Peptide aldehydes as inhibitors of HIV protease

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We have recently shown that α-MAPI, a peptidic aldehyde of microbial origin, inhibits the HIV protease with a potency comparable to pepstatin, having, differently from pepstatin, no activity on other aspartic proteases. In this study different peptide derivatives containing a C-terminal aldehyde have been tested to assess the potential of this function for the inhibition of HIV protease. The results of our analysis correspond with the recently published subsite preferences of the viral enzyme, indicating that aldehydes bind to the active site of the HIV protease. Our data suggest that peptide aldehydes can act in their hydrated forms as transition state analogues with the most potent inhibitor having an IC_{50} of 0.9 μM.

Human immunodeficiency virus; Aspartic protease; Microbial alkaline protease inhibitor; Peptide aldehyde; Transition state analogue

1. INTRODUCTION

The human immunodeficiency virus (HIV) aspartic protease is essential for the processing of the viral polyproteins, playing a crucial role in the HIV life cycle. It is therefore considered an appealing target for the treatment of AIDS [1,2].

We have recently reported the isolation and structural characterization of an HIV protease inhibitor from the fermentation broth of a *Streptomyces* strain [3,4]. The same molecule had been previously discovered as an inhibitor of subtilisin and other microbial serine proteases, with no activity on pepsin or other acidic proteases, and hence named α-microbial alkaline protease inhibitor (α-MAPI, [5,6]). However, the inhibition of HIV protease, an acidic protease, by α-MAPI ($IC_{50} = 2$ μM at pH 6.0) is comparable to pepstatin ($IC_{50} = 4.5$ μM at pH 6.0), a well-known natural inhibitor of the viral enzyme [3]. α-MAPI is a tetrapeptide derivative with a C-terminal phenylalanine in the aldehyde form [4,6]. This function is essential for the inhibition of HIV protease, as indicated by the complete loss of activity which follows its oxidation to carboxylic acid or reduction to alcohol [3,4].

Peptide aldehydes are well-known inhibitors of serine and cysteine proteases. They react with these proteases to yield, respectively, hemiacetals or hemithioacetals

which mimic the structure of the reaction transition state [7–10]. Conversely, little is known about peptide aldehydes as inhibitors of aspartic proteases, even though aldehydic inhibitors of renin have been reported [11,12]. These studies suggest that aspartic proteases may be inhibited by peptide aldehydes acting in their tetrahedral hydrated forms as transition state analogues. The inhibition of HIV protease by α-MAPI probably occurs by the same mechanism. This is supported by the observation that valine and phenylalanine, the two C-terminal amino acid residues of α-MAPI, are among the best suited to occupy, respectively, the S_2 and S_1 sub-sites of HIV protease [13–15].

In this study, to investigate the potential of the aldehyde function for inhibition of HIV protease, different peptide derivatives were synthesized and tested on the viral enzyme, pepsin and cathepsin D.

2. MATERIALS AND METHODS

2.1. Natural products and calpain inhibitors

α- and β-MAPI were isolated from the fermentation broth of a *Streptomyces* strain, as previously described [4]. Pepstatin, leupeptin and calpain inhibitors 1 and 2 were from Calbiochem (San Diego, CA); antipain and calpeptin were from Novabiochem (Switzerland); elastatinal was from Sigma (St. Louis, MO).

2.2. Chemical synthesis

The modified peptides described in this study were synthesized as follows.

The compounds listed in Table II (with the exception of calpeptin) were prepared starting from the respective N-protected dipeptide and following the procedure described in [16]. Briefly, the C-terminus was transformed into aldehyde by reaction with N,O-dimethyl-hydroxylamine and reduction of the corresponding N-methoxy-methyl-amide with lithium aluminium hydride.

The compounds listed in Table III were prepared by reaction of the dipeptide H_2N-Val-Phe-OH with the suitable acyl chlorides at pH 9.5

to produce the *N*-acylated dipeptides. These intermediates were then transformed into the corresponding aldehydes following the same procedure as above [16].

The structures of all compounds were confirmed by IR, NMR and MS spectra.

