subnanomolar to low nanomolar concentrations.[1] Stampidine was active against HIV-1 isolates with five thymidine analogue mutations at subnanomolar concentrations. 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.[4] In the feline immunodeficiency virus (FIV)-infected domestic cat model for AIDS, orally administered stampidine showed a dose-dependent anti-retroviral effect in chronically FIV-infected cats.[5] 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.[6,7] 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.

2. Results and discussion

The generation of ala-d4T-MP as the active metabolite of stampidine was originally proposed to require the esterase-mediated hydrolysis of the carbomethoxy group associated with the alanine side chain of stampidine.[7–33] We hypothesized that in various tissue microenvironments the metabolism of stampidine may occur through the action of hydrolytic enzymes other than esterases as well. In a recent study, we found that lipase-mediated stereoselective hydrolysis of stampidine and other phosphoramidates derivatives of stavudine play a key role for their metabolism.[34]

Modeling studies and comparison of the hydrolysis rate constants revealed a chiral preference of the lipase active site for the putative Sp-stereoisomer. The in vitro

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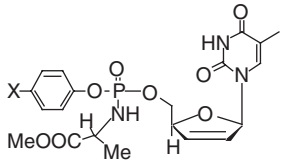
doi:10.1016/j.bmc.2004.12.024

anti-HIV activity of these compounds correlated with their susceptibility to lipase- (but not esterase-) mediated hydrolysis. Saboulard et al.[14] McGuigan et al.[10–13] Balzarini and co-workers,[23–26] Wagner and co-workers,[30,31,8] Meier et al.[45–48] have suggested an esterase-dependent biochemical pathway for the hydrolysis of phosphoramidate derivatives. Our recent studies provided experimental evidence that other enzymes including lipases and proteases[35] are also involved in the activation of phosphoramidate derivatives of nucleoside analogs. We propose that stampidine undergoes rapid enzymatic hydrolysis in the presence of lipase according to the following biochemical pathway: during the first step, hydrolysis of the ester group results in the formation of carboxylic acid. A subsequent step involves an intramolecular cyclization at the phosphorous center with simultaneous elimination of the phenoxy group to form a cyclic intermediate. In the presence of water, this intermediate is converted into the active metabolite ala-d4T-MP. We postulated that the lipase hydrolyzes the methyl ester group of the L-alanine side chain to form the cyclic intermediate in a stereoselective fashion. This hypothesis was supported by experimental data showing that chloroethylsubstituted derivatives of stampidine, which possess a chloroethyl linker unit instead of a methyl ester side chain, were resistant to lipase-mediated hydrolysis, which excludes the possibility of a direct hydrolysis of stampidine at the phosphorous center. However, caution should be exercised in that the inactivity of the chloroethyl analogs may be due to other reasons such as extra cellular instability or involvement of other enzymes. Thus, our model implies that the lipase-mediated formation of the cyclic intermediate is a key step in the metabolism of stampidine and relies on the initial configuration of the stereoisomers [Rp or Sp]. This is in accordance with the earlier notion by Subbord et al.,[14] and Balzarini and co-workers.[23–26] In an attempt to further confirm that the carboxymethyl ester side chain is the target site for the lipase-mediated hydrolysis, we replaced the L-alanine side chain with a D-alanine side chain, that does not fit the binding pocket of the lipase without spatial constraints. 'L' and 'D'-configured amino acid modification of phosphoramidate derivatives have been reported by McGuigan et al.[36] In their work these authors have prepared the unsubstituted phenyl phosphoramidate derivative of stavudine and examined its antiviral activity. Naesens et al.[37,38] have studied the metabolism and anti-HIV activity of phosphoramidate derivatives of d4T-MP with variations in the amino acid moiety and came to the conclusion that enzymes involved in the formation of the amino acyl d4T MP metabolite have specificity for L-alanine as the amino acid moiety. In a recent study, Siccardin et al.[39] have examined the stereospecific chemical and enzymatic stability of a phosphoramidate triester pro-drug in human plasma and came to the conclusion that there was no stereoselective preference for phosphate diastereoisomers. In our study, the D-amino acid substituted phosphoramidate isomers [Rp or Sp} were resistant to hydrolysis and they were substantially less active against HIV than corresponding L-amino acid substituted phosphoramidate isomers {Rp or Sp}. These experimental results suggest that lipase-mediated hydrolysis at the methyl ester side chain of the stampidine molecule plays an important role for the metabolic activation of this anti-HIV pro-drugs, which is consistent with and extends the work previously reported.[36,8,10,20–33]

Earlier studies on phosphoramidate derivatives of stavudine demonstrated that these compounds can be activated by esterases.[7–33] Recently we found that lipases as well as proteases can also activate these derivatives.[34,35] However, the position of enzymatic attack has not been deciphered. In order to establish the site of enzymatic attack we prepared the '*D*'-amino acid ester substituted phosphoramidate derivatives[39,40] and compared their activation by a lipase. During our synthesis of the phosphoramidate derivatives we obtained two isomers due to the chirality at phosphorus center of the molecule [Sp and Rp], consistent with previous reports.[8,10,23–33,35,38–47] Using HPLC, we were able to determine the ratios of each isomer. Table 1 shows the enantiomeric excess ratios obtained for four of these stavudine compounds having both 'L' and 'D' configuration at the amino acid terminal. It is interesting to note that in the case of the 'L'-amino acid methylester isomer we were able to obtain a ratio of approximately 40% of isomer 1, [Rp or Sp] and 60% of isomer 2, [Rp or Sp] but the 'D'-amino acid methylester substituted phosphorus isomers [Rp or Sp] were obtained almost in equal proportions in the majority of the compounds. This points out that the approach of nucleoside in the final segment of synthesis is affected by the configuration at the amino acid terminal of the phosphorochloridate. We have also observed similar results when the amino acid is changed in the structure of arylphosphoramidate derivatives of zidovudine (Venkatachalam, unpublished results).

