Inhibition of Human Skin Fibroblast Collagenase, Thermolysin, and *Pseudomonas aeruginosa* elastase by Peptide Hydroxamic Acids†

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Received March 31, 1992; Revised Manuscript Received May 5, 1992

ABSTRACT: The hydroxamic acid HONHCOCH$_2$CH(i-Bu)CO-L-Trp-NHMe, isomer **6A** (GM 6001), inhibits human skin fibroblast collagenase with K_i of 0.4 nM using the synthetic thiol ester substrate Ac-Pro-Leu-Gly-SCH(i-Bu)CO-Leu-Gly-OEt at pH 6.5. The other isomer, **6B**, which has the opposite configuration at the CH$_2$CH(i-Bu)CO α-carbon atom, has a K_i of 200 nM for this enzyme. GM 6001 is one of the most potent inhibitors of human skin fibroblast collagenase yet reported. GM 6001 has a K_i of 20 nM against thermolysin and *Pseudomonas aeruginosa* elastase. Isomer **6B** has a K_i of 7 nM against thermolysin and 2 nM against the elastase. **6A** and **6B** are the most potent hydroxamate inhibitors reported for these bacterial enzymes. The pattern of inhibition for all three enzymes suggests that isomer **6A** is the (*R*,*S*) compound, stereochemically analogous to the L,L-dipeptide, and isomer **6B** is the (*S*,*S*) compound, analogous to the DL-dipeptide. The tolerance of the D configuration by thermolysin and the elastase allows these inhibitors to discriminate between the human and bacterial enzymes simply by inversion of configuration at the CH$_2$CH(i-Bu)CO α-carbon atom. Substitution of the potential metal liganding groups carboxylate and hydrazide for the hydroxamate group yields much weaker inhibitors for all three enzymes.

The zinc metalloprotease human skin fibroblast collagenase is immunologically (Campbell et al., 1987) and catalytically (Welgus et al, 1986; Hasty et al., 1987) identical to human synovial collagenase. This enzyme is thought to be involved in the invasive phase of rheumatoid arthritis (Wize et al., 1984; Mullins & Rohrlich, 1983). Inhibitors could be useful in proving the role of this collagenase in experimental models of human disease and may ultimately be of therapeutic use. *Pseudomonas aeruginosa* elastase is also a collagenase and is thought to be the agent responsible for corneal ulceration in bacterial infections of the eye (Laibson, 1972). Thermolysin (*Bacillus subtilis*) is an endoprotease which is not known as a collagenase. However, the X-ray crystal structures of thermolysin–inhibitor complexes have been determined and have served as models for the catalytic sites of the medically significant endometalloproteases (Matthews, 1988).

Peptide hydroxamic acids are excellent inhibitors of zinc metalloproteases (Powers & Harper, 1986). Good binding to the enzyme is due to chelation of the active site zinc atom as shown by X-ray crystallography of thermolysin–hydroxamate complexes with K_i's as low as 0.43 μM (Holmes & Matthews, 1981). Peptide hydroxamates inhibit *P. aeruginosa* elastase with K_i's as low as 0.44 μM (Powers & Harper, 1986). Recently, the peptide hydroxamate HONHCOCH$_2$CH(i-Bu)CO-Tyr(OMe)-NHMe[1] (**5**) was reported to inhibit human rheumatoid synovial collagenase (Dickens et. al., 1986) and human skin fibroblast collagenase with an IC_{50} of 20 nM (Reich et al., 1988). A tripeptide hydroxamate HONHCOCH$_2$CH(i-Bu)CO-Leu-Ala-OEt has been reported to inhibit human synovial collagenase with a K_i of 5 nM (Johnson et al., 1987).

On the basis of our results with phosphoramidate inhibitors of human skin fibroblast collagenase (Kortylewicz & Galardy, 1989, 1990), and the good inhibition of many metalloproteases by phosphoramidon [rhamnosyl-phospho-Leu-Trp (Powers & Harper, 1986)], HONHCOCH$_2$CH(i-Bu)CO-Trp-NHMe (**6A** and **6B**) was synthesized as an inhibitor of human skin fibroblast collagenase, thermolysin, and *P. aeruginosa* elastase.

