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SUBSTITUTION OF PROLINE WITH PIPECOLIC ACID AT THE SCISSILE BOND CONVERTS A PEPTIDE SUBSTRATE OF HIV PROTEINASE INTO A SELECTIVE INHIBITOR

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The nonapeptide H-Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-NH_2 containing the retroviral Tyr-Pro cleavage site is a good substrate for the proteinase of human immunodeficiency viruses but it is not readily hydrolyzed by other nonviral proteinases including the structurally related pepsin-like aspartic proteinases. Replacing the Pro by L-pipecolic acid (2-piperidinecarboxylic acid) converted the substrate into an effective inhibitor of HIV-1 and HIV-2 proteinases with IC_{50} of ~ 1 μM. This compound showed a high degree of selectivity in that it did not inhibit cathepsin D and renin. © 1990 Academic Press, Inc.

During the maturation of retroviruses the virally encoded proteinase (PR) cleaves the Gag and Gag-Pol polyproteins into smaller units to produce the structural proteins and enzymes of the infectious virion. A striking feature of this proteolytic processing event is that it involves cleavage at the NH side of proline residue in all retroviruses. Without exception the N terminus of all retroviral capsid (CA) proteins is proline. In many retroviruses similar cleavages occur at more than a single site. For example, in the mammalian C-type viruses the Gag polyprotein is cleaved twice at proline residues and in the human immunodeficiency virus type-1 (HIV-1) the PR and reverse transcriptase (RT) have a proline N terminus (1,2). Since cellular proteinases have not been known to efficiently hydrolyze peptide bonds involving the proline nitrogen, it was this unusual proteolytic cleavage that suggested to us some time ago that a "unique" virus specified proteinase is involved in the proteolytic processing of retroviral polyproteins (3). Oligopeptides having the naturally occurring Phe-Pro or Tyr-Pro cleavage sites were found to be good substrates for both HIV-1 and HIV-2 proteinases (1,4,5).

Proline (2-pyrrolidinecarboxylic acid) and 4-hydroxyproline are the only imino acids naturally occurring in proteins. They have a five membered ring structure. A first approach to understand the role of proline residue in recognition and catalysis by the retroviral PR was to modify the size of the ring structure. The proline homolog, pipecolic acid (2-piperidinecarboxylic acid) has a six membered ring, hexahydropyridine.

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In this report we describe the conversion of a peptide substrate of HIV PR into an inhibitor by the replacement of proline with pipecolic acid.

MATERIALS AND METHODS

Synthetic Peptides: Peptides were synthesized on solid supports (6). Synthetic peptide SP-211 was described elsewhere (1). L-pipecolic acid and 2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile) (BOC-ON) were purchased from Aldrich Chemical Co. (Milwaukee, WI). L-pipecolic acid was protected at the N terminus using BOC-ON as recommended by the manufacturer. SP-346 was synthesized as SP-211 except that the coupling time for Boc-pipecolic acid was 3 times that of Boc-Pro. Purified peptides were taken for amino acid analysis and automated Edman N-terminal amino acid sequence determination in a gas phase sequenator (7).

Retroviral proteinases and their assays: Recombinant HIV-1 PR (8) and EIAV PR from virus were purified by subsequent chromatography on a hydrophobic interaction column, pepstatin A agarose, and cation exchange column (to be published elsewhere). HIV-2 PR was chemically synthesized (1), purified by RP-HPLC, refolded from guanidine-HCl and further purified by gel filtration. Proteinases were assayed with a synthetic nonapeptide substrate (SP-211) as described earlier (1,8).

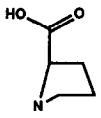
Pepsin, cathepsin D, renin, trypsin and chymotrypsin assays: These enzymes were purchased from Sigma and assayed according to published methods (9,10,11,12,13). SP-211 cleavage by these proteinases was assayed under the conditions described in the above references using RP-HPLC to detect cleavage products.

EIAV capsid: Capsids of EIAV were prepared as described earlier (14). These were incubated at 37°C in 10 mM Tris HCl 1 mM EDTA buffer at pH 7.6 containing 10 mM DTT. The extent of the nucleocapsid (NC) protein cleavage was determined by SDS-PAGE as described (14).

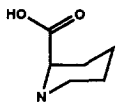
RESULTS AND DISCUSSION

In this study the proline residue of a nonapeptide substrate (SP-211) for HIV-1 and related retroviral proteinases, representing the cleavage site at the junction of the matrix (MA) and CA proteins in HIV-1 Gag precursor polyprotein was replaced by L-pipecolic acid. The structures of proline and pipecolic acid together with the amino acid sequences of the synthetic substrate (SP-211) and its analog (SP-346) are shown in Fig. 1. When tested with retroviral PRs the rate of cleavage of SP-346 as compared with that of SP-211, was reduced to such an insignificant level (100 fold or more) that the peptide could not be considered as a suitable substrate anymore. However, SP-346 was found to be an effective inhibitor of the SP-211 cleavage. The results are shown in Table 1. The IC_{50} values were determined to be 1.4 μM for HIV-1 PR, 0.6 μM for HIV-2 PR and 1.7 μM for the PR of the related lentivirus, equine infectious anemia virus (EIAV).

