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Enzymatic hydrolysis of stampidine and other stavudine phosphoramidates in the presence of mammalian proteases

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Abstract—Mammalian proteases have not been implicated in the metabolism of any nucleoside phosphoramidate prodrug. The results presented herein provide unprecedented and conclusive experimental evidence that mammalian proteases are capable of hydrolyzing stavudine phosphoramidates. Specifically, cathepsin B and Proteinase K are able to metabolize stampidine and other phosphoramidate derivatives of stavudine. Additionally, cathepsin B exhibits chiral selectivity at the phosphorus center. The elucidation of the metabolic pathways leading to activation of stampidine may provide the basis for pharmacologic interventions aimed at modulating the metabolism and thereby improving the therapeutic window of stampidine as an anti-HIV agent.
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1. Introduction

Stampidine is a 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 20 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] It was also 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 anti-retroviral activity in HIV-infected Hu-PBL SCID mice and FIV-infected cats warrants its further development as a new anti-HIV drug.

The analysis of the in vivo pharmacokinetics and metabolism of stampidine in mice revealed that stampidine forms two active Phase I metabolites after systemic administration, namely alaninyl-d4T-monophosphate (ala-d4T-MP) and d4T.[6] Subsequent studies of stampidine metabolism in mice, dogs, and cats revealed that in addition to these two active Phase I metabolites, *p*-bromophenol is generated as a metabolite.[7] The same metabolites were obtained by in vitro hydrolysis of stampidine using esterases and lipases,[8] implicating enzymatic hydrolysis as a major pathway for the metabolism of stampidine.

Generation of the active metabolite for the phosphoramidate derivatives of various nucleosides in biological systems was originally thought to require an

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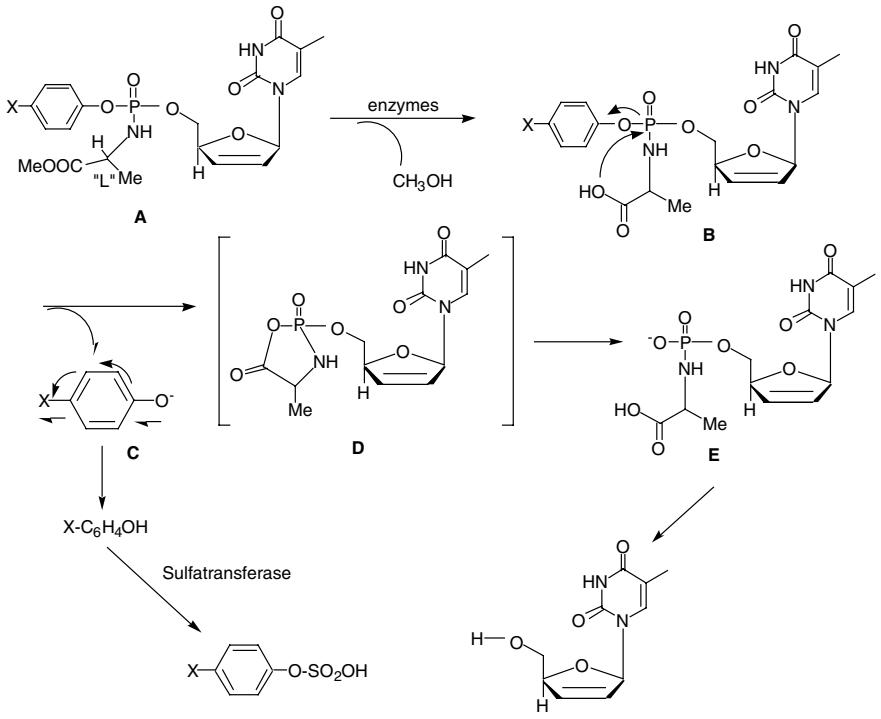


Scheme 1. Putative hydrolysis pathway for phosphoramidate derivatives of stavudine.