Hydroxamate **6A** (GM 6001), one of the diastereomers at the CH$_2$CH(i-Bu)CO α-carbon, is an excellent inhibitor of human skin fibroblast collagenase with a K_i of 0.4 nM when assayed with a synthetic thio ester substrate at pH 6.5. Its K_i is 50 times higher for thermolysin and *P. aeruginosa* elastase. The other diastereomer, **6B**, is a much weaker inhibitor of collagenase but about equipotent to **6A** against the bacterial enzymes. **6A** is one of the most potent inhibitors of human skin fibroblast collagenase and **6B** the most potent hydroxamate inhibitor of thermolysin and *P. aeruginosa* elastase reported. The strong inhibition of these three enzymes and of angiotensin-converting enzyme by peptides with Leu-Trp in the P_1'–P_2' positions suggests unexpected subsite similarites among these zinc metalloproteases. Inhibitors with carboxylate and hydrazide groups substituted for the hydroxamic acid group are weak against the three endoproteases with K_i's in the 1 μM to 1 mM range.

EXPERIMENTAL PROCEDURES

Synthesis of the Inhibitors. The synthesis and chemical characterization of the inhibitors is described in the supplementary material for this paper.

Enzyme Kinetics. Pure human skin fibroblast collagenase was a gift from Dr. John Jeffrey of Washington University School of Medicine, St. Louis, MO. Thermolysin was from the Calbiochem Corp., San Diego, CA. *Pseudomonas aerug-*

† Supported by National Institutes of Health Grant HL27368 and Glycomed Inc. (R.E.G.) and grants from the Cystic Fibrosis Foundation (L.P.).

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[1] Abbreviation: K_i, the enzyme–inhibitor dissociation constant.

0006-2960/92/0431-7152$03.00/0

Table I. Inhibitors of Human Skin Fibroblast Collagenase, Thermolysin, and *Pseudomonas aeruginosa* Elastase

inhibitor[a]	K_i/μM collagenase	thermolysin	elastase
$HONHCOCH_2CH$(i-Bu)CO-Tyr(OMe)-NHMe[e] (**5**)	0.002	0.05	0.05
$HONHCOCH_2CH$(i-Bu)CO-Trp-NHMe A[b] (**6A**)	0.0004	0.02	0.02
$HONHCOCH_2CH$(i-Bu)CO-Trp-NHMe B[c] (**6B**)	0.2	0.007	0.002
$HOCOCH_2CH$(i-Bu)CO-Tyr(OMe)-NHMe[d] (**7**)	1	16	
$H_2NNHCOCH_2CH$(i-Bu)CO-Tyr(OMe)-NHMe (**8**)	>500	>500	
$HOOCCH_2CH$(i-Bu)CO-Trp-Gly-OH[b] (**9A**)	1	30	400
$HOOCCH_2CH$(i-Bu)CO-Trp-Gly-OH[c] (**9B**)	60	3	70

[a] Compounds **5**, **7**, and **8** are diastereomeric pairs racemic at the Leu α-carbon. [b]One diastereomer. [c] The other diastereomer. [d] The synthesis but not the activity of **7** has been reported (Dickens et al., 1986). [e] IC_{50} = 0.1 μM for the mixture of diastereomers of **5** and 20 nM for the single diastereomer **5A** (Dickens et al., 1986; Reich et al., 1988). [f] K_i's were determined as described in the Materials and Methods section.

inosa elastase was from Nagase, Ltd., Tokyo, Japan. Rabbit lung angiotensin-converting enzyme was purified as described (Goli & Galardy, 1986). Human plasmin (Catalog No. P-4895) was from the Sigma Chemical Co., St. Louis, MO.

Collagenase was assayed using the synthetic thiol ester substrate Ac-Pro-Leu-Gly-SCH(i-Bu)CO-Leu-Gly-OEt at pH 6.5 exactly as described by Kortylewicz and Galardy (1990). The collagenase concentration was 1–2 nM, and the substrate concentrations were from 0.1 to 0.7 nM. K_m was found to vary between 1.5 and 4 mM. At the low substrate concentrations employed, it was not possible to accurately determine whether inhibition was competitive or not. Thermolysin was assayed using furanacryloyl-Gly-Leu-NH_2 (Sigma) at a concentration of 2, 4, 6, or 8 mM in 0.1 M Tris buffer, 0.5 M in sodium bromide, 2.5 mM in calcium chloride and 2.5% in dimethylformamide (Grobelny et al., 1989). The thermolysin concentration was 10–20 nM. *P. aeruginosa* elastase was assayed exactly as described for thermolysin. Angiotensin-converting enzyme was assayed at pH 7.5 using furanacryloyl-Phe-Gly-Gly (Sigma) according to Holmquist et al. (1979) at a substrate concentration of 0.1 mM. Plasmin was assayed at pH 8 with tosyl-Gly-Pro-Lys-*p*-nitroanilide (Sigma) according to Lottenberg et al. (1981) at a substrate concentration of 0.1 mM. All assays were at 25 °C.