We have previously found that the nonapeptide substrate SP-211 was not hydrolyzed by cellular enzymes present in lysates of *E. coli* cells (8). In the present study we examined further the resistance of SP-211 to a number of nonretroviral proteolytic enzymes. First we studied the susceptibility of SP-211 to cellular (pepsin-like) aspartic proteinases since the retroviral PR belongs to this family of enzymes. As shown in Table 1, the results indicate that neither renin nor cathepsin D cleaved SP-



Proline



Pipecolic Acid

P5 P4 P3 P2 P1 P1′ P2′ P3′ P4′

SP-211 H-Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-NH_2

SP-346 H-Val-Ser-Gln-Asn-Tyr-Pip-Ile-Val-Gln-NH_2

Figure 1. The structure of proline and pipecolic acid (Pip) and the amino acid sequence of nonapeptide substrate (SP-211) and inhibitor SP-346. Notations according to Schechter and Berger (16).

211. The value found for the substrate cleavage by pepsin is very small. As expected SP-211 was also resistant to serine proteinases such as trypsin and chymotrypsin (data not shown). The specificity of the SP-346 retroviral PR inhibitor was studied with the same set of cellular enzymes at the optimal pH in assays utilizing suitable substrates (see Table 1). The results presented in Table 1 clearly show that SP-346 did not

TABLE 1. The effect of SP-346 on retroviral and cellular (pepsin-like) aspartic proteinases

		Substrate Cleaved nmolxs^{-1} xmg^{-1}		Inhibition by SP-346
Enzyme	pH	SP-211	Other[a]	IC_{50} μM
HIV-1 PR	5.6	50	nt[b]	1.4
HIV-2 PR	5.6	185	nt	0.6
EIAV-PR	6.0	10	nt	1.7
Pepsin	2.0	0.04	14[c]	> 300
Cathepsin D	3.5	nc[d]	35	ni[e]
Renin	6.5	nc	6	ni

[a] Substrates other than SP-211 were: azocoll (Sigma) for pepsin, H-Phe-Ala-Ala-pNO_2-Phe-Phe-Val-Leu-4-hydroxy methyl pyridine ester (Bachem Bioscience) for cathepsin D and synthetic porcine renin substrate tetradecapeptide (Sigma) for renin.

[b] nt, not tested.

[c] Expressed as mg of substrate hydrolyzed per second per mg of enzyme.

[d] nc, not cleaved.

[e] ni, not inhibited at 300 μM, the highest concentration tested.

inhibit any of these nonviral proteinases with the exception of a very slight inhibitory effect against pepsin. The assayed serine proteinases were also not inhibited (data not shown). Thus, the nonapeptide SP-346 containing the L-pipecolic acid residue instead of L-proline in the P1' position is a highly selective substrate based inhibitor of the proteinase of HIVs and EIAV.

The synthetic peptide substrate used in these studies represents a maturation cleavage site. Our recent studies indicated that the retroviral PR in addition to its crucial role in virus maturation may also have an important function in the early phase of virus replication. This involves the *in situ* cleavage of the nucleocapsid (NC) protein inside the viral capsid (14). It was of interest to test the effect of the inhibitor SP-346 on this cleavage. For these studies purified capsids of EIAV were used and the results are shown in Fig. 2. It can be clearly seen that in the absence of inhibitor, incubation of the capsids in Tris-EDTA, pH 7.6 buffer containing DTT results in cleavage of the NC protein p11 to smaller peptides (band labeled p4) with p6 as the intermediate. The cleavage was inhibited by SP-346. These results demonstrate that SP-346 also effectively inhibits the cleavage of a natural substrate of EIAV PR.

The HIV PR is a potential target for chemotherapy (15). In order to design effective drugs, inhibitors must be specific. They must be able to inhibit the retroviral

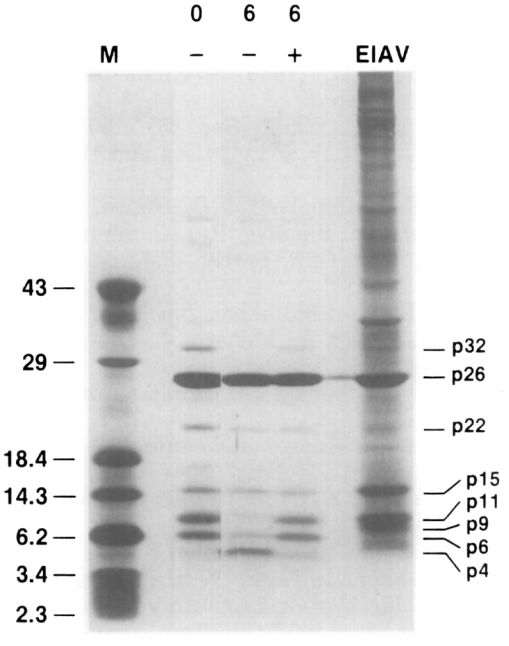


Figure 2. SDS-PAGE analysis of the protein components of EIAV capsids after incubation at 37°C in Tris-EDTA buffer containing 10 mM DTT, pH 7.6 for 0 hr, 6 hr without inhibitor (-) and 6 hr with inhibitor SP-346 (+). The total protein concentration of the capsids during the incubation was 300 μg/ml and that of the inhibitor ranged in various experiments from 100 μg - 750 μg/ml. The minimum concentration of SP-346 tested gave results similar to that shown here with 750 μg/ml. M: molecular weight markers; the numbers to the left are the respective molecular weights x 10^{-3}. EIAV (equine infectious anemia virus).

PR and yet not interfere with other essential cellular and extracellular proteinases. One such approach might be to include the proline homolog described in this report which possesses a high degree of specificity. Further modifications such as changing the scissile bond to nonhydrolyzable transition state analogs and shortening the peptide chain of this and similar substrate based inhibitors may lead to effective peptidomimetic inhibitors of HIV PR.

ACKNOWLEDGMENTS

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