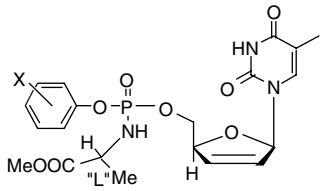
esterase-mediated hydrolysis of the carbomethoxy group associated with the amino acid side chain.[9–18] However, in probing the mechanism for the metabolism of amino acid phosphomonoester amidates, Wagner et al.[15] reported that the conversion of FLT phosphomonoester amidates to FLTMP follows an alternative pathway in which P–N bond cleavage by an unidentified phosphoramidate hydrolase occurs. We hypothesized that in various tissue microenvironments the metabolism of such compounds may occur through the action of hydrolytic enzymes in addition to esterases and phosphoramidate hydrolase. To support this proposal we recently examined the influence of other enzymes on the activation of stampidine. Our experimental results provided evidence that enzymes such as lipase, protease, and alanine racemase can also metabolize stampidine.[8,19] We observed that a change in nucleoside of these phosphoramidate derivatives resulted in considerable change in the enzymatic activation.[20] However, these studies were conducted using bacterial enzymes that may or may not be relevant to the in vivo models. This raises a question as to whether a similar trend is exhibited in cells. To address this, we recently examined the decomposition of the phosphoramidate derivative stampidine in presence of various cells (RAW264, Cos7, Jurkat Cells) and found that the cellular metabolism is very similar to that observed in vitro using bacterial enzymes. Additionally we have shown that the chiral selectivity of individual cell lines demonstrates that several enzymes/factors other than esterase are involved in the cellular metabolism of stampidine.

Originally, we hypothesized that in various tissue microenvironments, the metabolism of stampidine may occur through the action of hydrolytic enzymes other than esterases as well. Our hypothesis was confirmed by experimental results that provided additional evidence that stampidine as well as other halogen-substituted phosphoramidate derivatives of stavudine can also be metabolized by a lipase. Also, our earlier studies using modeling and comparison of the hydrolysis rate constants revealed a chiral preference of the lipase active site for the putative *S*-stereoisomer of these compounds.[8] Our model indicates that the lipase-mediated formation of the cyclic intermediate can be a key step in the metabolism of stampidine (Scheme 1). This step is dependent on the initial configuration of the stereoisomer. We have since demonstrated that proteases can also hydrolyze the phosphoramidate derivatives of stavudine.[19] The purpose of the present study was to further examine whether protease-mediated in vitro hydrolysis of stampidine can consistently yield one or more of the metabolites of stampidine observed during the in vivo metabolism of stampidine in animals.

2. Results and discussion

We first examined the hydrolysis of several of these phosphoramidate derivatives treated with cathepsin B. Cathepsin B is a lysosomal enzyme involved in physiological events such as intracellular protein catabolism and prohormone activation. Cathepsin B acts as a 'housekeeping' protein and is found in most mammalian tissues investigated. It belongs to the papain family of cyteine proteinases. Table 1 shows the hydrolysis rate constants obtained following cathepsin treatment of the compounds at room temperature. The table also provides the information on the substituents and the Hammett σ values for each of the compounds. The fastest rate (0.006 min^{-1}) was observed for the 4-bromo and the 2,5-dichloro derivatives of stavudine. The slowest

Table 1. Hydrolysis rate constants obtained for phosphoramidate derivatives treated with cathepsin B at room temperature



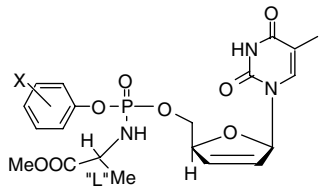
Compd #	Substituent (x)	σ value	Hydrolysis rate/min
113 (stampidine)	4-Br	0.26	0.006
604	4-F	0.15	0.002
607	H	0	0.004
599	3-N(Me)$_2$	−0.10	0.002
600	2,6-Di-OMe	−0.56	0.0007
601	4-CN	0.70	0.004
605	2-Br	0.26	0.004
602	3-Br	0.37	0.001
603	4-Br, 2-Cl	0.50	0.001
606	2-Cl	0.24	0.002
608	2,5-DiCl	0.61	0.006
598	4-OMe	−0.28	0.002
609	4-Cl	0.24	0.003

Repeated trials showed hydrolysis rate constants within ±5%. Sigma values for di-substituted compounds are additive values.

rate (0.0007 min^{-1}) was obtained for the 2,6-dimethoxy-substituted compound. This trend was similar to the trend observed using the bacterial proteases, Subtilisin Carlsberg, and Carcia Papaya.[19] The study was continued using proteinase K, a serine protease. Table 2 shows the hydrolysis rates obtained for all compounds after treatment with proteinase K. The rates of hydrolysis after treatment with proteinase K were approximately 10-fold faster that the rates of hydrolysis observed with cathepsin B (Table 2).