Table 1. Enantiomeric excess ratios of phosphorus isomers [Rp or Sp] obtained during synthesis of phosphoramidate derivatives of stavudine with L- and D-amino acid ester substitution



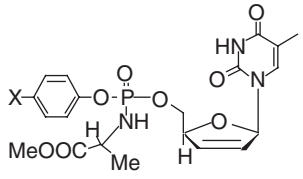
Compd	X	Amino acid configuration	Isomer #1 (%)	Isomer #2 (%)
(**1**)stamp	Br	'L'	40.0	60.0
(**2**)	Br	'D'	50.0	50.0
(**3**)	F	'L'	38.9	61.1
(**4**)	F	'D'	50.0	50.0
(**5**)	Me	'D'	43.0	57.0
(**7**)	OMe	'D'	51.0	49.0
(**8**)	OMe	'L'	41.5	58.5
(**9**)	H	'D'	50.0	50.0
(**10**)	H	'L'	36.0	64.0
(**11**)	Cl	'D'	52.0	48.0
(**12**)	Cl	'L'	46.0	54.0

Isomers 1 and 2 represents the chirality at the phosphorus center of the compound [Rp or Sp]. Ratios are measured using a HPLC assay for all the phosphoramidate derivatives under identical experimental conditions.

3. Lipase-mediated hydrolysis of D-amino acid substituted phosphoramidate isomers [Rp or Sp} of stampidine and other phosphoramidate derivatives of stavudine

'D'-Amino acid substituted phosphoramidate isomers [Rp or Sp} were prepared for stampidine and five other phosphoramidate derivatives of stavudine by replacing the L-alanine side chain with a D-alanine side chain (Table 2).[18,19] The compounds were further characterized using routine techniques and the results are presented in the experimental section. We compared the rate of lipase-mediated hydrolysis of these phosphoramidates using Candida B lipase as a model enzyme. The first order rate constants for disappearance of starting material during lipase-mediated hydrolysis were dependent on the configuration of the amino acid. Since multiple steps are involved for the formation of the metabolite and intermediates we were unable to determine the kinetic order for this process. However for simplicity we assumed that this process follows first order rate early in the kinetic cycle. The D-amino acid substituted isomers {Rp or Sp] were substantially more resistant to lipase-mediated hydrolysis, as reflected by markedly lower first order rate constants (Table 2). For example, the rate constants were 3.99 for the L-amino acid substituted isomer of stampidine, but only 0.41 for the corresponding D-amino acid substituted isomer (Figs. 1 and 2). The rate constants were calculated by integration of both peaks in the chromatogram. Our interest was not focused on the absolute rate constant values of Rp or Sp-isomers, but instead of a comparison with L- or D-amino acid substituted phosphoramidates under identical conditions to obtain a relative rate constant value. In the past we have calculated the rate constant values of individual isomers {Rp or Sp} and found that one of the isomers undergoes hydrolysis at a 4-times faster rate.[34] These results support our hypothesis that the hydrolysis occurs at the methyl ester side chain of stavudine phosphoramidates (Fig. 3). Likewise, both stereoisomers {Rp or Sp} of 'D'-amino acid configured compounds (compound #**2**) were analyzed for potential interaction with lipase. The conformational difference occurs at the C-alpha position of the amino acid, which is closer to the active site than that at the phosphate position, thus creating a greater energy barrier for stereoisomers {Rp or Sp}. Additionally our docking and subsequent energy analyses indicated that neither stereoisomer for the phosphate [Rp or Sp} of 'D'-amino acid configured compounds (compound #**2**) favorably fits the active site because of steric clash with the protein. With the chiral 'D'-amino acid configured compounds, the C-beta methyl group unfavorably stacks with the phenol group in an attempt to fit the active site. Overall the energy cost is high for both Rp- and Sp-isomers of 'D'-amino acid substituted compound #**2** (Fig. 4). As a result this model suggests that the 'D'-amino acid substituted derivatives {both Rp or SP] (compound #**2**) are poor substrates for lipase when compared to the 'L'-amino acid configured compound #**1**, which is consistent with our experimental data. Previously, by comparing this lipase active site model with a homologous model of the esterase active site, we observed a larger spatial opening near the entrance of the active site in the esterase. The larger opening corresponds to the roomier binding sites for both stavudine and the phenoxy group. The esterase thus appears to be more forgiving in accommodating the different structure of both enantiomers and thus less discriminative. These observations hold true for 'D'-amino acid configured compound #**2**. The overall result is that our model is in agreement with the hypothesis suggesting that the hydrolysis rate is determined by the stereo-selection by the active site of the enzyme. The observed differences in the rate of lipase-mediated hydrolysis of L- versus D-amino acid substituted isomers [Rp or Sp] of stampidine and other stavudine phosphoramidates indicate that the lipase 'prefers' the L-amino acid configured [Rp or Sp] isomers. Consequently, D-amino acid substituted isomers [Rp or Sp] are poor substrates for the lipase, which is in agreement with our experimental data.

Table 2. First order rate constants for disappearance of starting material using lipase and arylphosphoramidate derivatives of stavudine at room temperature



Compound	X	Amino acid configuration	Rate constant for lipase-mediated hydrolysis	HTL VIII$_B$ (nM)
1(stamp)	Br	'L'[18,19]	3.99	1 ± 0*
2	Br	'D'	0.41	212 ± 155
3	F	'L'[18,19]	2.59	1 ± 0
4	F	'D'	0.27	851 ± 720
6	Me	'D'	0.16	427 ± 107
7	OMe	'L'[18,19]	2.2	6 ± 3
8	OMe	'D'	0.18	1290 ± 313
9	H	'L'[18,19]	2.20	2 ± 0.6
10	H	'D'	0.14	1787 ± 215
11	Cl	'L'[18,19]	5.80	1 ± 0.3
12	Cl	'D'	0.18	5837 ± 2304
d4T	—	—	—	18 ± 2
AZT	—	—	—	4 ± 1

Rate constants are expressed per hour. Repeated trials gave rate constant values within ±5%.

* Prep HPLC separated individual isomers {Rp or Sp] showed identical activity of 1 nM (Venkatachalam et al., to be published).

