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PEPTIDE HYDROXAMIC ACIDS INHIBIT SKIN COLLAGENASE

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Summary: A number of peptide hydroxamic acids have been synthesized and have been shown to be inhibitors of human skin collagenase. One of these, Z-Pro-Leu-Gly-NHOH, has an IC_{50} value of 4×10^{-5}M. Corresponding peptides with different C-terminal functional groups, such as amide, carboxylate and aldehyde, showed little or no inhibition, indicating the importance of the hydroxamate functional group. In addition, the peptide sequence of this effective inhibitor corresponds closely to that of the cleavage site of native collagen, the substrate for the enzyme. Thus, substrate analogs incorporating a suitable metal coordinating group serve as potential inhibitors of human collagenase.

Human collagenases cleave undenatured collagen at a single point three quarters the distance from the amino terminal end. While this highly specific enzyme is now recognized to play a critical role in a number of normal and pathogenic states (1,2), there have been few attempts to synthesize small organic inhibitors as a means to modulate its activity. In this brief report we show that peptide hydroxamic acids can block the activity of the enzyme, and further, the most effective inhibitor of this kind contains the peptide sequence which most closely resembles that around the cleavage site in native collagen.

MATERIALS AND METHODS

Collagenase: Skin fibroblasts (CRL 1224) were purchased from American type cell culture and cultured in 150 cm^2 T-flasks. When the flasks became overgrown with cells, the media was collected and the enzyme purified as described by Stricklin (3).

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Collagenase Assays: [^{14}C]-Collagen was prepared by reductive methylation of calf skin collagen using [^{14}C]-formaldehyde and sodium borohydride. Collagenase activity was measured using the method of Terato (4). Briefly, 50 µl of [^{14}C]-collagen solution (4 mg/ml) were incubated with 50 µl of 1.0 M glucose, 0.01 M Tris, 0.40 M NaCl, 0.02 M $CaCl_2$ pH 7.5 for ten minutes at 35°C, and the reaction was initiated by the addition of 100 µl of enzyme solution. Enzyme activity was quenched after 30 minutes by the addition of 1,10-phenanthroline and the uncleaved collagen was precipitated with 200 µl of dioxane. Following centrifugation, an aliquot was added to Picofluor 30 to determine radioactivity. To determine the level of inhibition, aliquots of inhibitors (0-85 µl) and buffer were added to 100 µl of the glucose-collagen solution to give a total volume of 185 µl and the reaction was initiated with 15 µl of purified enzyme.

Peptide Hydroxamic Acids: Zincov was purchased from Calbiochem. Peptides were synthesized by first preparing the succinimide ester of the N-blocked peptide and then the amino acid or amino acid derivative was added in bicarbonate. Hydroxamic acids were synthesized by adding an excess of neutralized hydroxylamine hydrochloride in dimethylformamide to the peptide succinimide ester. Compounds were characterized by amino acid analysis after acid hydrolysis; TLC (silica gel 60 F-254; $CHCl_3$:CH_3OH::3:1); melting point; IR (1% KBR pellets); and extinction coefficient of the corresponding Fe^{3+} complex at 540 nm.

RESULTS AND DISCUSSION

Small organic inhibitors of metallo-proteases have been designed by incorporating a negatively charged metal binding agent onto a substrate-like chain (5). Interaction between inhibitor and active site metal was proven from spectral perturbations which occur when inhibitors of this type are added to Co^{2+} substituted thermolysin or carboxypeptidase, demonstrating the utility of this technique for this enzyme class. Since mammalian collagenases are inhibited by general chelating agents, such as 1,10-phenanthroline, it has been inferred that they too are metalloenzymes; and therefore, this methodology of inhibitor design should be appropriate for these highly specific proteases. Therefore, the activity of human skin collagenase was measured in the presence of highly specific metalloprotease inhibitors, each of which contained a different metal coordinating group, to determine which functional group would be most suitable for collagenases. As shown in Table I, at 5 x 10^{-4} M only Zincov, [2-(N-Hydroxycarboxamido)-4-methyl pentanoyl-L-alanyl-glycine amide], showed meaningful inhibition, indicating that the hydroxamic acid coordinating group would be a good choice for further study.

TABLE I: INHIBITION OF HUMAN SKIN COLLAGENASE BY METALLO PROTEASE INHIBITORS

INHIBITOR	% INHIBITION[a]
Amastatin	0
Captopril	0
Phosphoramidone	0
Zincov	27

[a]Measured at 5 X 10^{-4} M.