In order to ensure that the K_i's measured represented equilibrium binding of inhibitor to enzyme, the time course of inhibition was examined. For thermolysin and *P. aeruginosa* elastase, enzyme and each inhibitor were incubated together at 25 °C at concentrations which would give about 50% inhibition when diluted one to one with substrate solution. No change in the amount of inhibition was observed over a period of 4 h compared to a control enzyme solution incubated in the absence of inhibitor. For collagenase, no change in the amount of inhibition was observed under similar conditions except that the total incubation time was not longer than 20 min.

K_i's whose values were well above the enzyme concentration were determined from Dixon plots using 0.1 and 0.3 mM substrate for collagenase and 2 mM substrate for thermolysin and *P. aeruginosa* elastase at up to eight different concentrations of each inhibitor. Where K_i was in the range of the enzyme concentration, Henderson plots were used to calculate K_i (Henderson, 1972). K_i's were determined at least twice and averaged to give the mean reported K_i. Standard deviations from the mean were always less than ±50%. For **5** and **6A**, K_i values reported are means of values calculated from Henderson plots of each of three independent experiments.

RESULTS AND DISCUSSION

We have demonstrated the increasing affinity of phosphonamidate inhibitors for human skin fibroblast collagenase with

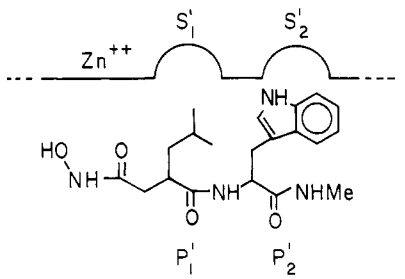

FIGURE 1: Inhibitor $NHOHCOCH_2CH$(i-Bu)CO-Trp-NHMe (**6A**, GM 6001) aligned with a simple diagram of the active site of human skin fibroblast collagenase. The S_1' and S_2' subsites on the enzyme are occupied by the P_1' and P_2' amino acid side chains of the inhibitor (Schechter & Berger, 1968).

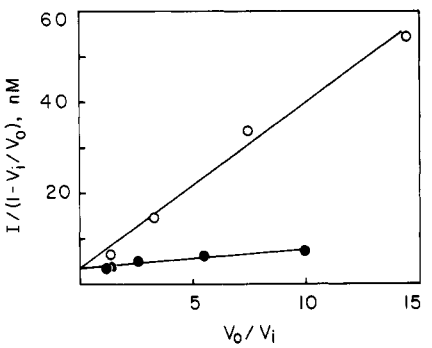

FIGURE 2: Henderson plot of the inhibition of human skin fibroblast collagenase by $HONHCOCH_2CH$(i-Bu)CO-Tyr(OMe)NHMe (**5**) (○) and $HONHCOCH_2CH$(i-Bu)CO-Trp-NHMe (**6A**) (●). The slope of each line is equal to $K_i/(1 + S/K_m)$, assuming that inhibition is competitive (Henderson, 1972).

increasing bulkiness of aromatic substituents in the P_2' subsite (see Figure 1) of the inhibitor (Kortylewicz & Galardy, 1990). This effect was shown for this enzyme by the inhibitor *N*-[3-[(*N*-benzyloxycarbonyl)amino]-1-(*R*)-carboxypropyl]-L-Leu-L-Tyr(OMe)-NHMe (Delaisse et al., 1985). On the basis of our results with the phosphonamidates and the good inhibition of many metalloproteases by phosphoramidon [rhamnosylphospho-Leu-Trp (Powers & Harper, 1986)], $HONHCOCH_2CH$(i-Bu)CO-Trp-NHMe (**6A**, GM 6001) was synthesized as an inhibitor of human skin fibroblast collagenase. Surprisingly, **6A** was found to be a selective inhibitor of collagenase compared to thermolysin and *P. aeruginosa* elastase.

Table I gives the K_i values for seven inhibitors against human skin fibroblast collagenase, thermolysin, and *P. aeruginosa* elastase. Figure 2 shows Henderson plots (which correct for the depletion of free inhibitor concentration by the enzyme) for inhibition of collagenase by **6A** and **5**. When K_i approaches or is less than the total enzyme concentration, methods of calculating K_i (such as Dixon or Lineweaver–Burk plots) which do not correct for free inhibitor depletion by the enzyme will

give K_i values which are too high. Inhibitor **5**, with *O*-methyltyrosine substituted for tryptophan, was isolated as the mixture of its two diastereomers. Since diastereomer **5A** must be more potent that **5B** (Dickens et al., 1986), dividing the K_i for the mixture **5** in Table I by 2 gives a K_i of 1 nM for **5A**. **6A**, with a K_i of 0.4 nM, is thus one of the most potent inhibitors of human skin fibroblast collagenase reported to date.