3.1. General mechanism of hydrolysis by lipase or esterase enzymes

After having established that enzymes are responsible for the hydrolysis of this series of phosphoramidate derivatives of stavudine, we propose the following mechanism of hydrolysis, which is consistent with the earlier reports.[8,10,14,15,17,27–33] In the first step, the ester side chain is hydrolyzed by the enzyme to the carboxylic acid that subsequently cyclizes and eliminates the phenoxy moiety resulting in a cyclic intermediate (Fig. 3). This unstable intermediate is further hydrolyzed by a water

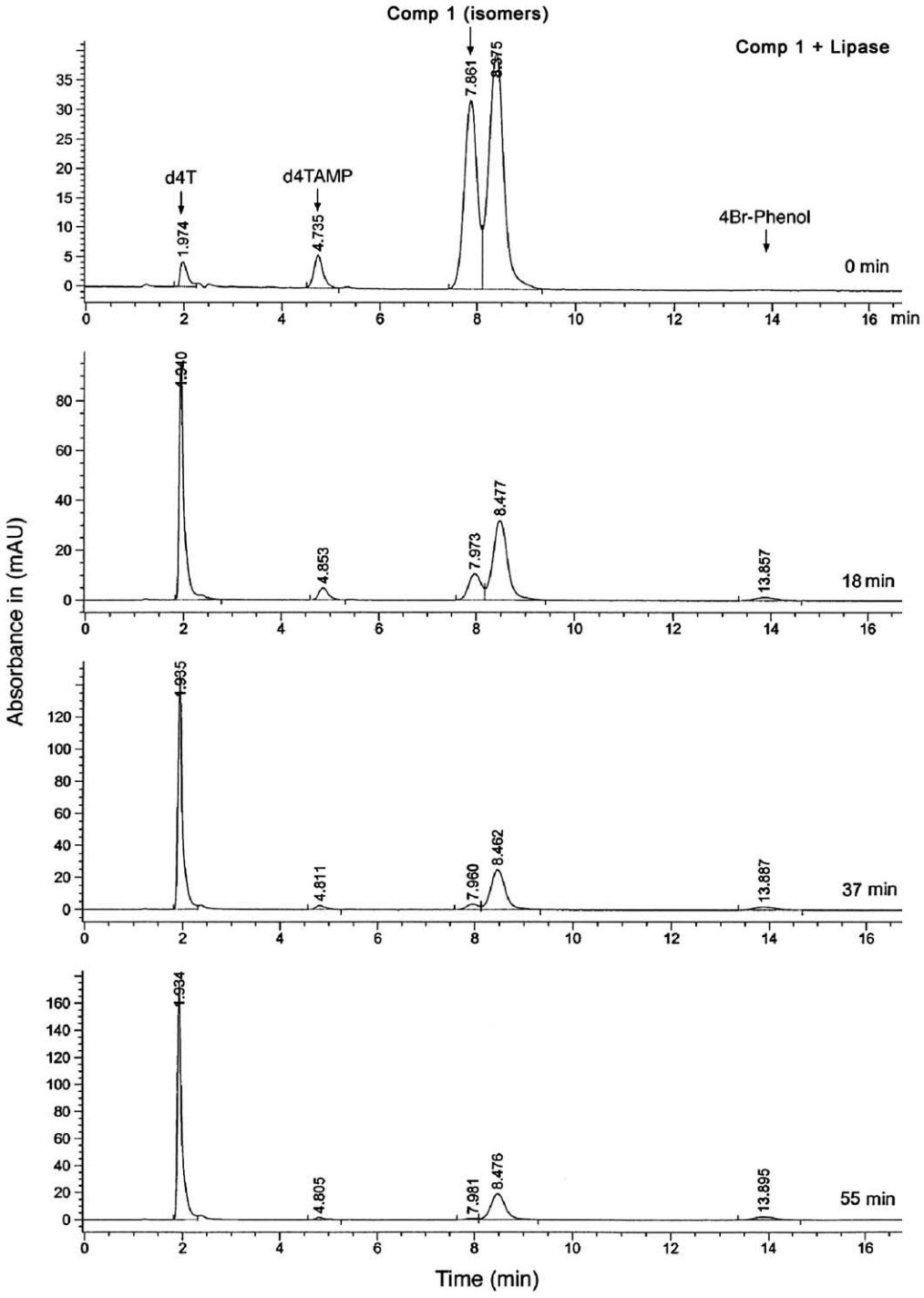


Figure 1. Lipase-mediated hydrolysis profile of compound **1** at various time intervals. The two peaks represent the phosphorus diastereoisomers {Rp and Sp}.

molecule to yield the active metabolite. In the case of phosphoramidate derivatives containing the 'L'-configured amino acid terminal, the initial step of hydrolysis of the carboxy ester side chain is faster as compared to that of the 'D'-amino acid configured derivatives. This result supports our hypothesis that these enzymes attack at the methylester side chain of the phosphoramidate derivatives and not at the phosphorus center or another site. Since we have only altered the configuration at the methylester side chain, one would conclude that any changes in the enzyme activity must be associated with this alteration. Based on these experimental results, we have demonstrated the site at which these enzymes act during hydrolysis of these phosphoramidate derivatives of stavudine.

3.2. Proof of hydrolysis of phosphoramidate derivatives with lipase

In our earlier discussion we postulated that lipases are also involved in hydrolysis of these phosphoramidate analogs and proposed a mechanism for the hydrolysis.

