Inhibition of Matrix Metalloproteinases by N-Carboxyalkyl Peptides

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An extensive study of the requirements for effective binding of N-carboxyalkyl peptides to human stromelysin, collagenase, and to a lesser extent, gelatinase A has been investigated. These efforts afforded inhibitors generally in the 100–400 nM range for these matrix metalloproteinases. The most significant increase in potency was obtained with the introduction of a β-phenylethyl group at the P_1' position, suggesting a small hydrophobic channel into the S_1' subsite of stromelysin. One particular compound, N-[1(R)-carboxyethyl]-α(S)-(2-phenylethyl)glycyl-L-leucine, N-phenylamide (**79a**), is relatively selective for rabbit stromelysin with a K_i = 6.5 nM and may prove useful for elucidating the role of endogenously-produced stromelysin in lapine models of tissue degradation.

The matrix metalloproteinases (MMP's) are a family of zinc-containing mammalian proteinases that are capable of degrading the extracellular matrix of connective tissues and basement membranes. They are empirically characterized as follows:[1] metal dependent in that they contain an essential zinc for catalysis and calcium for stability and maximum activity. Each member of the family has high homology to interstitial collagenase (MMP-1), one of the earliest described members of the family. The MMP's are secreted as "inactive" zymogens which may be activated by organomercurials. Each is inhibitable by tissue inhibitor of metalloproteinases (TIMP), α_2-macroglobulin, ovostatin, and 1,10-phenanthroline. These enzymes also have an extracellular (or membrane) site of proteolytic action. The discovery of these enzymes, their relationships, and their individual characteristics have been categorized in several reports.[2-6] There are now believed to be 12 members of this family of proteinases that fit these criteria and can be loosely associated into three groups: the collagenases which have triple helical interstitial collagen as a substrate, the gelatinases which are efficient proteinases of denatured collagen and Type IV collagen, and the stromelysins which were originally characterized as proteoglycanases but now appear to have a broader proteolytic spectrum.

As agents capable of remodeling and degrading the extracellular matrix, these metalloproteinases have been implicated in a wide variety of biological processes including diseases characterized by connective tissue or basement membrane degradation or penetration as well as normal repair and maintenance of these tissues. Increased levels of collagenase and stromelysin have been observed in synovium and cartilage in several arthritic diseases and the levels correlated with the severity or advancement of the disease.[7-13] Increased levels of these enzymes have also been reported in models of septic arthritis.[14,15] Many members of this family of metalloproteinases were originally described in malignant cell lines and suggested that they played a role in tumor metastasis.[16-19] Collagenase and stromelysin activities have been reported to be associated with periodontal disease.[20,21] Other conditions where matrix metalloproteinase activities may play a key role in connective tissue integrity include corneal ulceration following alkali burns,[22] proteinuria associated with kidney malfunction,[23] atherosclerotic plaque rupturing,[24] aortic aneurysms,[25] and skin lesions associated with dystrophic epidermolysis bullosa.[26,27]

Collagenase and stromelysin gene expression and protein levels appear increased in cells involved in fetal implantation and development and during ovulation.[28-30] Stromelysin is capable of degrading the endogenous inhibitor, α_1-proteinase inhibitor (α_1-PI), and may therefore influence the balance between α_1-PI and other proteases, such as neutrophil elastase.[31]

The development of specific inhibitors for the matrix metalloproteinases would aid in delineating the role of these enzymes in ongoing processes of tissue development, remodeling, and repair as well as in models of some of the diseases noted above. The proteolytic activity of these enzymes is closely regulated *in vivo* by endogenous inhibitors, such as α_2-macroglobulin and tissue inhibitors of metalloproteinases (TIMP-1 and -2). An imbalance toward excess proteinase activity might lead to the tissue degradation observed in many of these diseases. Such an imbalance of protease and inhibitor has been observed in arthritic conditions[8,32] and is reminiscent of a "protease–antiprotease" hypothesis proposed as a cause of emphysema involving neutrophil elastase and α_1-PI.[33] Low molecular weight synthetic inhibitors for the matrix metalloproteinases might help to restore the balance to levels of proteolytic activity that may be needed for normal tissue maintenance and repair. However, given the high sequence homology among the members of this family, the development of inhibitors specific for each metalloproteinase appears to be a difficult task. Moreover, in light of the sometimes discoordinate regulation of these enzymes under different conditions, it might be difficult to know which enzyme to target. In this case, a nonspecific inhibitor or a "cocktail" of inhibitors may be more effective.

Research to design inhibitors of angiotensin-converting enzyme (ACE) stimulated an enormous effort in many laboratories to discover unique inhibitors of this metallodipeptidase.[34-39] The same strategy of incorporating a zinc ligand into a scaffold containing other binding substituents has also been effective in the design of potent

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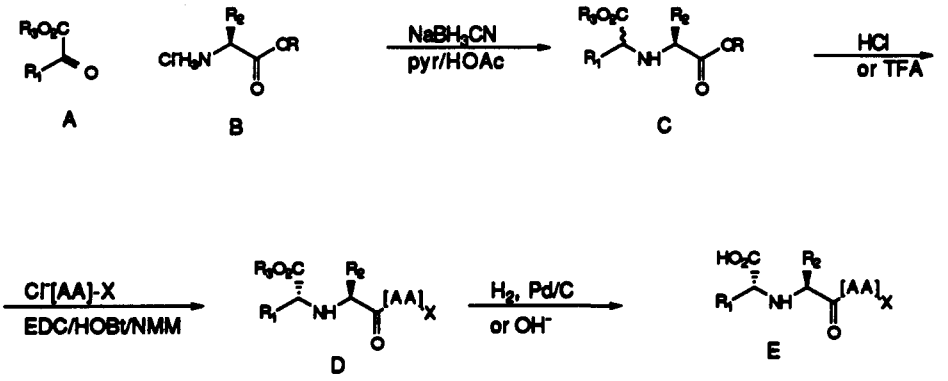
Scheme I



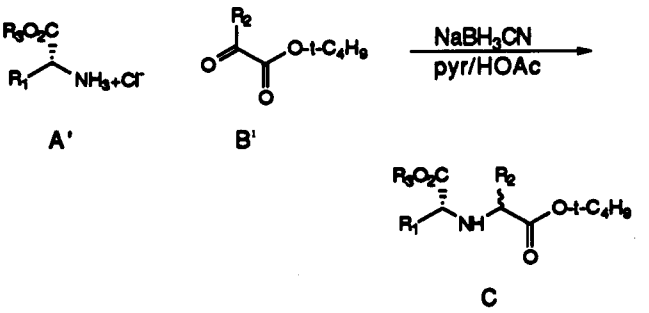
inhibitors of neutral endopeptidase (NEP).[40-43] Inhibitors of collagenase and stromelysin have been designed along similar strategies and are the subject of several reviews.[44-48]

We have chosen to focus on developing selective inhibitors of stromelysin-1 (MMP-3) for a number of reasons. Increased levels of stromelysin mRNA, protein, and/or activities have been found in many of the diseases stated above along with varying levels of other matrix metalloproteinases. Initially named "proteoglycanase" for its ability to degrade proteoglycan, stromelysin has been found to degrade a wider variety of connective tissue substrates including laminin, fibronectin, nonhelical domains of collagen types II, IV, IX, X, and XI and the propeptides of type I procollagen.[49,50] Elevated levels of stromelysin in human rheumatoid synovium[51,52] and the differential expression of stromelysin relative to collagenase in human articular cartilage have been reported.[53,54] Also, the role of stromelysin as a potential activator of other matrix metalloproteinases may imply a cascade of proteolytic processing, with stromelysin playing a major part.[55-60] Thus, in addition to modulating the role of stromelysin in the degradation of connective tissue, a specific inhibitor of stromelysin may prevent processing of the proforms of other matrix metalloproteinases, thereby reducing their proteolytic activities as well. Herein, we report our initial efforts to identify specific binding sites for inhibitors of stromelysin-1 as well as two other matrix metalloproteinases using a series of *N*-carboxylalkyl peptides containing both natural and synthetic amino acid side chains.