GM 6001 (**6A**) is selective for collagenase compared to the two bacterial enzymes, with a K_i of 20 nM for each of the latter. This selectivity is exactly mirrored by **5**. GM 6001 showed high selectivity for human collagenase compared to human plasmin, a serine protease (no inhibition at 0.5 mM **6A**), and rabbit lung angiotensin-converting enzyme, a metalloprotease (approximately 50% inhibition at 0.1 mM).

The other diastereomer, **6B**, is a much weaker inhibitor of collagenase with a K_i of 200 nM but inhibits the two bacterial enzymes with K_i's of 7 and 2 nM, respectively. Diastereomer **6A** presumably has the L configuration at the CH_2CH(i-Bu)CO α-carbon, and diastereomer **6B** and D configuration. Thermolysin favors the binding of $HONHCOCH_2CH$-(Bzl)COAla-Gly-*p*-nitroanilide with the D configuration at the CH_2CH(Bzl)CO α-carbon atom compared to its L diastereomer (Holmes & Matthews, 1981). The present study confirms this preference for the D configuration at the P_1, position, with **6B** being bound about 3 times more strongly than **6A**. **6B** is the most potent hydroxamate inhibitor of thermolysin and *P. aeruginosa* elastase reported. The strong inhibition of these three enzymes and of angiotensin-converting enzyme by peptides with Leu(or Val)-Trp in the P_1'–P_2' positions (Powers & Harper, 1986) suggests unexpected subsite similarites among these zinc metalloproteases. The preference for a bulky hydrophobic amino acid residue in the P_2' position (for human skin fibroblast collagenase inhibitors (Delaisse et al., 1985; Kortylewicz & Galardy, 1990) is surprising since alanine occupies this position in the hydrophilic collagen substrate.

Inhibitors with carboxylate and hydrazide groups substituted for the hydroxamic acid are weak with K_i's in the 1 μM to 1 mM range (see Table I). Carboxylate inhibitors of these and other metalloproteases have K_i's in this same range. However, *N*-(1-carboxyalkyl)peptides can have K_i's in the nanomolar and picomolar range for other metalloproteases [see Powers and Harper (1986) for examples]. The increased potency of hydroxamate [and presumably *N*-(1-carboxyalkyl) peptides] compared to simple carboxylates must be due to stronger, bidentate chelation of the active site zinc atom or the more extensive hydrogen-bonding capability of the former [see Powers and Harper, (1986)]. The weak inhibition of collagenase and thermolysin by the hydrazide **8** could be due to its very high pK_a. For loss of a proton to give an anion, the pK_a of hydrazide **8** must be within a few pH units of that of an amine or amide [pK_a >20 (March, 1968)] since, for acceptance of a proton to give a cation, the pK_a's of acetamide (–0.51) and acetohydrazide (3.24) are similar within a few pH units. [Fasman, 1976 (p 344)]. The pK_a for loss of a proton from acetohydroxamic acid to give the anion is 9.4 [Fasman, 1976 (p 315]. Since it cannot ionize to give an anion near neutral pH, the hydrazide would be a very poor ligand for the active site zinc atom of metalloproteases [See Grobelny et al., (1989) for a discussion of the effect of pK_a on binding of inhibitors which ionize around neutral pH to the active site zinc atom of thermolysin].

In summary, GM 6001 (**6A**) is one of the most potent inhibitors of human skin fibroblast collagenase yet reported. It is surprisingly selective for collagenase when compared to thermolysin, *P. aeruginosa* elastase, angiotensin-converting enzyme, and plasmin. Thermolysin and the elastase recognize **6B**, the diastereomer of **6A** at the CH_2CH(i-Bu)CO α-carbon, better than **6A**. When the carboxylate or hydrazide functionality is substituted for hydroxamate, potency decreases by over 3 orders of magnitude.

ACKNOWLEDGMENT

We thank Regina Reynolds for determining the K_i's of the inhibitors againt human skin fibroblast collagenase.

SUPPLEMENTARY MATERIAL AVAILABLE

Synthesis and chemical characterization of the inhibitors (7 pages). Ordering information is given on any current masthead page.

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