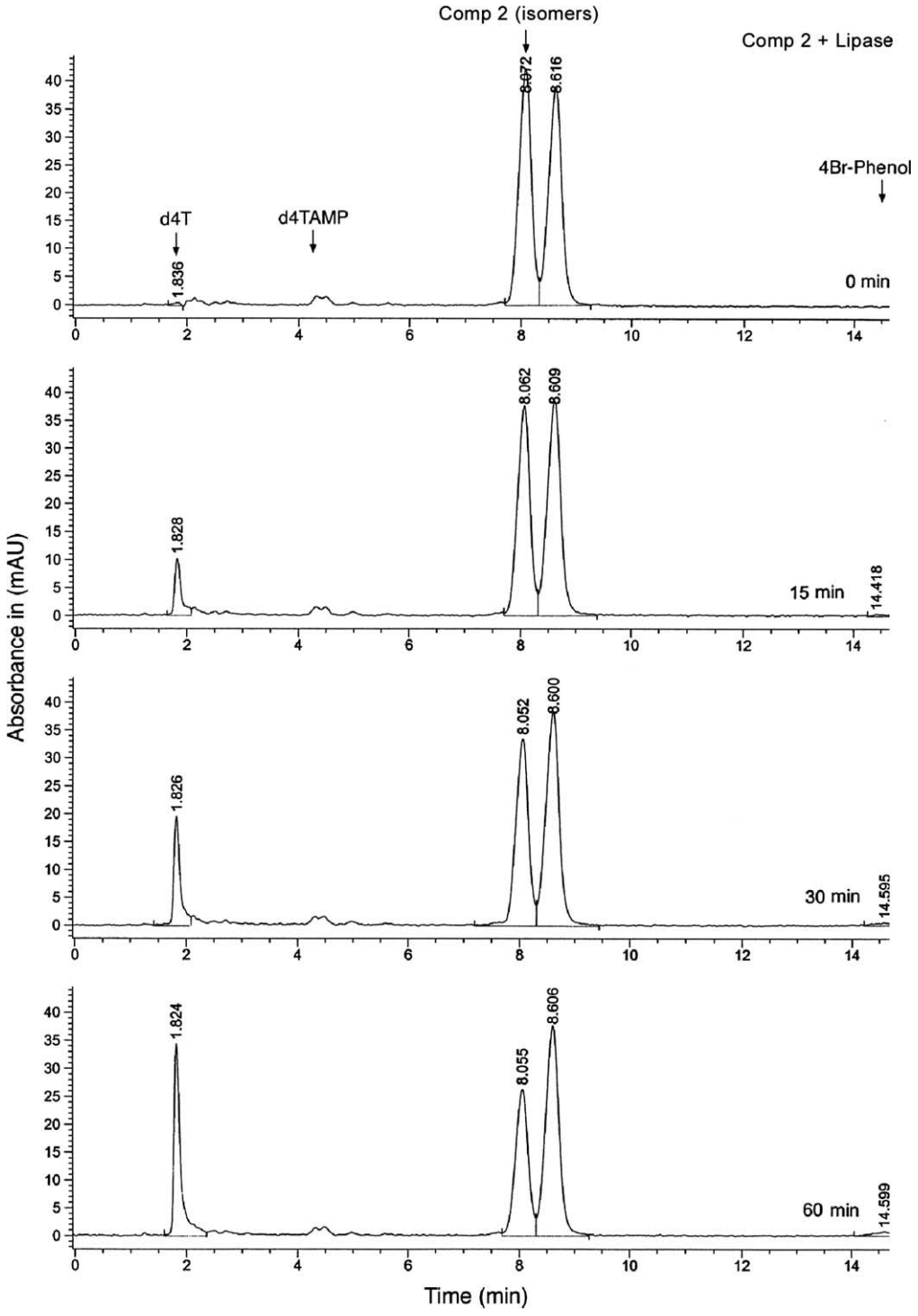


Figure 2. Lipase-mediated hydrolysis profile of compound **2** at various time intervals. The two peaks represent the phosphorus diastereoisomers {Rp and Sp}.

(Fig. 3). There are three pathways through which the hydrolysis product could result from the starting materials. In the first pathway, the attack of the enzyme takes place at the phosphorus center eliminating the phenoxy group in the structure, yielding d4T alaninyl methylester monophosphate. However, the final product of the reaction was d4T alaninyl monophosphate indicating that the ester side chain has been hydrolyzed. Based on this evidence one can rule out the first pathway.

For the second pathway we propose a simultaneous attack on the phosphorus site as well as at the methyl ester side chain. In this mechanism, formation of d4T alanine monophosphate can result as a final product. However, we can rule out the attack on the phosphorus center as discussed previously based on the experimental results with 'D'-amino acid substituted stavudine derivatives, which showed resistant towards hydrolysis. Additionally if the attack occurred at the phosphorus center the