2.3. Protease assays

The inhibition of HIV protease activity was assayed using the solid-phase immunoassay previously described [3]. The only difference was the pH of the incubation solution (5.6 instead of 6.0), as explained in section 3.1.

The inhibition of pepsin and cathepsin D activities was assayed with the same solid-phase immunoassay, at pH 5.6, using, respectively, pepsin (2 μg/ml) or cathepsin D (0.2 U/ml), both from Sigma, in place of the HIV protease. This was possible as it was previously found that the same substrate used for HIV protease, the gal-gag110 fusion protein [3], contains cleavage sites for both proteases (unpublished results).

The values shown in the Tables are derived from at least two separate experiments for each compound.

3. RESULTS

3.1. Natural products and calpain inhibitors

As a first approach, a number of known peptide aldehydes were tested for inhibition of HIV protease, pepsin and cathepsin D. Table I shows the results obtained with five natural products and two synthetic compounds, calpain inhibitors 1 and 2 (CI-1 and CI-2, [17]), compared with pepstatin, used as control. The same pH (5.6) was used for all three proteases in order to avoid any complication due to the pH dependence of inhibitors (reported, for instance, for pepstatin in [18,19]). At this pH the HIV protease has maximum activity [18–21] and both pepsin and cathepsin D are still quite active.

The data shown in Table I indicate that: (a) the C-terminal amino acid of α-MAPI has a crucial role for the inhibition of HIV protease. Comparison of α-MAPI and antipain shows that the activity is completely lost when only the C-terminal, aldehyde-containing amino acid residue is substituted. A similar effect is observed comparing leupeptin with CI-1 and CI-2; (b) the requirement for phenylalanine for the C-terminal position is not absolute. The inhibitory activities of CI-1 and CI-2 show that other relatively large hydrophobic amino acids can replace it; (c) not only the identity, but also the stereochemistry of the C-terminal amino acid is important, as shown by the different activities of α-MAPI and β-MAPI.

3.2. Cbz-dipeptide aldehydes

The results shown in Table I led us into a more systematic study on the effects of amino acid substitutions in peptide aldehydes as inhibitors of HIV protease. A panel of carbobenzoxy (Cbz)-protected dipeptide aldehydes were synthesized, in which either the phenylalanine in P_1 or the valine in P_2 were substituted. Table II shows the inhibitory activity of these compounds, together with Cbz-Leu-Nle-H, or calpeptin, another calpain inhibitor [22].

These data indicate that: (a) the replacement of the Phe-ureido-Arg-portion of α-MAPI with Cbz has minimal effect on HIV protease inhibition, as shown by the IC_{50} values of α-MAPI (2.7 μM, Table I) and Cbz-Val-Phe-H (2.9 μM, Table II); (b) in the Cbz-Val-**Xaa**-H series, the substitution of phenylalaninal with tyrosinal does not significantly affect the activity on the HIV protease while tryptophanal has a more pronounced effect. With the alaninal analogue the inhibitory activity is completely lost; (c) in the Cbz-**Yaa**-Phe-H series, the substitution of valine with isoleucine causes a slightly lower activity on HIV protease and the appearance of some activity on cathepsin D. If instead Yaa is either a bulky residue, like phenylalanine, or one too small, like alanine, the IC_{50} on HIV protease is one order of magnitude higher.

3.3. R-Val-Phe-aldehydes

Once established that the C-terminal sequence -Val-

Table I

Inhibition of aspartic proteases

	IC_{50} (μM)		
	HIV protease	Pepsin	Cathepsin D
NATURAL PRODUCTS			
Iva-Val-Val-Sta-Ala-Sta	2.0	<0.005	<0.04
Pepstatin			
Phe O=C Arg-Val-L-Phe-H *α-MAPI*	2.7	>200	>200
Phe O=C Arg-Val-D-Phe-H *β-MAPI*	45	>200	>200
Phe O=C Arg-Val-Arg-H *Antipain*	>200	>200	>200
Leu O=C Cpd-Gln-Ala-H *Elastatinal*	>200	>200	>200
Ac-Leu-Leu-Arg-H *Leupeptin*	>200	>200	>200
CALPAIN INHIBITORS			
Ac-Leu-Leu-Nle-H *CI-1*	5.2	24	35
Ac-Leu-Leu-Met-H *CI-2*	4.9	>200	>200