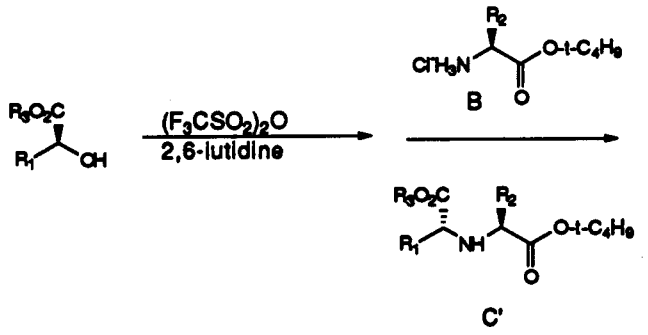
Chemistry

The synthesis of the *N*-carboxyalkyl peptides basically followed procedures originally described for the preparation of angiotensin-converting enzyme inhibitors.[61] Reductive amination with sodium cyanoborohydride in acetic acid/pyridine of the imine intermediate formed from keto esters and amino acid esters afforded the *N*-carboxyalkyl amino acid esters C as separable mixtures of diastereomers[62] (Schemes I and II). The purity of the separated isomers was ≥95% as determined by ^{1}H-NMR (200 MHz). Alternatively, displacement of the triflate derived from an (*S*)-α-hydroxy ester with an esterified (*S*)-amino acid afforded C′ as a single isomer with the desired *R,S* configuration at P_1 and P_1', respectively (Scheme III).[63] The *tert*-butyl esters (C or C′) were removed with either trifluoroacetic (TFA) or hydrochloric acid (HCl), and the resulting acids coupled to derivatized amino acids or peptides in the presence of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxybenztriazole (HOBt), and *N*-methylmorpholine (NMM). Catalytic hydrogenation or basic hydrolysis of

Scheme II



Scheme III



the benzyl ester afforded the *N*-carboxyalkyl peptides listed in Tables I and II (and Tables III–VII in the supplementary material). Generally, commercially available *tert*-butyl esters of amino acids of ≥95% reported enantiomeric purity were used. Commercially unavailable amino acids were prepared by the stereoselective azide transfer method described by Evans.[64]

Enzymology

Inhibition of Human Fibroblast Stromelysin. Initially, compounds were evaluated as inhibitors of stromelysin-catalyzed hydrolysis of Substance P Nle^{11} (SP-Nle^{11}; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle) at the underlined Gln–Phe bond at pH = 7.5. HPLC methodology for this assay has been described.[65] Briefly, peak areas for the hydrolysis product SP-Nle$^{7-11}$ in the presence of inhibitor was compared with a control sample and the ratio plotted as a function of inhibitor concentration to give the IC_{50} values reported in the tables.

Mechanistic studies revealed that many of the compounds exhibited time-dependent or "slow-binding" inhibition.[66] Therefore, the assay to determine inhibitor potency needed to be modified. Ideally, the characterization of the interaction of these compounds with stromelysin should be determined by obtaining complete

Table I. Inhibition of Human Stromelysin, Collagenase, and Gelatinase A by Selected *N*-Carboxyalkyl Peptides[a]

no.	structure[b,c]	stromelysin IC_{50}, μM[d] (± SE)	stromelysin K_i, μM[d] (± SE)	collagenase IC_{50}, μM[e] (± SE)	collagenase K_i, μM[e] (± SE)	gelatinase A K_i, μM[f] (± SE)
1b	(*R*)-Leu-[N]-Leu-PheNH$_2$	~100		>100		
7b	(*R*)-Val-[N]-Leu-PheNHCH$_2$Ph	>100		2		
8b	(*R*)-Leu-[N]-Leu-PheNHCH$_2$Ph	10.8(0.4)		0.5		
20b	(*R*)-Leu-[N]-Trp-LeuNHCH$_2$Ph	27.3(1.6)		>60		
25	(*R*)-Ala-[N]-Trp-LeuNHPh	6.5(0.9)		10.6(0.5)		
26	(*R*)-Ala-[N]-Trp-LeuNHCH$_2$Ph	28.0(2.0)		23.9(4.3)		
27	(*R*)-Ala-[N]-Trp-LeuNHCH$_2$CH$_2$Ph	42.0(2.0)		17.8(0.5)		
28	(*R*)-Ala-[N]-Trp-LeuNH-(*S*)-CH(CH$_3$)Ph	12.0(1.0)		7.5(1.1)		
29	(*R*)-Ala-[N]-Trp-LeuNH-(*R*)-CH(CH$_3$)Ph	47.0(6.0)		>60		
59	(*R*)-Ala-[N]-Trp-nLeu-LeuNH$_2$	27.0(4.0)		>60		
60	(*R*)-Ala-[N]-Trp-nLeu-Leu-nLeuNH$_2$	22.0(3.0)		>60		
77a	(*R*)-Ala-[N]-PhGly-LeuNHPh	19.0(3.0)	34(5)	5.9(0.7)		12%
78a	(*R*)-Ala-[N]-Phe-LeuNHPh	>100		52.1(14.8)		
79a	(*R*)-Ala-[N]-hPhe-LeuNHPh	0.32(0.02)	0.47(0.08)	0.061(0.003)	0.76(0.22)	0.20(0.04)
81a	(*R*)-Ala-[N]-(Ph(CH$_2$)$_3$)Gly-LeuNHPh		1.6(0.2)		2.9(0.7)	77%
136	(*R*)-Ala-[N]-hPhe-(*R*)-AlaNHPh		23% at 6.5[g]		17% at 10	13%
165	(*R*)-Ala-[N]-hPhe-(H$_2$NCH$_2$)GlyNHPh		>10		3.18(0.45)	25%
166	(*R*)-Ala-[N]-hPhe-(H$_2$N(CH$_2$)$_2$GlyNHPh		5.3(0.9)		1.74(0.09)	
167	(*R*)-Ala-[N]-hPhe-OrnNHPh		1.30(0.10)		0.82(0.20)	46%
168	(*R*)-Ala-[N]-hPhe-LysNHPh		0.64(0.05)		0.85(0.04)	71%
169	(*R*)-Ala-[N]-hPhe-hLysNHPh		1.6(0.3)		0.51(0.04)	67%
170	(*R*)-Ala-[N]-hPhe-(H$_2$N(CH$_2$)$_6$GlyNHPh		0.54(0.07)		0.37(0.03)	83%
171	(*R*)-Ala-[N]-hPhe-(H$_2$N(CH$_2$)$_9$GlyNHPh		0.11(0.01)		1.00(0.04)	87%
185	(*R*)-Ala-[N]-hPhe-LeuNHPh-(2-OCH$_3$)		>10		>10	0%
186	(*R*)-Ala-[N]-hPhe-LeuNHPh-(3-OCH$_3$)		0.59(0.17)		1.1(0.7)	68%
187	(*R*)-Ala-[N]-hPhe-LeuNHPh-(4-OCH$_3$)		0.23(0.05)		1.2(0.1)	70%

[a] A complete tabulation of all compounds and enzyme inhibition data is provided in tables available in the supplementary material. [b] All amino acids are presumed to be of the natural (*S*)-configuration unless otherwise noted. [c] Satisfactory elemental analyses were obtained (±0.4% of calculated values). [d] Stromelysin assays were performed at pH = 7.5 and 25 °C. [e] Collagenase assays were performed at pH = 6.5 and 25 °C for IC_{50} determinations and at pH = 7.5 and 25 °C for K_i determinations. [f] Gelatinase A assays were performed at pH = 7.5 and 25 °C. A value followed by a percent % sign indicates percent inhibition at 1 μM. [g] Percent inhibition at highest tested concentration (e.g. 80% inhibition at 32 μM).

