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Design and Synthesis of Acidic Dipeptide Hydroxamate Inhibitors of Procollagen *C*-Proteinase

ANNABEL OVENS[1,2], JOHN A. JOULE[2] and KARL E. KADLER[1,*]

[1] Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, Manchester M13 9PT, UK
[2] Department of Chemistry, University of Manchester, Brunswick Street, Manchester M13 9PL, UK

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Abstract: Procollagen *C*-proteinase (PCP) is essential for the cleavage of procollagen to collagen in the extracellular matrix of animals and is, therefore, of major relevance to studies of ectopic deposition of collagen during fibrosis. In this study, we describe the design and synthesis of acidic side chain hydroxamate dipeptide inhibitors of PCP having IC_{50} values in the range 0.1–10 μM that mimic the location of aspartic acid residues in the P1′ and P2′ positions (i.e. immediately *C*-terminal) of the PCP cleavage site in procollagen. Assays of PCP using purified human type I procollagen (a natural substrate of PCP) showed that the structure activity relationship of the inhibitors was improved with a glutamic acid mimic at the P1′ position. The results also showed that the presence of an acidic side chain at the P2′ position was not necessary for PCP inhibition. Marimastat and BB3103, which are highly effective inhibitors of matrix metalloproteinases and ADAMS proteinases, respectively, did not inhibit PCP. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: BMP-1; collagen; fibrosis; matrix

INTRODUCTION

Procollagen *C*-proteinase (PCP) cleaves the *C*-propeptides of fibrillar procollagens [1] to initiate spontaneous self-assembly of collagen fibrils in the extracellular matrix of animal tissues (for review see [2]). The fibrils, that are the principal source of tensile strength of tissues such as tendon, skin and bone, can be millimetres in length. Consequently, excessive or ectopic fibril assembly can have devastating effects, as occurs after repeated injury or insult when the injury becomes encapsulated with fibrosis tissue. When this occurs in vital organs such as the liver, fibrosis can lead to cirrhosis, often with fatal consequences (for review see [3]). The ability of PCP to cleave procollagen during the formation of tough fibrous tissues and in the pathogenesis of fibrosis relies on the enzyme cleaving the *C*-propeptides of procollagen at specific glycine or alanine–aspartic acid residues in the trimeric procollagen molecule. The identification of inhibitors to PCP would have value in studying the structure activity relationship of PCP and in devising novel strategies to combat fibrosis.

PCP activity is exhibited by bone morphogenetic protein (BMP)-1, which is a zinc endopeptidase [4–6] having a metalloproteinase domain homologous to that of astacin [7]. It is a member of the metzincin family of metalloproteinases that contain a common signature, HExxH, which is involved in penta-coordination of a zinc cation in the catalytic site. The requirement of the active site zinc cation for

Abbreviations: *C*, carboxyl; *N*, amino; PAGE, polyacrylamide gel electrophoresis; PCP, procollagen *C*-proteinase; proα chains, the alpha chains of procollagen; *pN*α chains, the alpha chains of *pN*collagen; P1, P2, P3 etc., P1′, P2′, P3′ etc., designate substrate/inhibitor residues amino terminal ('non-primed side') and carboxyl-terminal ('primed side') to the scissile bond, respectively.

* Correspondence to: Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, Stopford Building 2.205, Oxford Road, Manchester M13 9PT, UK; e-mail: karl.kadler@man.ac.uk

catalytic activity has led to the development of hydroxamate inhibitors [8–10], which takes advantage of the high affinity of the hydroxamate group for the active site zinc cation. Inhibitor studies of matrix metalloproteinases (MMPs) have shown that the most effective compounds are peptidyl hydroxamates that mimic the primed (right-hand) side of the scissile bond in the substrate [11,12].