Table II

Inhibition of aspartic proteases

	IC$_{50}$ (μM)		
	HIV protease	Pepsin	Cathepsin D
Cbz-dipeptide-aldehydes			
Cbz-Val-Phe-H	2.9	>200	>200
Cbz-Val-Tyr-H	3.8	>200	>200
Cbz-Val-Trp-H	21	>200	>200
Cbz-Val-Ala-H	>200	>200	>200
Cbz-Ile-Phe-H	4.8	>200	20
Cbz-Phe-Phe-H	37	>200	>200
Cbz-Ala-Phe-H	40	>200	>200
Cbz-Leu-Nle-H Calpeptin	160	>200	>200

Phe-H is the most active among the peptide aldehydes tested, we addressed the question of whether different N-substituents of valine might yield a higher activity on the HIV protease.

Table III shows the inhibition of the three aspartic proteases by a series of R-Val-Phe-H compounds. When compared with α-MAPI (Table I) or Cbz-Val-Phe-H (Table II), small alkyl groups in the R- position give lower activity on HIV protease. In addition, some activity on cathepsin D appears.

The observation that the substitution of Cbz in calpeptin (Table II) with N-Ac-Leu- in CI-1 (Table I) results in a 30-fold lower IC$_{50}$ on HIV protease, led us to synthesize the tripeptide N-Ac-Leu-Val-Phe-H. As shown in Table III, the IC$_{50}$ value of this compound on the viral enzyme is about 3-fold lower than α-MAPI (Table I) or Cbz-Val-Phe-H (Table II). However, specificity is decreased as the activity on cathepsin D is also higher and even some activity on pepsin appears.

4. DISCUSSION

The observations derived from this study yield valua-

Table III

Inhibition of aspartic proteases

	IC$_{50}$ (μM)		
	HIV protease	Pepsin	Cathepsin D
R-Val-Phe-aldehydes			
Me-CO-Val-Phe-H	5.4	>200	40
nPr-CO-Val-Phe-H	12	>200	60
iPr-CO-Val-Phe-H	17	>200	100
nNon-CO-Val-Phe-H	17	>200	72
N-Ac-Leu-Val-Phe-H	0.9	100	37

ble information on the inhibition of the HIV protease by peptide aldehydes. Such observations are particularly interesting if examined in the light of recent studies on the viral enzyme [13–15]. These studies, based on substrate analogs, show that hydrophobic/aromatic moieties are preferable in the S$_1$ subsite of the enzyme, with Phe, Tyr and Met as best residues. Small polar or apolar, preferably β-branched, amino acids are instead well suited for the S$_2$ position, while a large variety of residues, but not proline, can occupy the S$_3$ subsite.

Our results indicate that α-MAPI and the other peptide aldehydes of this study show the structure–activity relationship expected from inhibitors acting as substrate analogs. As a consequence, the aldehyde function or its hydrated form is located exactly in the S$_1$/S$_1^*$ catalytic site.

Fluorine nuclear magnetic resonance studies have shown that in aqueous solutions N-Ac-p-F-Phe-H is about 90% in the hydrated form [23]. For compounds like α-MAPI the ratio between the two forms is probably not very different. It is reasonable to suppose that the real inhibitors of the HIV protease are the hydrated forms of our peptide aldehydes.

Aspartic proteases are believed to work by a general acid–base mechanism, in which a water molecule attacks the carbonyl of the scissile bond with the active-site Asp residues mediating the appropriate proton transfers [24,25]. The tetrahedral hydrated forms of peptide aldehydes can inhibit this reaction acting as transition state analogues. This mechanism can explain the relatively high inhibitory activities shown by compounds with only two residues for subsite recognition (Tables II and III). Indeed, Table III shows that the addition of a third residue results in a further improvement of this activity.

In conclusion, peptide aldehydes are reagents relatively easy to prepare and at the same time very useful for the study of the inhibition of the HIV protease.

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