Due to the stereochemistry of its phosphorous chiral center, stampidine exists as a mixture of two diastereomers.[8,13] We next sought to determine if the two stereoisomers of stampidine are preferentially recognized and hydrolyzed by cathepsin. Figure 1 shows a representative HPLC profile of the distereoisomeric mixture of stampidine treated with cathepsin B at various time intervals. At 0 min immediately after addition of cathepsin B, three peaks are observed in the chromatogram. The peaks at the elution times 7.6 and 8.1 min represent the individual isomers of stampidine. The peak at 1.6 min represents the formation of d4T in the enzyme treated system. Within 20 min, the isomer eluting at 7.6 min is converted into d4T, as reflected by the decrease in the size of the isomer peak at 7.6 min and the increase in the size of the d4T peak at 1.6 min as well as the emergence of the *p*-bromophenol peak with an elution time of 14.4 min. At 160 min, cathepsin-mediated hydrolysis of stampidine is documented by a decrease of the size of the isomer peaks, increased d4T peak and increased *p*-bromophenol peak in the HPLC chromatogram. Overall, the isomer eluting at 7.6 min appeared to be more sensitive to cathepsin B than the isomer eluting at 8.1 min.

Table 2. Hydrolysis rate constants obtained for phosphoramidate derivatives treated with proteinase K at room temperature



Compd #	Substituent (x)	σ value	Hydrolysis rate/min
113 (stampidine)	4-Br	0.26	0.01
604	4-F	0.15	0.01
607	H	0	0.02
605	2-Br	0.26	0.03
602	3-Br	0.37	0.04
603	4-Br, 2-Cl	0.50	0.02
606	2-Cl	0.24	0.02
598	4-OMe	−0.28	0.01
609	4-Cl	0.24	0.01

Repeated trials showed hydrolysis rate constants within ±5%. Sigma values for di-substituted compounds are additive values.

Mammalian proteases have not been implicated in the metabolism of any nucleoside phosphoramidate prodrug. The results presented herein provide unprecedented and conclusive experimental evidence that mammalian proteases are capable of hydrolyzing stampidine, thereby both confirming and extending our recent work on the metabolic activation pathways of stampidine.[19] We previously reported that protease inhibitors block the hydrolysis of stavudine phosphoramidates by bacterial proteases.[19] Thus, besides esterases and lipases, proteases may play a role in activating the metabolism of stampidine and other stavudine phosphoramidates. We postulate that the methoxy ester group on the side chain of these compounds is first converted into the corresponding carboxylic acid derivative, which then undergoes intramolecular cyclization to form the active metabolite. Our model indicates that the protease-mediated formation of a cyclic intermediate is the key step in the metabolism of stampidine and may be affected by the initial configuration of the stereoisomers. The elucidation of the metabolic pathways leading to activation of stampidine may provide the basis for pharmacologic interventions aimed at modulating the metabolism and thereby improving the therapeutic window of stampidine as an anti-HIV agent.

3. Conclusions

The ability of cathepsin B and proteinase K to hydrolyze stampidine implicates mammalian proteases in the metabolic activation of this potent stavudine phorphoramidate. Further work is in progress to decipher the metabolic pathways and specific enzymes engaged in intracellular and extracellular metabolism of stampidine.

4. Compounds

All the compounds in the study were prepared using known literature procedures and were characterized