In this study, we show that the MMP inhibitors marimastat and BB3103 are ineffective inhibitors of PCP. We designed a new series of hydroxamate dipeptide inhibitors based on the occurrence of aspartic acid on the primed side of the cleavage site in type I procollagen by PCP. Seven of these compounds are reported here, the most effective of which had an IC_{50} value of 0.1 μM against purified human type I procollagen. The IC_{50} was the concentration of inhibitor needed to inhibit PCP by 50% during the time course of the assay.

EXPERIMENTAL PROCEDURES

Inhibitor Synthesis

4-Pentenoic and 5-hexenoic acids were converted to their acid chlorides using thionyl chloride then the acid chlorides condensed with the sodium salt of the Evans auxiliary 4S-4-benzyloxazolidin-2-one. *C*-Alkylation of each of the products with *t*-butyl bromoacetate by deprotonation using sodium *bis* (trimethylsilyl) amide then cleavage of the auxiliary with aqueous lithium hydroxide and hydrogen peroxide gave 2-*R*-2-*t*-butyloxycarbonylmethylpent-4-enoic and 2-*R*-2-*t*-butyloxycarbonylmethylhexenoic acids, respectively. The alkene acids were converted into 1,1,1-trichloroethyl esters by reaction with 1,1,1-trichloroethanol with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP). Oxidative cleavage of the alkene groups using sodium periodate, in combination with ruthenium (III) chloride was followed by formation of the benzyl esters of the newly formed carboxylic acid groups using benzyl bromide in the presence of potassium carbonate.

Deprotection of the trichlorethyl esters proved troublesome using zinc powder in aqueous acetic acid until sonication was applied when efficient removal was achieved. Coupling with Asp(OBzl)-NHMe, again with the combination of EDC with HOBt was followed by removal of the *T* Butyl ester group using trifluoroacetic acid (TFA) giving 3-benzyloxycarbonyl-2*R*-2-carboxymethylpropanoyl-Asp-(OBzl)-NHMe and 4-benzyloxycarbonyl-2*R*-2- carboxymethylbutanoyl -Asp(OBzl)-NHMe, respectively.

In each of these, the free carboxyl group was converted into the hydroxamic acids function by reaction with *O*-benzylhydroxylamine hydrochloride in the presence of EDC, 1-hydroxybenzotriazole (HOBt) and *N*-methylmorpholine followed by hydrogenolysis of all benzyl groups over Pd-C. All compounds were fully characterized by 300 MHz ^{1}H nuclear magnetic resonance (NMR) spectroscopy and high resolution mass spectrometry.

Purification and Assay of PCP from MG63 Cells

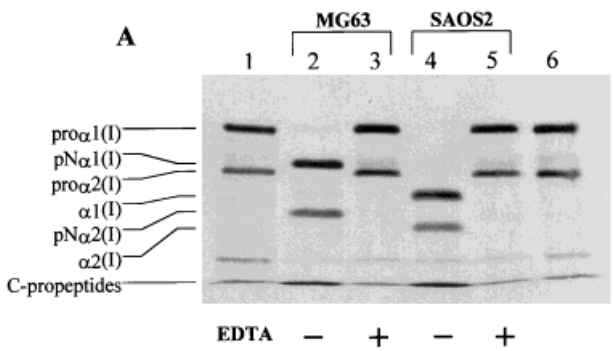
The same protocol which worked well to purify the chick PCP from 17-day chick embryos [13] was also used successfully to purify human PCP from the culture medium of MG63 cells. Approximately 10^4 units of PCP were obtained from 7 l of culture medium. The preparation had 400 units/ml of activity. Uniformly-labelled human ^{14}C-L-type I procollagen was purified as described [14]. In assays of PCP, 1 μg of purified human type I procollagen was incubated with 50 Units of PCP in 50 mM Tris–HCl buffer (pH 7.4) containing 0.12 M NaCl, 5 mM $CaCl_2$ and 0.01% (w/v) Brij-35, at 37 °C. Proteins were separated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and visualized by exposing dried gels to a phosphoimager plate. Cleavage was measured as the percent conversion of procollagen α (proα)-chains to *pN*collagen α (*pN*α)-chains. In inhibitor studies, the inhibitors were dissolved in dimethylsulphoxide (DMSO) and used in assays of PCP at concentrations ranging from 0 to 500 μM. The concentration of DMSO in the assay did not exceed 1% (v/v). Inhibitors were incubated with purified PCP at 37 °C for 0–15 min prior to the addition of type I procollagen. The duration of pre-incubation of inhibitor with PCP did not appear to influence the outcome of the assay.