Table II. Inhibition of Human and Rabbit Matrix Metalloproteinases by *N*-[1(*R*)-Carboxyethyl]-α-(*S*)-(2-phenylethyl)glycyl-L-leucine, *N*-Phenylamide (79a)[a]

species	stromelysin	collagenase	gelatinase A	gelatinase B
human	0.47(0.08)	0.70 (0.10)	0.20(0.04)	0.45(0.03)
rabbit	0.0065(0.00012)	ND[b]	0.41(0.12)[c]	0.76(0.08)

[a] Data reported as K_i in μM (± SE). [b] ND = not determined. [c] Rabbit gelatinase A was a mixture (~1:1) of 72 kDa and 95 kDa gelatinases.

inhibition progress curves at several concentrations of inhibitor and then, using the kinetic constants obtained from those curves, calculate K_i as well as the kinetic constants for the attainment of inhibition. This was done with selected inhibitors but could not be performed routinely on the many compounds described herein. Alternatively, various concentrations of compounds and activated stromelysin were incubated for 4 h to establish equilibrium before substrate (Arg-Pro-Lys-Pro-Leu-Ala-Phe-TrpNH$_2$)[67] was added, and the reaction was allowed to proceed for 18 h. In this assay, the unreacted substrate is separated from the products by HPLC, and the area counts for starting substrate are determined by detection of tryptophan fluorescence. Since the reactions were run under first-order conditions ([S] ≪ K_m), the pseudo-first-order rate constant k_{obs} was determined for each inhibitor concentration by taking a ratio of rate constants for inhibited and control sample (no inhibitor). This ratio is plotted as a function of inhibitor concentration.

Inhibition of Human Fibroblast Collagenase. The *N*-carboxyalkyl peptides were initially assayed against recombinant human fibroblast collagenase by the spectrophotometric method of Weingarten.[68,69] The IC_{50} determinations in the tables were obtained from the collagenase-catalyzed cleavage of the thioester-peptide *N*-acetyl-Pro-Leu-Gly-SCH(i-C$_4$H$_9$)CO-Leu-GlyOC$_2$H$_5$ at pH = 6.5. As these compounds were found to be time-dependent inhibitors of collagenase, the assay was modified analogously to the one developed for stromelysin using the substrate of Stack and Gray.[70] Compounds were incubated with collagenase for 4 h at pH = 7.5 prior to the addition of the fluorogenic substrate DNP-Pro-Leu-Gly-Leu-Trp-Ala-dArgNH$_2$. The products of cleavage were separated by HPLC and peak area counts obtained by fluorometric detection. K_i values reported in the tables were obtained by the same method used for stromelysin.

Inhibition of Human Gelatinase A. Inhibition of human 72 kD gelatinase by *N*-carboxyalkyl peptides was determined by the same conditions and substrate used for collagenase, except added substrate was incubated with enzyme for 2 h at pH = 7.5. The K_i values or percent inhibition at 1 μM are reported.

Results and Discussion

Our starting point for these studies was the *N*-carboxyalkyl dipeptide Leu-[N]-Leu-Phe-NH$_2$ (1) originally reported to be a 1.9 μM inhibitor of rabbit articular chondrocyte proteoglycanase (presumably rabbit stromelysin).[71,72] However, the more active isomer **1b** exhibited an IC_{50} ~100 μM against human fibroblast stromelysin. Given the limited structure–activity relationship for stromelysin reported for this class of inhibitors, we investigated them as a means to explore various potential binding domains in human stromelysin. The first significant improvement in stromelysin inhibitory activity was discovered with the addition of a benzyl amide onto **1b** into what would purportedly be the P_3' position.[73] Leu-[N]-Leu-PheNHCH$_2$Ph (**8b**) had an IC_{50} = 11 μM against stromelysin but was even more potent against human fibroblast collagenase (IC_{50} = 0.5 μM). We were unable

to significantly improve upon the original stromelysin activity or selectivity of **8b** by replacing the P_1 Leu with a variety of amino acids. Compounds with small aliphatic and aryl amino acids at P_1 (alanine **6b**, methionine **9a**, homophenylalanine **10b**, tyrosine **11b**, tryptophan **12b**) had comparable activity to **8b** (IC_{50}'s 11–50 μM) while charged (histidine **13b**, lysine **19b**, aspartic acid **14–15**) or polar (glutamine **16**) groups were poorly tolerated (IC_{50}'s $\geq 80\ \mu$M). Both enantiomers of proline (**17–18**) were also inactive (>100 μM). Of particular interest with respect to selectivity was the apparent loss of stromelysin activity ($IC_{50} > 100\ \mu$M) relative to collagenase ($IC_{50} = 2\ \mu$M) by the P_1 valine analog, Val-[N]-Leu-PheNHCH$_2$Ph, **7b**. Generally, compounds that were active in the low micromolar range against stromelysin were more potent collagenase inhibitors. However, as noted below, this could be a result of the different assay conditions, especially pH.

Results from a study of peptide substrate cleavage by several MMP's by Van Wart suggested that selectivity between stromelysin and collagenase could be achieved by replacement of a P_1' isoleucine with tryptophan or phenylalanine.[74] By incorporating a tryptophan side chain at P_1' into our *N*-carboxyalkyl dipeptides and replacing the P_2' phenylalanine with leucine, we were able to greatly increase our selectivity for stromelysin over collagenase. Leu-[N]-Trp-LeuNHPhCH$_2$Ph, **20b**, had IC_{50}'s of 27 and >60 μM against stromelysin and collagenase, respectively, as compared to 11 and 0.5 μM for compound **8b**. Interestingly, replacement of the P_2' alanine with a tryptophan in the octapeptide cleavage study greatly enhanced cleavage by all MMP's studied. Replacement of the leucine at P_2' of **20b** with a tryptophan resurrected collagenase activity in Leu-[N]-Trp-TrpNHCH$_2$Ph, **22b** (IC_{50} = 20 μM for collagenase), with minimal effect on stromelysin activity ($IC_{50} = 20\ \mu$M). Replacement of the isobutyl group at the P_1 position of **20b** with a methyl group (Ala-[N]-Trp-LeuNHCH$_2$Ph, **26**) restored significant collagenase activity ($IC_{50} = 24\ \mu$M vs $IC_{50} = 28\ \mu$M against stromelysin).