A series of hydroxamic acids was then synthesized, and as shown in Table II, all inhibited skin collagenase; however, the degree of inhibition depended on both the presence and nature of a peptide chain. Thus, aceto-hydroxamic acid was about 75 times less effective than Z-glycine hydroxamic acid, the simplest possible peptide hydroxamic acid. The most potent inhibitor found in the series was Z-Pro-Leu-Gly-NHOH with an IC_{50} value of 4 X 10^{-5} M. It is interesting that this peptide sequence is similar to that of the cleavage site in native collagen (6), indicating that this inhibitor is most likely behaving like a metal coordinating substrate analog.

Peptide hydroxamic acids have also been shown to be inhibitors of the collagenase from Clostridium histolyticum (7). In this case, the most effective compound reported was Z-Gly-Pro-Leu-NHOH, with a K_I of 2.4 X

TABLE II: INHIBITION OF SKIN FIBROBLAST COLLAGENASE BY HYDROXAMIC ACID DERIVATIVES

HYDROXAMIC ACID	IC_{50} (mM)[a]
$CH_3-\overset{\overset{O}{\|}}{C}-NHOH$	40
Z-Gly-NHOH	0.60
Z-Leu-Gly-NHOH	0.48
Z-Pro-Leu-Gly-NHOH	0.04

[a]IC_{50} determined at 10.0 μM collagen.

10^{-4} M. Once again, the effectiveness of this inhibitor was postulated to be due to the correspondence of its sequence to that of the cleavage site in native collagen, especially the presence of a proline residue in P_2.[1] To confirm this explanation, Z-Pro-Leu-Gly-NHOH was incubated with the bacterial enzyme at a concentration which totally inhibits the human skin enzyme (5 X 10^{-4} M) and little or no inhibition was observed (data not shown). This finding undoubtedly reflects the different manner in which these two enzymes degrade native collagen. Thus, the bacterial enzyme cleaves the -Y-Gly-bond in -Pro-Y-Gly-Pro-sequences, producing many peptide fragments containing N-terminal glycine (8). On the other hand, the mammalian enzyme produces a single cleavage at a -Gly-Leu (Ile) sequence, resulting in a C-terminal glycine. As might be expected, this different substrate specificity should require different peptide sequences for effective inhibitor design for the two enzymes, and as shown above, this is indeed the case.

The importance of the hydroxamic acid to the observed inhibition was demonstrated by attaching different functional groups to the Z-Pro-Leu peptide core. As shown in Table III, the glycinal and glycinamide derivatives showed no inhibition, indicating that the peptide core itself is necessary but not sufficient for inhibition to occur.

The only other Z-Pro-Leu species which showed any inhibition was the glycine derivative itself. As shown in Table III, at 5.3 x 10^{-3}M there was 15% inhibition, much less than that shown by the corresponding hydroxamic acid. This finding is consistent with the proposed mechanism of interaction of hydroxamic acid inhibitors with thermolysin, another metalloenzyme (9). From crystallographic studies, it was found that the anionic form of the hydroxamic acid could function as a bidentate ligand to the active site zinc (10). Similar binding could be occurring between the hydroxamate inhibitor described here and human collagenase. The weak

[1] This notation is the same as that introduced by Schechter and Berger (11).

TABLE III: INHIBITION OF SKIN FIBROBLAST COLLAGENASE BY Z-Pro-Leu-X ANALOGS

X	CONCENTRATION (X 10^4)[a]	% I
$-NH-CH_2-\overset{O}{\overset{\|\|}{C}}-NHOH$	1.15	70
$-NH-CH_2-\overset{O}{\overset{\|\|}{C}}-OH$	53	15
$-NH-CH_2-\overset{O}{\overset{\|\|}{C}}-H$	1	0
$-NH-CH_2-\overset{O}{\overset{\|\|}{C}}-NH_2$	53	0

[a]The hydroxamic acid, carboxylic acid and amide were dissolved in 5% dioxane, 0.50 M NaCl, 0.050 M Tris, 0.01 M $CaCl_2$ pH 7.5. The assay mixture contained 50 µl [^{14}C]- collagen, 50 µl Tris-glucose, 10 µl enzyme, X µl inhibitor, (90-X) µl inhibitor buffer. The aldehyde was dissolved in dimethylformamide and controls run with equivalent amounts of dimethylformamide.

binding of Z-Pro-Leu-Gly occurs because only a monodentate complex can be formed with the carboxylate anion.

In conclusion we have shown that hydroxamic acids are effective inhibitors of human skin collagenase. These inhibitors should be useful as affinity ligands or as potential drug candidates in diseases like rheumatoid arthritis where excess collagenase production presents a clinical problem.

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