RESULTS

Purification and Characterization of Human PCP

Initial studies showed that cultured human fibroblasts, MRC5 lung fibroblasts, HT1080 fibrosarcoma cells, C6B5 adrenal fibroblasts, and HOS osteosarcoma cells secrete metalloproteinases with

C-proteinase-like activities. The most abundant and reliable sources of activity were MG63 and $SaOS_2$ cells (Figure 1(A)). Incubation of purified human type I procollagen with the conditioned culture medium of these cells resulted in cleavage of the *C*-propeptides by MG63 medium, and both the *N*- and *C*-propeptides by $SaOS_2$ medium. We wanted to avoid contamination of *C*-proteinase preparations with procollagen *N*-proteinase (PNP), the enzyme that cleaves the *N*-propeptides of procollagen. Thus, *C*-proteinase was purified from the medium of cultured MG63 cells. To characterize the human enzyme, purified human and chick type I procollagens were incubated separately with the purified *C*-proteinase and the reaction products were examined on SDS-polyacrylamide gels under reducing conditions. The cleaved *C*-propeptides were transferred to PVDF membrane and partially sequenced, separately, at their *N*-termini. The sequences corresponded precisely to the first ten



B

C1	Hu	--DDANVMRARA
	Ch	--DDANVMRDRD
C2	Hu	--DQPNVMR
	Ch	--DQPSLRPKRY

Figure 1 Characterization of PCP from MG63 cells. (A) 1 μg of human type I procollagen was incubated with 100 μl MG63 or $SaOS_2$ conditioned medium at 37 °C for 8 h. Lane 1, normal procollagen; Lane 2, procollagen incubated with MG63 medium; Lane 3, procollagen incubated with MG63 medium in the presence of 25 mM EDTA; Lane 4, procollagen incubated with $SaOS_2$ medium; Lane 5, procollagen incubated with $SaOS_2$ in the presence of 25 mM EDTA; Lane 6, procollagen incubated at 37 °C alone. (B) *N*-terminal amino acid sequences of cleaved *C*-propeptides. Purified human and chick type I procollagen was incubated with purified MG63 PCP and the cleaved *C*-propeptides were analysed by *N*-terminal sequencing. C1, *C*-propeptides of proα1(I) chains. C2, *C*-propeptides of proα2(I) chains. Hu, human; Ch, chick. The data show that the enzyme cleaves at the expected site for a PCP.