Replacement of the P_3' benzyl amide in **26** resulted in a modest improvement of stromelysin inhibition with a compound containing an anilide at P_3' (Ala-[N]-Trp-LeuNHPh, **25**, IC_{50} = 6.5 μM). Nearly all other amino acid side chains with the $\alpha(S)$-configuration were tolerated by stromelysin at this position, except proline. There was also a slight preference for the $\alpha(S)$-configuration in the 1-aryl- and 1-cyclohexylethyl series (Ala-[N]-Trp-Leu-NHCH(*R*- or *S*-CH$_3$)(aryl or cyclohexyl), **28–33**). The (*R*)-alanine amide Ala-[N]-Trp-Leu-(D)AlaNH$_2$ (**36**) was ~19 times less active than its diastereomer Ala-[N]-Trp-Leu-AlaNH$_2$ (**35**), but the P_3' (*R*)-alanine methyl esters **38** and **45** were only ~3–6 less potent than their respective (*S*)-alanine isomers **37** and **44**. The esters are more lipophilic than the corresponding amides, and the unnatural (*R*)-configuration may put the esters into a site previously occupied by the alkyl side chains where the configuration was (*S*). The (*R*)-amide in **36** being more hydrophilic may not be accommodated in this putative site as well as the ester. These results suggest a slight preference for hydrophobic side chains with the natural (*S*)-configuration at this position.

Interestingly, collagenase activity was much more sensitive to substitution at the P_3' position. In the series, with (*R*- and *S*)-1-aryl- and 1-cyclohexylethyl groups **28–33**, collagenase inhibition was nearly eliminated with the $\alpha(R)$-configuration. It also appeares that longer alkyl (norvaline (**40**), norleucine (**41**)) or branching (leucine (**43**, **44**), isoleucine (**46**)) or aryl (phenylalanine (**48**), tyrosine (**49**), tryptophan (**50**)) amino acid side chains at the P_3' position are not well tolerated by collagenase (IC_{50}'s of 38 to >100 μM). However, amino acid amides with polar side chains such as Ala-[N]-Trp-Leu-ThrNH$_2$ (**52**) and Ala-[N]-Trp-Leu-HisNH$_2$ (**53**) can offer significant inhibition (IC_{50} = 36 and 16 μM, respectively, vs collagenase). In summarizing the results of the P_3' position, stromelysin appears generally tolerant of a wide range of substitution whereas collagenase seems to prefer smaller amino acid side chains. Both enzymes prefer the $\alpha(S)$-configuration, perhaps implying a backbone binding similar to peptidyl substrates.

The extension of amino acid amides into the P_4' and P_5' positions deserve special mention. In the study of amino acid substitution into a series of octapeptide substrates, Van Wart noted a significant increase in relative cleavage rates by stromelysin when the P_3' glycine was replaced by methionine (100 to 810).[74] In our inhibitors, the norleucine methyl ester **41** was slightly more potent as a stromelysin inhibitor than the alanine ester **37** and comparable to the ethyl and *n*-propyl glycine esters **39** and **40**. It was thought that perhaps additional backbone binding may be necessary to orient the *n*-butyl side chain into a more favorable interaction. The tetra- and pentapeptide inhibitors Ala-[N]-Trp-Leu-nLeu-LeuNH$_2$ (**59**) and Ala-[N]-Trp-Leu-nLeu-LeuNH$_2$ (**60**) with norleucine as an isosteric replacement for methionine at P_3' were not significantly more active than the tripeptide analogs having a small amino acid amide at P_3'. Both compounds were inactive against collagenase. It appears that inhibitors for stromelysin in this class have no important binding interactions in the P_4' or P_5' domains, which bodes well for the design of inhibitors of relatively low molecular weights. Similar results were reported for a series of truncated peptide substrates of stromelysin.[67]

The evolution of our understanding of the mechanisms of inhibition is exemplified by the reporting of inhibition data obtained early in the program as IC_{50}'s and then later as K_i's.[66,75] With the P_1 and P_3' terminal positions initially optimized for stromelysin activity and selectivity, the "inner" P_1' and P_2' positions were explored. Screening against human gelatinase A (72 kDa gelatinase, MMP-2) was also added. All three assays were run under similar conditions of incubation time, temperature, and pH.

The P_1' tryptophan of **25** was replaced with a large number of natural and unnatural amino acids. Aliphatic side chains larger than ethyl (Ala-[N]-hAla-LeuNHPh **64a**) at P_1' provide stromelysin inhibitions similar to that observed for **25**. Straight or branched propyl and butyl side chains at this position (norvaline **65a**, valine **66a**, leucine **67a**, isoleucine **68a**) were comparable to the tryptophan in **25** as were analogs with cyclohexylglycine (**70a**) or cyclohexylpropylglycine (**73a**) groups. Interestingly, the P_1' cyclohexylalanine (**71a**) and cyclohexylethylglycine (**72a**) analogues were considerably less active than **25**. The P_1' *tert*-butylglycine analog **69** was inactive. The most dramatic result from an SAR perspective was discovered in the series containing aryl substituents. The phenylglycine analog Ala-[N]-(*S*-Ph)Gly-LeuNHPh (**77a**) was comparable (IC_{50} = 19 μM) to **25** but the compound with a P_1' phenylalanine Ala-[N]-Phe-LeuNHPh (**78a**) was approximately 10-fold less potent (IC_{50} ~200 μM). Extending the phenyl ring by an additional methylene group gave the homophenylalanine analog Ala-[N]-hPhe-LeuNHPh (**79a**) with greatly enhanced potency for

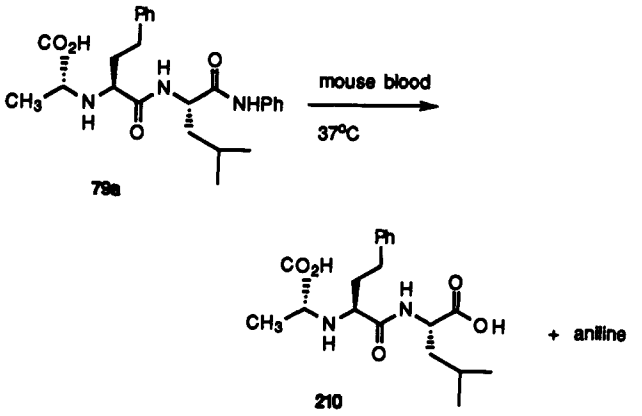


Figure 1. Hydrolysis of the P_3' amide bond by mouse blood.

stromelysin ($IC_{50} = 0.3\ \mu M$, $K_i = 0.5\ \mu M$). The difference in the collagenase IC_{50} and K_i for **79a** highlights the importance of running the assays under the same conditions ($IC_{50} = 0.061\ \mu M$; $K_i = 0.76\ \mu M$). The phenylpropylglycine derivative **81a** was only slightly less active ($K_i = 1.6\ \mu M$). This same trend of the arylmethyl analog being the least active against stromelysin was observed in the β-naphthylalkyl analogs (**83a**, **85a**, **87a**). The α-naphthyl derivatives were uniformly inactive, suggesting a well-defined binding orientation for arylethyl side chains. 2-Thienylglycine (**131**) and 2-furylethylglycine (**132**) at P_1' were equipotent to **79a**. Polar or charged side chains gave mostly inactive compounds (**88a–130**). These results are consistent with the initial reports of rabbit stromelysin having a preference for hydrophobic substituents at the P_1' position.[72] Generally, the more potent stromelysin inhibitors were also more potent against collagenase and gelatinase-A, and inactive compounds were inactive against all three enzymes.