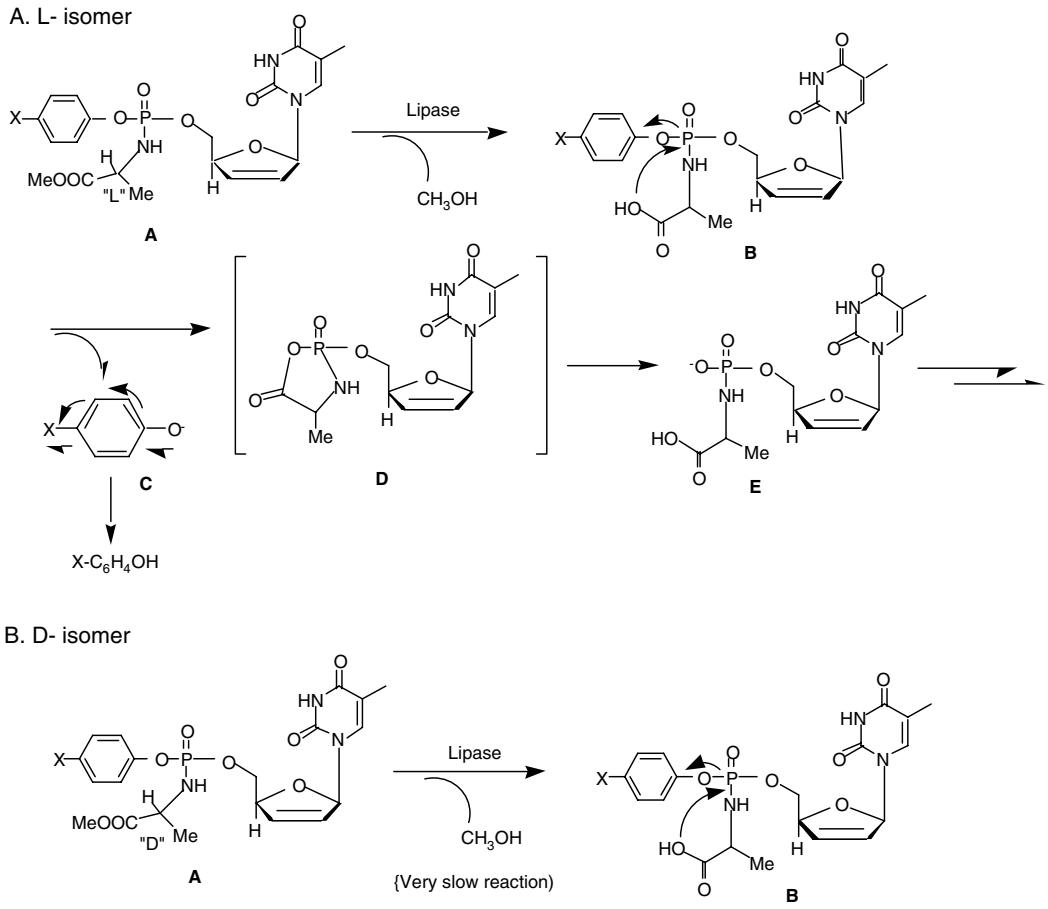


Figure 3. Enzyme mediated hydrolysis of both 'L'- and 'D'-amino acid substituted isomers of [Rp or Sp] phosphoramidate derivatives of stavudine.

'D'-amino acid configured isomer of the phosphoramidate {Rp or SP} should have reacted and formed the hydrolysis product. From our results, we found that a 'D'-amino acid substituted compound is not hydrolyzed by lipase. Therefore, we can rule out the second pathway.

In the third pathway, the ester side chain of the phosphoramidate is hydrolyzed to form the carboxylic acid, which then eliminates the phenoxy moiety yielding a unstable cyclic intermediate. This intermediate is then hydrolyzed in the presence of water to yield d4T alaninyl monophosphate as the final product. This is further supported by the experimental data as only the 'L'-amino acid substituted phosphoramidate isomers {Rp or Sp}, but not 'D'-amino acid substituted isomers {Rp or Sp} were susceptible to hydrolysis by lipase. This may be owing to the change in the binding characteristics as well as the stereochemical geometry of the binding pocket. Hence we conclude that similar to the esterase[8,10,14,17,27–33,45–50] activated mechanism, these phosphoramidate derivatives undergo hydrolysis by lipase through the ester side chain of the molecule.

3.3. Intracellular metabolism of 'L'- versus 'D'-amino acid substituted isomers of stampidine {Rp or Sp}

In order to evaluate whether stereochemistry at the amino acid chain imparts a differential hydrolytic profile we treated Cos 7 cells with 'D'- versus 'L'-amino acid substituted isomers [Rp or Sp] of stampidine. The cellular extracts were subjected to HPLC analysis to compare the metabolic rates of the compounds (Fig. 5A and B). The 'D'-amino acid substituted isomer [Rp or Sp] did not hydrolyze even after 3 h demonstrating that a change of stereochemistry at the amino acid side chain of these phosphoramidate derivatives of stavudine results in poor intracellular metabolism.

3.4. Antiviral activity of phosphoramidate derivatives of stavudine

We examined the antiviral activity of stavudine phosphoramidate derivatives with 'L'- versus 'D'-configurations at the amino acid side chain. In general, D-amino acid substituted phosphoramidate isomers [Rp or Sp] were substantially less active than the corresponding 'L'-amino acid substituted phosphoramidate isomers [Rp or Sp]. This is in accordance with and extends the results obtained by McGuigan et al.[36] for 'D'-amino acid substituted phenyl phosphoramidate derivatives. Preparative HPLC separated individual isomers of compound **#1** that showed identical anti-HIV activity of 1 nM. This result demonstrates that chirality change at the phosphorus center did not alter the antiviral potency of the compound (Venkatachalam et al., to be published). This experimental finding further supports the

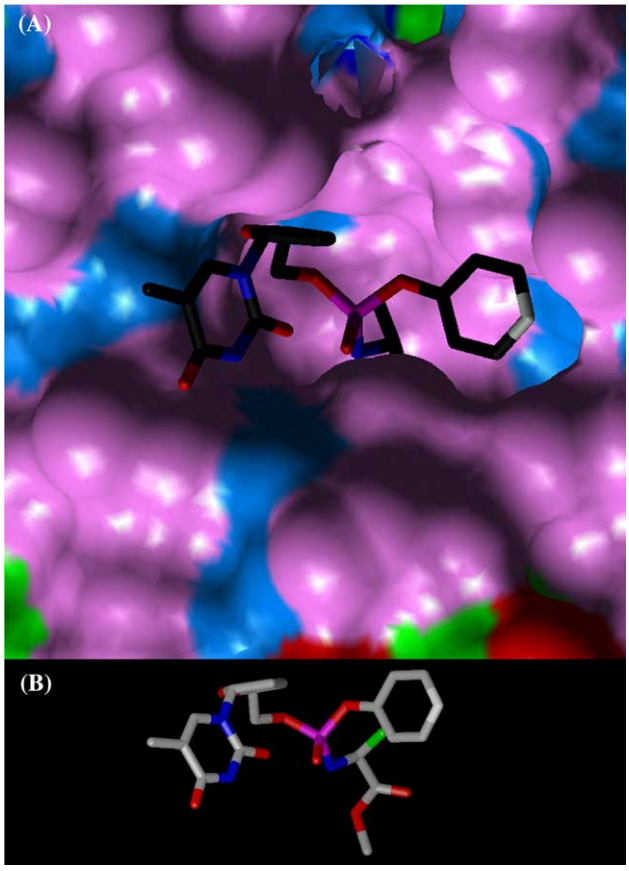


Figure 4. Model of 'L'-amino acid substituted compound **1** and 'D'-amino acid substituted compound **2** in the active site of lipase. (A) A 'L'-amino acid substituted derivative is docked into the active site of lipase with favorable conformation. (B) A 'D'-amino acid substituted compound, which is modeled in the same active site with unfavorable conformation. Prepared with Insight II.

notion that the lipase-mediated hydrolysis at the methyl ester side chain of stampidine and other stavudine phosphoramidates plays an important role for the activation of these anti-HIV pro-drugs.