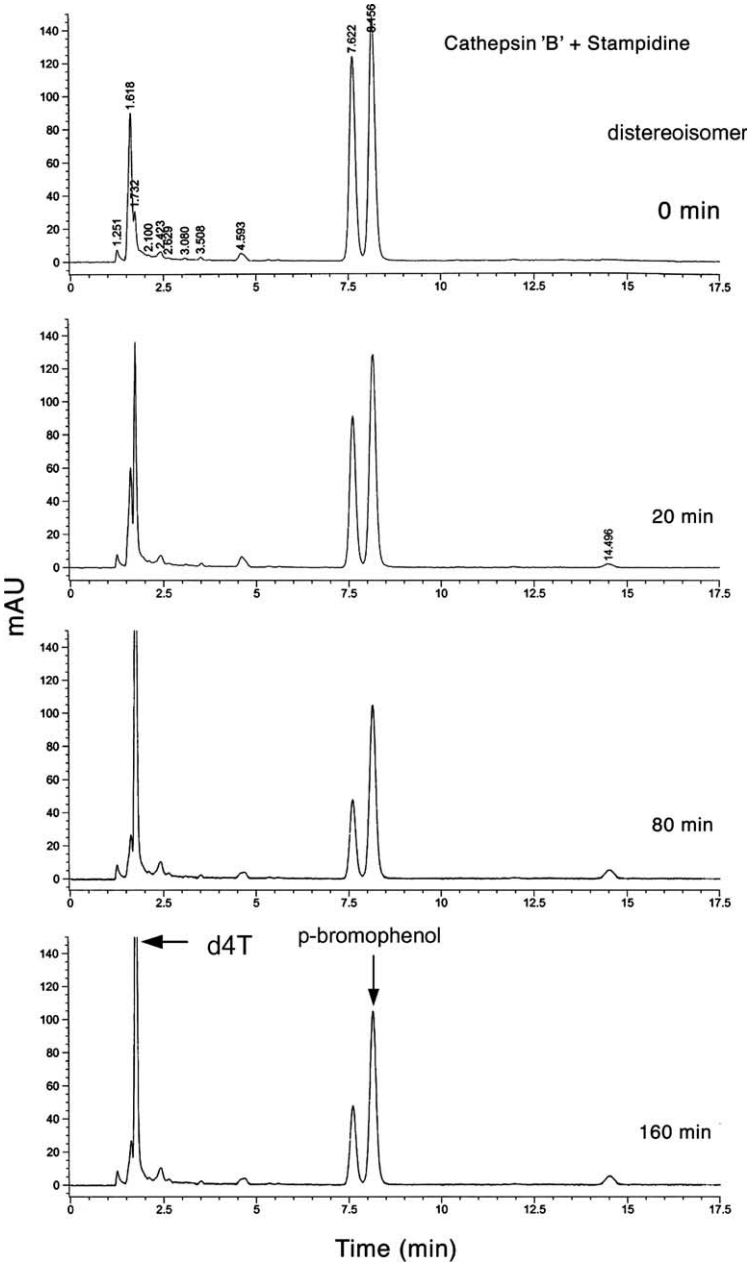


Figure 1. HPLC profile of stampidine treated with cathepsin B at various time intervals. Peaks corresponding to Rt 1.62 represents d4T, 7.82 and 8.16 min; peaks are due to two distereoisomers of stampidine. Peak at 14.4 min corresponds to *p*-bromophenol. One of the stereoisomer peaks of stampidine is disappearing forming both d4T and *p*-bromophenol.

using various analytical techniques and the details are reported in our earlier publications.[3,16]

5. Experimental conditions for cathepsin study

For the kinetic study, a known amount of the phosphoramidate derivative was carefully weighed (5–7 mg) using an analytical balance and transferred into a 20 mL scintillation glass vial. Using a pipetteman, 3 mL of methanol was added and the contents were vortexed for 2 min until a homogenous solution resulted. 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 lyophilized powder of cathepsin B (100 units) from Bovine spleen that was dissolved in 2 mL of water. From the stock solution of the compound 150 μL of the phosphoramidate derivative was pipetted out into another glass vial and to this 150 μL of enzyme stock solution was added. The contents were shaken to form a homogenous solution. From this reaction mixture 20 μL was used for HPLC analysis. The column used was a Lichrospher RP-18 (5μ) column 0.007/min 4×250 mm. The eluent used for HPLC was water (0.1% TFA and 0.1% 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, and the detection wavelength was 265 nm with a reference wavelength of 400 nm. 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.

6. Experimental conditions for proteinase K 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 pipetteman, 3 mL of methanol was added and the contents were vortexed for 2 min until a homogenous solution resulted. 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 solid proteinase K and transferred to a volumetric flask, diluted with water and the contents were shaken to dissolve the enzyme. From the compound stock solution, 150 μL was pipetted into another glass vial. To this, 150 μL of protease stock solution was added and the contents were shaken to form a homogenous solution. From this reaction mixture 20 μL was used for HPLC analysis as previously described in the manuscript.

7. 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/min.

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