The P_2' position was explored within the **79a** series by substituting the (*S*)-leucine with a large variety of amino acids. All three matrix metalloproteinases were found to tolerate a wide variety of amino acids at this position including aliphatic, aromatic, polar, acidic, and basic side chain substituents. The α(*R*)-configuration in Ala-[N]-hPhe-D-AlaNHPh (**136**) was not tolerate. These results suggest that the side chain at this position is not interacting with any of the enzymes and is oriented towards solvent. Only a few examples within this series merit comment. The inhibitor with a tryptophan side chain (Ala-[N]-hPhe-TrpNHPh **156**) was the most potent, and tryptophan has been reported as a P_2' potency-enhancing group for collagenase inhibition.[69] The only SAR observed at this position are seen in the aminoalkyls of various chain length (**165–171**). As the protonated amino group was brought closer to the enzyme at the P_2' position, the activity began to drop with the aminomethyl and aminoethyl side chains in **165** and **166**. The P_2' histidine analog (Ala-[N]-hPhe-HisNHPh **180**) was also inactive against stromelysin and collagenase. Two possible explanations for the loss of activity seen in **165**, **166**, and **180** are that a positively charged species cannot approach too close to the enzyme's surface or that these side chains induce a conformational change in the inhibitor backbone that disfavors effective binding to the enzymes.

Amide bond hydrolysis is a potential problem with many peptide-containing structures. The *N*-carboxyalkyl peptide inhibitors described herein are no exception. Incubation of **79a** in mouse blood at 37 °C resulted in loss of the anilino group and the formation of corresponding diacid, Ala-[N]-hPhe-Leu **210** (Figure 1).[77] Obviously, the formation of aniline as a potential product of metabolism from these inhibitors is unacceptable. The processes responsible for the anilide cleavage are unknown, but inhibitors with Lys and Arg at P_2' (**168** and **178**, respectively) were cleaved faster in blood than **79a**. This may imply a "tryptase-like" activity for cleavage of **168** and **178** but no direct proof of the nature of this process is offered.

The P_3' anilide of **79a** was replaced with substituted anilides, aminoheteroaryls, and alkyl groups. Among the anilides containing hydroxy, methoxy, methyl carboxylate, carboxylate, and trifluoromethyl substituents (**185–198**), meta and para substitution gave comparable inhibition to **79a** with the para substituents being slightly more active. Interestingly, there was little sensitivity to the type of functionality at these two positions. The ortho substituents were generally much less active. The benzyl amide (Ala-[N]-hPhe-LeuNHCH$_2$Ph, **199**) and the α(*S*)-methyl analog (Ala-[N]-hPhe-LeuNHCH(*S*-CH$_3$)Ph, **200**) were slightly less active than **79a** against stromelysin and gelatinase but comparable against collagenase. The heteroaryl analogs (2-, 3-, and 4-pyridyls, 4-pyrimidinyl, 2-benzothiazolyl, 2-benzimidazolyl **201–206**) were also generally well tolerated by each of the enzymes with those having basic groups closer to the amide linkage being less active. Finally, the simple alkyl amides containing a cyclohexyl group (Ala-[N]-hPhe-LeuNH-cycloC_6H_{11}, **208**) and a *n*-butyl group (Ala-[N]-hPhe-LeuNH-*n*-C_4H_9, **209**) were significantly less active than the anilide **79a**. N-Methylation of the aniline (Ala-[N]-hPhe-LeuN(CH$_3$)-Ph) gave no inhibition of any of the enzymes and was not pursued (data not shown).

A summary of the structure–activity relationship for inhibition of the matrix metalloproteinases described here by *N*-carboxyalkyl peptides is represented in Figure 2. The S_1 subsite appears to be a loosely defined domain in stromelysin and collagenase with significant preference for hydrophobic side chains. Collagenase will tolerate β-branched side chains such as valine while stromelysin

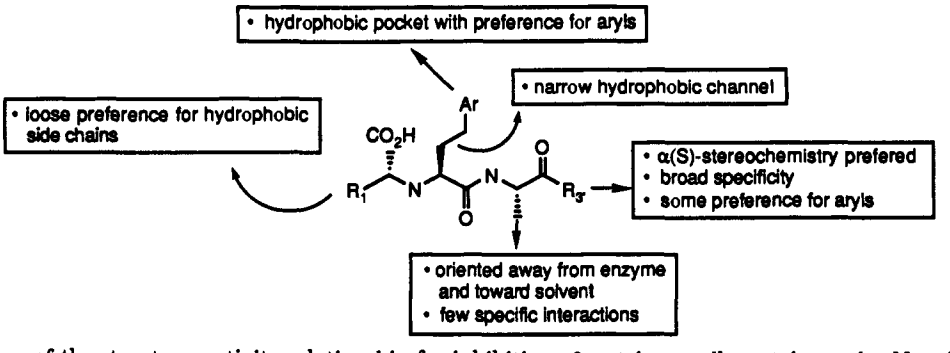


Figure 2. Summary of the structure–activity relationship for inhibition of matrix metalloproteinases by *N*-carboxyalkyl peptides.

will not. The S_1' subsite for each of the enzymes seems to contain a sharply defined hydrophobic "channel" that opens into a broader hydrophobic region that binds aryl groups more tightly. Similar results have been claimed in a series of P_1' arylalkyl substituted hydroxamic acid inhibitors of gelatinase A.[78] The S_2' subsites will tolerate a very broad range of functionality implying that this position is not important for binding of inhibitors. Thus, the P_2' position may be useful as a location for attachment of functionality designed to modify the chemophysical characteristics of a potent inhibitor. The terminal P_3' position has broad specificity among L-amino acid amides and esters, but seems to have a preference for aryl groups. Stromelysin is more tolerant of branching and chain extensions in this region than is collagenase.

An extensive study of the requirements for effective binding of *N*-carboxyalkyl peptides by human stromelysin, collagenase, and, to a lesser extent human, gelatinase A has been explored. While the absolute potencies of the inhibitors described herein are modest compared to those reported by other groups[44-48] and are far from being potent or selective enough to be considered as potential therapeutic agents, they have greatly expanded our knowledge of the binding requirements for these matrix metalloproteinases. In addition, compounds such as **79a** represent important tools to further our understanding of the roles of these enzymes in cartilage degradation. Table II summarizes both the human and rabbit enzyme inhibition data for **79a**. As a selective inhibitor of rabbit stromelysin (K_i = 6.5 nM), **79a** has been useful in defining a role for endogenously-produced stromelysin in a model of IL-1β-induced cartilage degradation.[79,80]

Experimental Section

Chemistry. General Procedures. Proton NMR spectra were recorded on a Varian XL-200 NMR spectrometer. Chemical shifts are given on the δ scale. Spectra were measured at ambient temperature for solutions in chloroform-*d* or methanol-d_4. Infrared spectra were obtained as thin films on sodium plates on Perkin-Elmer 295 or 1310 spectrophotometers. Mass spectra were determined on a LKB 9000 mass spectrometer. Analytical results for compounds followed by elemental symbols were ±0.4% of calculated values unless otherwise indicated and were determined by the Micro-Analytical Laboratory of Merck & Co., Inc., or Robertson-Microlit Laboratories, Inc., Madison, NJ. Thin-layer chromatography was performed on precoated Silica Gel-GHLF$_{254}$ plates (Analtech) or Kieselgel-60, and visualization was effected with UV light, iodine, or ceric sulfate (1%)–sulfuric acid (10%) spray. Preparative flash column chromatography was performed on silica gel 60 (E. Merck, 40–63 μm).