4. General experimental

NMR spectra were obtained at ambient temperature in deuterated solvent. Chemical shifts are reported as δ values in parts per million downfield and referenced to the solvent. Coupling constants (J) are reported in Hz. HPLCs were obtained using an analytical RP–18 Lichrospher column, (4.6×250 mm) and CH_3CN/H_2O as the eluent. The flow rate was maintained at 1.0 mL/min and the detection wavelength was set at 266 nm. The column was maintained at room temperature throughout the analysis. Column and TLC chromatography was performed using silica gel 60, 230–400 mesh.

5. Synthetic procedure

Triethylamine (4.2 mL, 30 mmol) was added dropwise to a stirred solution of $POCl_3$ (1.53 g, 10 mmol) and substituted phenol (10 mmol) in anhydrous chloroform (40 mL) at 0 °C. The reaction was allowed to warm to room temperature and stirred for 15 h. Then the reaction mixture was cooled to −70 °C and D-alanine methyl ester hydrochloride (1.39 g, 10 mmol) was added to the reaction flask; the reaction was allowed to warm to room temperature and stirred overnight. 1,2,4- Triazole (1.80 g, 25 mmol) was then added to the above reaction flask. After stirring at room temperature for 6 h, d4T (0.45 g, 2.0 mmol) was added to the above reaction flask and the reaction solution was stirred for 4 days. The solvent was removed under reduced pressure. The crude product was purified by column chromatography (100% $CHCl_3$ followed by 5% MeOH in $CHCl_3$) and it was further purified using preparative TLC to obtain analytically pure compound.

6. Physical constants of new compounds[3]

6.1. d4T-5′-*p*-Bromophenyl methyl-D-alaninyl phosphate (2)

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 7.0 Hz), 1.81 (d, 3H, J = 6.6 Hz), 3.58–4.06 (m, 2H), 3.70 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.90 (m, 1H), 6.33 (s, 1H), 7.02 (s, 1H), 7.08 (d, 2H, J = 9.0 Hz), 7.24 (s, 1H), 7.43 (d, 2H, J = 8.1 Hz), 8.61 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.3, 50.3, 53.0, 66.7, 67.6, 84.8, 89.9, 111.5, 118.4, 122.1, 127.6, 133.0, 133.5, 136.0, 150.9, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.56, 2.97; UV (MeOH) λ_{max}: 266 nm. $[\alpha]_D$ {MeOH} −25.2: HPLC: 12.72, 12.92; % purity: >98.

6.2. d4T-5′-*p*-Fluorophenyl methoxy-D-alaninyl phosphate (4)

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (q, 3H, J = 4.2 Hz), 1.86 (d, 3H, J = 7.5 Hz), 3.50–4.06 (m, 2H), 3.70 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.89 (m, 1H), 6.98–7.04 (m, 3H), 7.13–7.17 (m, 2H), 7.28 (s, 1H), 8.41 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.3, 50.3, 53.0, 66.6, 67.5, 84.8, 89.9, 111.5, 116.6, 121.7, 127.6, 133.4, 135.9, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.96, 3.34; UV (MeOH) λ_{max}: 266 nm. $[\alpha]_D$ {MeOH} −22.2: HPLC: 10.25, 10.35 min. % Purity: 98.0.

6.3. d4T-5′-*p*-Methylphenyl methoxy-D-alaninyl phosphate (5)

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32 (t, 3H, J = 7.2 Hz), 1.85 (d, 3H, J = 6.6 Hz), 2.30 (s, 3H), 3.44–4.05 (m, 2H), 3.69 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.89 (m, 1H), 6.33 (s, 1H), 7.00–7.12 (m, 5H), 7.30 (s, 1H), 8.35 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.7, 21.3, 30.0, 50.3, 53.0, 66.4, 67.3, 84.8, 89.9, 111.5, 120.0, 127.5, 133.4, 133.5, 136.1, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.78, 3.27; UV (MeOH) λ_{max}: 266 nm. $[\alpha]_D$ {MeOH} −24.6: HPLC: 11.33, 11.44 min. % Purity: >98.

6.4. d4T-5′-*p*-Methoxyphenyl methoxy-D-alaninyl phosphate (8)

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, J = 8.7 Hz), 1.81 (d, 3H, J = 6.0 Hz), 3.44–4.06 (m, 2H), 3.70 (s, 3H),