Method A. *N*-[1(*R*)-(Benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenylethyl)glycine, *tert*-Butyl Ester. To a solution of 10.5 g of (*S*)-α-(2-phenylethyl)glycine, *tert*-butyl ester in 50 mL each of acetic acid and pyridine at 0 °C was added 27.2 mL of a 1.0 M solution of sodium cyanoborohydride ($NaBH_3CN$) in tetrahydrofuran (THF) followed by 18.1 mL of benzyl pyruvate. Additional $NaBH_3CN$ solution (50 mL, 1.0 M) was then added over 5 h via syringe pump. The mixture was allowed to warm slowly to room temperature during the first 30 min of this addition. The reaction mixture was then poured into a slurry of ice and 100 mL of concentrated hydrochloric acid (HCl) and extracted with 500 mL of 30% ether/hexane (discarded).

The aqueous slurry (containing the desired HCl salt) was then extracted three times with ethyl acetate, and the combined organic layers were washed three times with saturated aqueous sodium bicarbonate. The mixture was dried over anhydrous sodium sulfate and concentrated *in vacuo* to give 20 g of a yellow oil. The mixture was purified by flash column chromatography on silica gel (120- × 200-mm column, 10% ethyl acetate/hexane) to give *N*-[1(*R*)-(benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenylethyl)glycine, *tert*-butyl ester as a colorless oil (6.40 g): R_f = 0.25 (10% ethyl acetate/hexane); ^{1}H-NMR (CD_3OD) δ 7.13–7.37 (m, 10 H), 5.12 (AB q, J = 5.1 Hz, 2 H), 3.36 (q, J = 7.0 Hz, 1 H), 3.15 (t, J = 6.4 Hz, 1 H), 2.65 (m, 2 H), 1.90 (m, 2 H), 1.45 (s, 9 H), 1.26 (d, J = 7.0 Hz, 3 H).

***N*-[1(*R*)-(Benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenethyl)glycyl-L-leucine, *N*-Phenylamide.** *N*-[1(*R*)-(Benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenethyl)glycine, *tert*-butyl ester (6.40 g) was dissolved in a small amount of ethyl acetate, and 250 mL of a saturated solution of anhydrous HCl in ethyl acetate was added. The mixture was held for 7 h at 50 °C and then cooled and concentrated *in vacuo* to give *N*-[1(*R*)-(benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenethyl)glycine hydrochloride as a colorless solid which was used without further purification: ^{1}H-NMR (CD_3OD) δ 7.41–7.20 (m, 10 H), 5.25 (AB, J = 12.1 Hz, 2 H), 4.27 (q, J = 7.3 Hz, 1 H), 4.06 (t, J = 6.6 Hz, 1 H), 2.9–2.7 (m, 2 H), 2.24 (m, 2 H), 1.55 (d, J = 7.4 Hz, 3 H).

The crude *N*-[1(*R*)-(benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenethyl)glycine hydrochloride from above, L-leucine, *N*-phenylamide hydrochloride (3.71 g, 18.0 mmol), and *N*-hydroxybenzotriazole (3.31 g, 24.5 mmol) were dissolved in 250 mL of dimethylformamide (DMF). *N*-Methylmorpholine (NMM) (3.6 mL, 32.7 mmol) was added and the mixture cooled to 0 °C. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 3.76 g, 19.6 mmol) was added and the mixture stirred for 16 h at ambient temperature. The solution was diluted with 1 L of ethyl acetate and washed three times with saturated aqueous sodium bicarbonate and three times with water. The mixture was then dried over anhydrous sodium sulfate and concentrated *in vacuo* to give a pale yellow oil. The product was purified by MPLC (35- × 350-mm column, 30% ethyl acetate/hexane) to afford 8.31 g of *N*-[1(*R*)-(benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenethyl)glycyl-L-leucine, *N*-phenylamide as a colorless glass: ^{1}H-NMR (CD_3OD) δ 7.57–7.08 (m, 15 H), 5.13 (AB q, J = 5 Hz, 2 H), 4.57 (m, 1 H), 3.47 (q, J = 7.1 Hz, 1 H), 3.25 (t, J = 6.4 Hz, 1 H), 2.67 (t, J = 8.1 Hz, 2 H), 2.1–1.6 (m, 5 H), 1.31 (d, J = 7.0 Hz, 3 H), 0.98 (d, J = 6.5 Hz, 3 H), 0.96 (d, J = 6.0 Hz, 3 H).

***N*-[1(*R*)-Carboxyethyl]-α-(*S*)-(2-phenylethyl)glycyl-L-leucine, *N*-Phenylamide (79a).** To a solution of 8.31 g of *N*-[1(*R*)-(benzyloxycarbonyl)ethyl]-α-(*S*)-(2-phenethyl)glycyl-L-leucine, *N*-phenylamide in 125 mL of methanol was added 500 mg of Pearlman's catalyst. After 3 h of vigorous stirring under an atmosphere of hydrogen, the mixture was filtered through Celite filter aid and concentrated *in vacuo* to give 6.50 g of amino acid **79a** as a colorless solid: ^{1}H-NMR (CH_3OD) δ 7.59–7.08 (m, 10 H), 4.65 (m, 1 H), 3.96 (t, J = 6.8 Hz, 1 H), 3.58 (q, J = 7.4 Hz, 1 H), 2.9–2.6 (m, 2 H), 2.15 (m, 2 H), 1.52 (d, J = 7.4 Hz, 3 H), 1.02 (d, J = 6.0 Hz, 3 H), 1.00 (d, J = 6.0 Hz, 3 H).

Method B. *N*-[1(*R*)-(*tert*-Butyloxycarbonyl)ethyl]-α-(*S*)-(3-phenylpropyl)glycine, Benzyl Ester. To a solution of benzyl 5-phenyl-2-oxopentanoate (1.83 g, 6.48 mmol) in 3 mL each of pyridine and acetic acid at 0 °C was added alanine, *tert*-butyl ester hydrochloride (1.18 g, 6.48 mmol). Sodium cyanoborohydride (4.32 mL, 1.0 M solution in THF, 4.32 mmol) was added *via* syringe pump over 10 h. The mixture was poured into a slurry of ice and excess concentrated hydrochloric acid, and the resulting mixture was extracted three times with ethyl acetate. The combined organic layers were diluted 1:1 with hexane and washed three times with saturated sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The individual diastereomers were purified by MPLC on silica gel (35- × 350-mm column, eluted with 10% ethyl acetate in hexane) which afforded 644 mg of the title compound as a colorless oil: ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.4–7.1 (m, 10H), 5.11 (AB, 2H, J = 12.5 Hz), 3.29 (m, 1H), 3.17 (q, 1H, J = 7.05 Hz), 2.56 (m, 2H), 1.8–1.5 (m, 4H), 1.41 (s, 9H), 1.20 (d, 3H, J = 7.06 Hz). Only the diastereomer that led to the active isomer **81b** is reported in this example.

***N*-[1(*R*)-(*tert*-Butyloxycarbonyl)ethyl]-α-(*S*)-(3-phenylpropyl)glycine Hydrochloride.** *N*-[1(*R*)-(*tert*-Butyloxycarbonyl)ethyl]-α-(*S*)-(3-phenylpropyl)glycine, benzyl ester (644 mg) was dissolved in ethyl acetate and acidified with a cold solution of hydrochloric acid in ethyl acetate. The mixture was concentrated *in vacuo* to afford the hydrochloride salt. This salt was dissolved in 10 mL of methanol, and 20 mg of Pearlman's catalyst was added. After 1 h under 1 atm of hydrogen, the mixture was filtered and concentrated to afford 598 mg of the title compound as a colorless solid: ^{1}H-NMR (200 MHz, CD_3OD) δ 7.4–7.1 (m,

5H), 4.1–3.95 (m, 2H), 2.69 (t, 2H, J = 7.3 Hz), 2.1–1.7 (m, 4H), 1.53 (d, 3H, J = 7.06 Hz), 1.51 (s, 9H).

N-[1(R)-(*tert*-Butyloxycarbonyl)ethyl]-α-(S)-(3-phenylpropyl)glycyl-L-leucine, N-Phenylamide. To a solution of N-[1(R)-(*tert*-butyloxycarbonyl)ethyl]-α-(S)-(3-phenylpropyl)-glycine hydrochloride (196.6 mg, 0.55 mmol), L-leucine N-phenylamide (147 mg, 0.60 mmol), and hydroxybenzotriazole (149 mg, 1.10 mmol) in 3 mL of DMF at 0 °C was added 4-methylmorpholine (127 mL, 1.15 mmol) followed by ethyl[(dimethylamino)propyl]carbodiimide (158 mg, 0.825 mmol). After 16 h at ambient temperature, the mixture was diluted with ethyl acetate and washed successively with saturated sodium bicarbonate (3×) and water (3×). The organic layer was dried over sodium sulfate, filtered, and concentrated *in vacuo*. The product was purified by MPLC on silica gel (21- × 300-mm column eluted with 25% ethyl acetate in hexane) to afford 255 mg of the title compound as a colorless oil: ^{1}H-NMR (200 MHz, CD_3OD) δ 7.6–7.1 (m, 10H), 4.57 (m, 1H), 3.2–3.05 (m, 2H), 2.59 (m, 2H), 1.8–1.6 (m, 7H), 1.44 (s, 9H), 1.25 (d, 3H, J = 6.95 Hz), 0.96 (t, 6H, J = 5.99 Hz).

N-[1(R)-Carboxyethyl]-α-(S)-(3-phenylpropyl)glycyl-L-leucine, N-Phenylamide Trifluoroacetate (81b). N-[1(R)-(*tert*-Butyloxycarbonyl)ethyl]-α-(S)-(3-phenylpropyl)glycyl-L-leucine, N-phenylamide (250 mg) was dissolved in 6 mL of trifluoroacetic acid and stirred for 4 h. The mixture was concentrated *in vacuo*, diluted with toluene and methanol, and concentrated *in vacuo* to afford **81b** as a colorless foam: ^{1}H-NMR (200 MHz, CD_3OD) δ 7.6–7.1 (m, 10H), 4.64 (m, 1H), 4.1–3.8 (m, 2H), 2.62 (m, 2H), 2.0–1.4 (m, 7H), 1.56 (d, 3H, J = 6.95 Hz), 1.00 (d, 6H).

Method C. N-[1(R)-(Benzyloxycarbonyl)ethyl]-α-(S)-(2-phenylethyl)glycine, *tert*-Butyl Ester. To a solution of benzyl (S)-lactate (3.75 g, 20.8 mmol) in dry methylene chloride (75 mL) cooled to 0 °C was added trifluoromethanesulfonic anhydride (3.75 mL, 22.3 mmol) dropwise over 5 min with stirring under an inert atmosphere. After 5 min at 0 °C, 2,6-lutidine (2.77 mL, 23.8 mmol) was added in one portion. After stirring for 10 min, at 0 °C, N,N-diisopropylethylamine (4.0 mL, 23.0 mmol) was added, followed immediately by a solution of L-homophenylalanine, *tert*-butyl ester (4.97 g, 21.1 mmol) in methylene chloride (40 mL) dropwise over 15 min with stirring. The cooling bath was then removed, and the mixture was stirred for 24 h at room temperature. The mixture was diluted with methylene chloride (150 mL) which was successively washed with water, saturated aqueous sodium bicarbonate solution, and saturated salt solution. After drying over anhydrous magnesium sulfate, the solvent was removed *in vacuo*. Purification was achieved by means of flash column chromatography on silica gel eluted with 5–10% ethyl acetate in hexane. N-[1(R)-(Benzyloxycarbonyl)ethyl]-α-(S)-(2-phenylethyl)glycine, *tert*-butyl ester was obtained as an oil in 83% yield (6.90 g) and converted to **79a** by the methodology described in method A.

Inhibition Assays. Materials. All reagents and buffer salts were from Sigma Chemical Co., St. Louis, MO, and of the highest purity available. Trypsin and soybean trypsin inhibitor bound to agarose were from Sigma. All substrates were prepared by Bachem, Philadelphia, PA.

Chromatographic Conditions. The chromatographic apparatus was described elsewhere.[1] Separations were carried out on a Whatman RAC II C8 column using isocratic elution. The mobile phase consisted of an aqueous solution of 0.1% trifluoroacetic acid and acetonitrile at a flow rate of 1.3 mL/min. The mobile phase compositions were as follows: stromelysin, 33% acetonitrile; collagenase, 25% acetonitrile; gelatinase, 25% acetonitrile. Peak detection was by either UV absorbance at 215 nm or fluorescence (λ_{exit} = 280 nm, λ_{emit} = 343 nm).

Inhibition of Human Fibroblast Stromelysin. Activation. Human recombinant stromelysin was purchased from Celltech (Slough, U.K.) as a proenzyme of 55 kD in a buffer consisting of 20 mM Tris, 10 mM $CaCl_2$, 0.05% Brij-35, and 0.2% NaN_3, pH = 7.5. The material was activated as described.[65] Briefly, to 1.0 mL of a 2.2 μM solution of prostromelysin was added 20 μL of a 1.0 μM solution of trypsin in assay buffer (20 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35, pH = 7.5, $[trypsin]_{final}$ = 20 nM). The solution was incubated at 37 °C for 30 min. The reaction was quenched by addition of a 50-fold molar excess of soybean trypsin inhibitor bound to agarose (Sigma), and the solution was centrifuged to remove the trypsin:inhibitor complex.