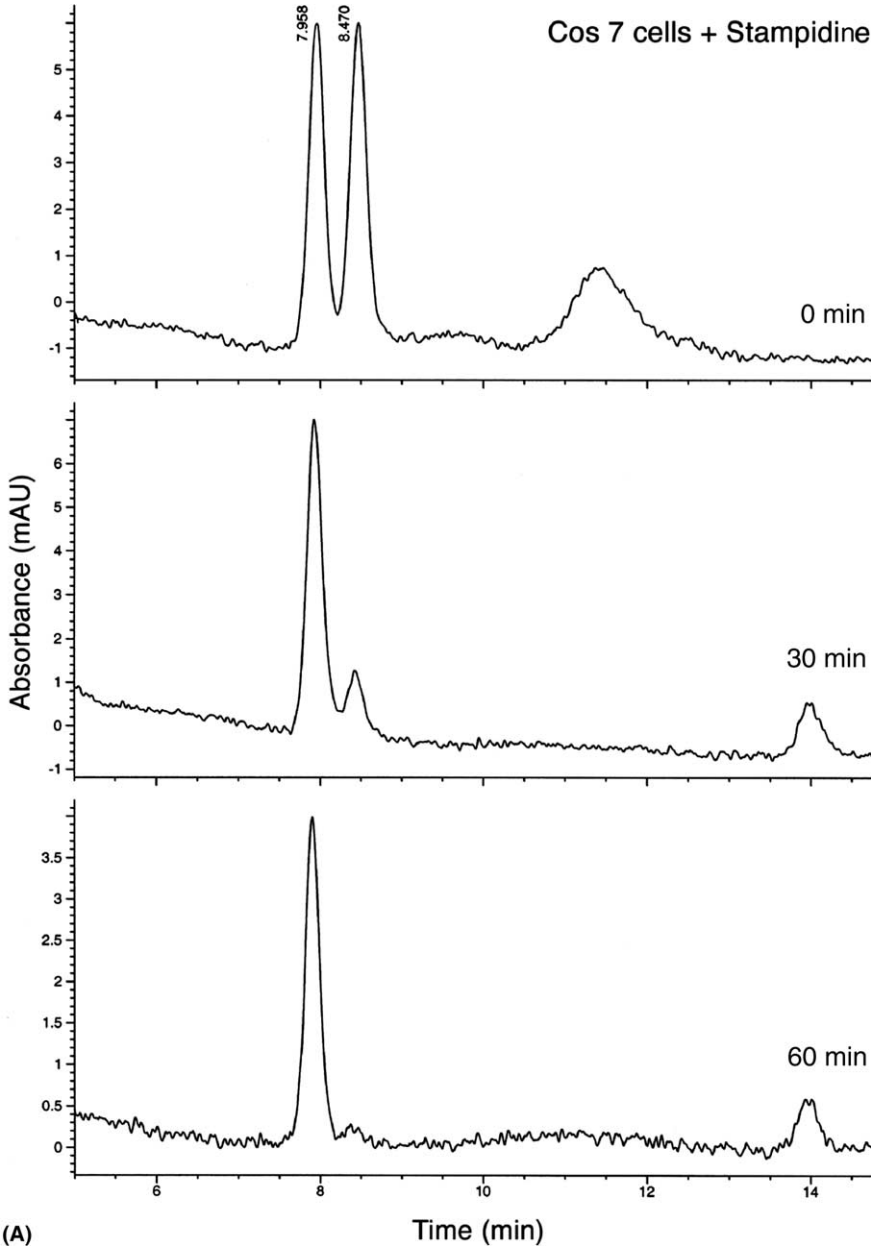


Figure 5. (A) HPLC profiles of stampidine ('L'-amino acid substituted compound **1**) and its hydrolyzed products in Cos 7 cells at various time intervals of exposure. The two HPLC peaks represent the phosphorus diastereoisomers [Rp and Sp]. (B) HPLC profiles of stampidine ('D'-amino acid substituted compound **2**) and its products in Cos 7 cells at various time intervals of exposure. The two peaks represent phosphorus diastereoisomers {Rp and Sp].

3.78 (s, 3H), 4.28 (s, 2H), 4.99 (s, 1H), 5.88 (m, 1H), 6.34 (s, 1H), 6.83 (d, 2H, $J = 9.0$ Hz), 7.02 (s, 1H), 7.10 (d, 2H, $J = 10.5$ Hz), 7.30 (d, 1H, $J = 8.4$ Hz), 8.41 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.4, 50.3, 52.9, 55.9, 66.9, 84.8, 89.8, 111.5, 114.9, 121.3, 127.6, 133.6, 136.2, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 3.11, 3.55; UV (MeOH) λ_{max}: 266 nm. HPLC: 10.20, 10.27 min.

6.5. d4T-5′-Phenyl methoxy-D-alaninyl phosphate (10)

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, $J = 7.6$ Hz), 1.86 (d, 3H, $J = 6.6$ Hz), 3.44–4.06 (m, 2H), 3.70 (s, 3H), 4.30 (m, 2H), 5.00 (s, 1H), 5.89 (m, 1H), 6.33 (m, 1H), 7.02 (s, 1H), 7.19 (d, 2H, $J = 7.5$ Hz), 7.28–7.36 (m, 6H), 8.27 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.4, 50.4, 53.0, 66.5, 67.4, 84.8, 89.9, 111.5, 120.3, 125.4, 127.5, 133.0, 133.5, 136.0, 150.8, 163.5. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.17, 2.64; UV (MeOH) λ_{max}: 266 nm. HPLC: 12.71, 12.88 min.

6.6. d4T-5′-*p*-Chlorophenyl methoxy-D-alaninyl phosphate (12)

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.33 (t, 3H, $J = 7.5$ Hz), 1.86 (d, 3H, $J = 7.8$ Hz), 3.55–4.06 (m, 2H), 3.70 (s, 3H), 4.28 (s, 2H), 5.00 (s, 1H), 5.91 (m, 1H), 6.33 (m, 1H), 7.02 (s, 1H), 7.14 (d, 2H, $J = 8.1$ Hz), 7.24 (s, 1H),

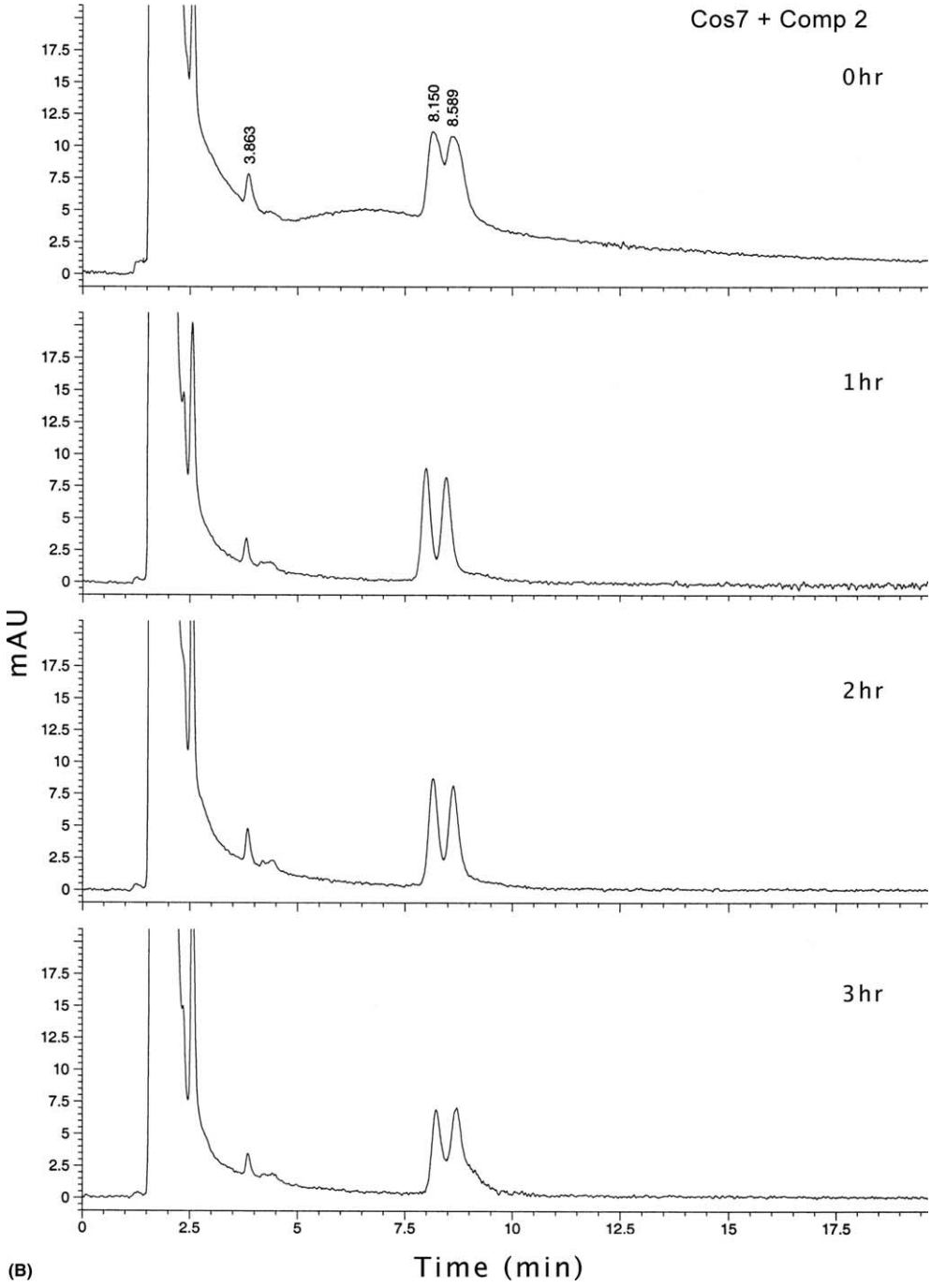


Figure 5. (*continued*)

7.29 (d, 2H, $J = 8.7$ Hz), 8.55 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.8, 21.3, 50.3, 53.0, 66.7, 67.6, 84.8, 89.9, 111.5, 121.7, 127.6, 133.0, 133.4, 136.0, 150.8, 163.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 2.77, 3.16; UV (MeOH) λ_{max}: 266 nm. HPLC: 10.00 min.

7. Experimental conditions for lipase study

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (5–7 mg) using a Metller analytical balance and transferred into a 20 mL scintillation glass vial. Using a pipetman, 3 mL of methanol was added and the contents were vortexed for 2 min until a homogenous solution resulted. Using another pipetman, 100 mL of this stock solution was transferred into another scintillation vial, and to this was added 900 mL of water and the contents vortexed. In the meantime, 5 mg of solid lipase powder was weighed and transferred to a volumetric flask. To this was added 8 mL of water and the contents were shaken to dissolve the enzyme. From the stock solution of the compound as mentioned above, 500 mL of the methanolic solution of the phosphoramidate derivative was pipetted out into another glass vial and to this 500 mL

of lipase solution was added and the contents were shaken to form a homogenous solution. From this reaction mixture 50 mL was used for HPLC analysis. The column used was a Lichrospher (RP-18) analytical column of 4×250 mm. The eluent used for HPLC was water/TFA and TEA (0.1%) and CH_3CN in the ratio of 65:35. The column was maintained at room temperature. The flow rate was maintained at 1 mL/min, the detection wavelength was adjusted to 265 nm and the reference wavelength was kept at 400 nm. An 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.

8. Estimation of products

The amount of products observed during the reaction of these phosphoramidates were estimated from the area obtained from the HPLC profiles. Authentic samples of the products, when possible, were run to identify the peaks observed during the reaction. Further confirmation of the product structures were obtained using a LC/mass instrument. 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 refers to rate/hr as some of the reactions were too slow to obtain meaningful results.

9. In vitro assays of anti-HIV activity

Normal human peripheral blood mononuclear cells (PBMNC) from HIV-negative donors were cultured for 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 d4T phosphoramidates and aliquots of culture supernatants were removed from the wells on the 7th day after infection for p24 antigen assays, as previously described.[51] The applied p24 enzyme immunoassay (EIA) was an unmodified kinetic assay commercially available from Coulter Corporation/Immunotech. Inc. (Westbrooke, ME), which utilizes a murine mAb to an 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).

10. T-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%; 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. 140 million cells were used for the enzyme experiments. 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 resuspended 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 140 million cells. The cells were collected by centrifugation at 1000 rpm for 5 min, subsequently washed thrice in $1 \times$ PBS and then resuspended in 1 mL of $1 \times$ PBS.

For the HPLC measurements, approximately, 0.5 mL of the suspended cell mixture in PBS buffer was taken and treated with compound **1** (stampidine) or compound **2** and incubated at 37 °C. At various time intervals, an aliquot (clear supernatent) was drawn and assayed using the HPLC. Authentic samples were used to identify the products during T-lymphocyte mediated hydrolysis of stampidine. A lichrospher-RP-18 column 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/min and the column was maintained at room temperature throughout the analysis. An aliquot of the samples were drawn at various time intervals and assayed using a diode-array detector.

11. Conclusions

Stampidine and other halogen substituted stavudine phosphoramidates can be activated by lipase-mediated hydrolysis. The target site for the lipase appears to be the methyl ester group of the 'L'-alanine side chain. Molecular modeling results indicate that the 'L'-amino acid substituted isomer [Rp or Sp] is preferred in the lipase binding pocket. Accordingly, the 'D'-amino acid substituted phosphoramidate isomers [Rp or Sp} are resistant to lipase-mediated hydrolysis and exhibit substantially less anti-HIV activity.

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