IC_{50} Determinations. For each inhibitor concentration, to 100 μL of a 525 μM solution of substance P Nle[11] (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle, cleavage at the Gln–Phe bond, K_m = 0.5 mM) in assay buffer was added 50 μL of inhibitor in assay buffer containing 5% DMSO. Next, 25 μL of a 0.25 μM solution of trypsin activated stromelysin was added, and the reactions were allowed to proceed for 4 h. In the reaction mixture [S] = 300 μM, [Sln] = 35 nM. The reactions were terminated by the addition of 100 μL of 0.15 M phosphoric acid, and 100 μL of the reaction mixture was injected onto the HPLC. The peak areas corresponding to the hydrolysis product, SP-Nle$^{7-11}$ (Gln-Gln-Phe-Phe-Gly-Leu-Nle) were calculated using a Nelson Turbochrome Data Station (Cupertino, CA). The ratio of the peak area for the inhibited sample divided by the control sample (no inhibitor) was plotted as a function of the inhibitor concentration. The IC_{50} was calculated from a least squares fit of the data to the following equation:

$$\frac{\text{area}_{\text{inhib}}}{\text{area}_{\text{control}}} = \frac{[\text{I}]}{[\text{I}] + \text{IC}_{50}}$$

K_i Determinations. Stock solutions of inhibitors were prepared by dissolving the compounds in DMSO. The inhibitors were further diluted in assay buffer to eight different concentrations encompassing the approximate K_i, and covering a 200-fold range. To 50 μL of each of the inhibitor solutions was added 25 μL of an 8 nM solution of trypsin-activated stromelysin, [DMSO] = 1.8%. The solution was allowed to incubate for 4 h to each equilibrium. To this solution was added 60 μL of a 12.8 μM solution of the substrate Arg-Pro-Lys-Pro-Leu-Ala-Phe-Trp-NH_2 (k_c/K_m = 12 000 M^{-1} s^{-1}),[67] and the reaction was allowed to proceed for 18 h. In the reaction [S] = 5.7 μM and [E] = 1.5 nM. The reaction was quenched by addition of 50 μL of 0.15 M phosphoric acid, and 100 μL of the reaction mixture was injected onto the HPLC. Since the reactions were run under first order conditions, ([S] ≪ K_m, K_m = 0.5 mM), the pseudo-first-order rate constant, k_{obs}, was determined for each of the inhibitor concentrations from the peak area corresponding to unreacted substrate in the inhibited sample, and the peak area for substrate at time = 0:

$$\ln \frac{\text{area}_{\text{inhib}}}{\text{area}_{t=0}} = -k_{\text{obs}}t$$

Values of K_i were determined from the ratio of the rate constants for inhibited and control sample (no inhibitor) plotted as a function of the inhibitor concentration and fit to the following equation:

$$\frac{k_{\text{inhib}}}{k_{\text{control}}} = \frac{1}{1 + [\text{I}]/K_i}$$

Inhibition of Human Fibroblast Collagenase. Activation. Human fibroblast collagenase was purchased from Celltech (Slough, U.K.). The material was received as a proenzyme of 54 kD at a concentration of 1.2 μM in a buffer consisting of 20 mM Tris, 5 mM $CaCl_2$, 0.15 M NaCl, and 0.01% NaN_3. The material was activated with trypsin using the same procedure as for stromelysin, with the addition that the activation buffer contained 40 nM prostromelysin.

IC_{50} Determinations. The methods of Weingarten[68] were followed to determine IC_{50} values for inhibition of collagenase.

K_i Determinations. Stock solutions of inhibitors were prepared by dissolving the material in DMSO. The inhibitors were further diluted in assay buffer to eight different concentrations encompassing the approximate K_i and covering a 200-fold range. Final DMSO concentration was 2.8%. To 50 μL of the inhibitor solutions was added 25 μL of a 108 nM solution of trypsin activated collagenase. The solution was allowed to incubated for 4 h to reach equilibrium. To this solution was added 60 μL of a 56 μM solution of the substrate DNP-Pro-Leu-Gly-Leu-Trp-Ala-dArg-NH_2 (k_c/K_m = 270 000 M^{-1} s^{-1}), and the reaction was allowed to proceed for 18 h. In the incubation mixture [S] = 25 μM, [E] = 20 nM. The reaction was quenched by addition of 50 μL of 0.15 M phosphoric acid. The reaction mixture was injected onto the HPLC. The calculation of K_i was the same as for stromelysin.

Inhibition of Human Gelatinase A. Activation. Human 72 kD gelatinase was purchased from Celltech (Slough, U.K.) as a proenzyme at a concentration of 1.5 μM in a buffer consisting of 20 mM Tris, 5 mM $CaCl_2$ 150 mM NaCl, 0.01% Brij, 0.02% NaN_3, pH = 7.5. The proenzyme was activated by incubation of 500 μL of proenzyme with 50 μL of a 11 mM solution aminophenyl mercuric acetate in NaOH (pH = 11) at 25 °C for 120 min.

K_i Determinations. Determination of K_i values for gelatinase A were identical to that of collagenase and stromelysin with the exception that incubation of the enzyme–inhibitor mixture with the substrate was performed for only 2 h. [S] = 25 μM, [E] = 20 nM.

Supplementary Material Available: Complete tabulation of compounds and enzyme inhibition data cited in the text (11 pages). Ordering information is given on any current masthead page.

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(75) As noted above in the Enzymology section, the assays for stromelysin and collagenase were eventually modified to account for the possibility of time-dependent inhibition by these compounds.[66] Given sufficient time, a solution of enzyme and time-dependent inhibitor will reach equilibrium even at low concentrations of enzyme and inhibitor. Once equilibrium is established, total enzyme will be distributed between free enzyme, E, and enzyme:inhibitor complex, E:I. The relative concentrations of E and E:I will be dictated by K_i, the dissociation constant for E:I, and $[I]_{total}$. If free enzyme concentration [E] can be determined, its dependence on $[I]_{total}$ can be used to calculate K_i. [E] can be determined by enzyme-activity measurements, but only under conditions where $[S]_{total} \ll K_m$. If $[S]_{total}$ approaches or exceeds K_m, then the addition of substrate at this high concentration would perturb the equilibrium between enzyme and inhibitor. In such a case, the dependence of enzyme activity on $[I]_{total}$ will not reflect the dependence of [E] on $[I]_{total}$, and thus, the IC_{50} extracted from this dependence will not have a clear relationship to K_i. In an enzyme assay in which $[S]_{total} \ll K_m$, progress curves for loss of substrate are first-order and governed by the pseudo-first-order rate constant, k, where

$$k = (k_c/K_m)/[E]_{total} \quad (1)$$

In the presence of inhibitor, a corresponding rate constant, k_i, can be defined as

$$k_i = \frac{k_c}{K_m(1 + [I]/K_i)}[E]_{total} \quad (2)$$

The ratio of these rate constants provides eq 3:

$$\frac{k_i}{k} = \frac{K_i}{K_i + [I]_{total}} \quad (3)$$

Nonlinear least-squares regression analysis of the dependence of k_i/k on $[I]_{total}$ according to eq 3 will yield values for K_i. Ideally, to obtain values of k and k_i, complete progress curves for substrate loss would be needed and fit to eqs 4 and 5:

$$[S]_t = [S]_{total}e^{-kt} \quad (4)$$

$$[S]_{t,i} = [S]_{total,i}e^{-k_i t} \quad (5)$$

where $[S]_t$ and $[S]_{t,i}$ are the substrate concentrations at a time, t, in the absence and presence of inhibitor, respectively. Rearrangement of these equations and solving for k and k_i yields

$$k = (-\ln([S]_t/[S]_{total}))/t \quad (6)$$

$$k_i = (-\ln([S]_{t,i}/[S]_{total}))/t \quad (7)$$

The following relationship then holds:

$$\frac{(-\ln([S]_{t,i}/[S]_{total}))}{(-\ln([S]_t/[S]_{total}))} = \frac{k_i}{k} = \frac{K_i}{K_i + [I]_{total}} \quad (8)$$

Thus, a plot of k_i/k vs $[I]_{total}$ will be a simple titration from which K_i can be extracted by nonlinear least-squares fit to eq 8. In cases where $[I]_{total}$ is similar to $[E]_{total}$, Morrison's equation for tight-binding inhibition can be used to fit the data.[76] Note that an assay designed according to these considerations provides a K_i value that reflects the true dissociation of the final, stable E:I complex.
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