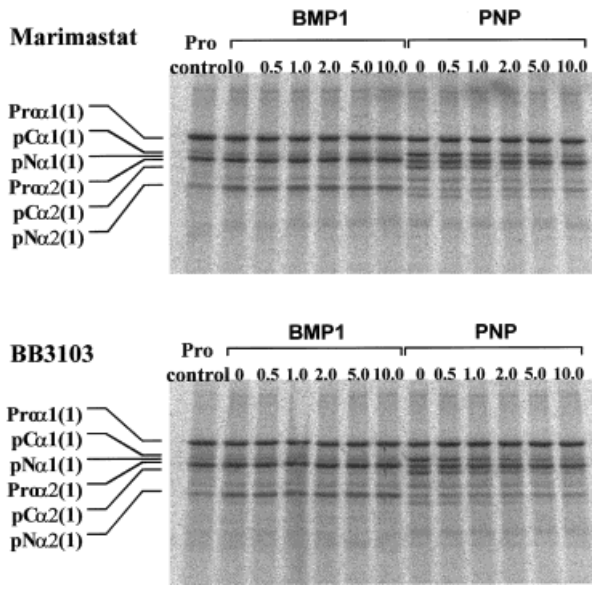


Figure 2 Marimastat and BB3103 do not inhibit PCP *in vitro*. Human type I ^{14}C-U-L-procollagen (12 μg/ml) was incubated with purified PCP (50 Units/ml) or PNP (50 Units/ml) in 50 mM Tris–HCl buffer (pH 7.4 at 20°C) containing 5 mM $CaCl_2$, 0.12 M NaCl, 0.01% Brij-35 and 0.01% NaN_3 at 37 °C for 30 min. Samples were examined by SDS-PAGE under reducing conditions and the procollagen cleavage products detected using a phosphorimager. Top panel, marimastat was added to the assays at the concentrations shown (μM). Pro control, procollagen incubated in the absence of enzymes. Bottom panel, BB3103 was added to the assays at the concentrations shown (μM). Pro control, procollagen incubated in the absence of enzymes.

residues of each the α1(I) and α2(I) *C*-propeptides (Figure 1(B)). The purified human enzyme was similar to the chick PCP in that it was inhibited 100% by 100 mM ethylenediamine-tetraacetic acid (EDTA), 100% by 3 mM *o*-phenanthroline, 4% by phenylmethylsulphonyl fluoride (PMSF) and was not inhibited by soybean trypsin inhibitor or by *N*-ethylmaleimide.

Identification of a Consensus Cleavage Site in Fibrillar Procollagens

Initial experiments showed that the hydroxamate compounds marimastat and BB3103 (which are effective inhibitors of MMPs) were ineffective against the human *C*-proteinase (Figure 2). Although the results of these experiments were of no surprise to us, they encouraged us to make hydroxamate compounds that closely mimicked the cleavage site in procollagen. Marimastat and BB3103 were weak inhibitors of the PNP.

We prepared a multiple sequence alignment of cleavage sites in fibrillar procollagens cleaved by PCP (Figure 3). The alignment shows an aspartic acid in the P1′ position in type I, II and III procollagen, which is consistent with the known cleavage site in these substrates, and either an aspartic acid, glutamic acid or glutamine residue in the P2′ position. The alignment suggested that the consensus cleavage site for PCP of procollagens is **M/Y-R-A/G-↓-D-D/E/Q-A/P**, in which the aspartic acid in the P1′ position (underlined) is conserved.

Inhibition of PCP by Acidic Side Chain Dipeptide Hydroxamic Acids

Figure 4 shows the compounds synthesized. The numbered code is arbitrary. The compounds were examined in assays of PCP, using purified human ^{14}C-labelled type I procollagen as the substrate, and the results examined by SDS-PAGE and quantitative phosphorimaging (Table 1). A typical gel is shown in Figure 5. One of the first compounds we synthesized was the di-aspartic acid peptide, compound 27, and a dipeptide glutamic acid-aspartic acid (not shown). These compounds were not inhibitory at a concentration of 500 μM. However, the hydroxamate derivative of di-aspartic acid (compound 37) inhibited the PCP with an IC_{50} of 10 μM. Clearly, the replacement of the *N*-terminal amino group with a hydroxamate group greatly increased the efficacy of the compound in inhibiting PCP. We wanted to know the contribution of the $-CH_2-$ group that linked the hydroxamate to the backbone of the compound. We chose to synthesize compound 28, in which the $-CH_2-$ that linked the zinc-binding hydroxamate group to the compound was replaced with –NH–. This compound was only moderately effective at inhibiting PCP (having an IC_{50} of 100 μM), presumably because the addition of a nitrogen group decreased the efficacy of the hydroxamate group to bind to the catalytic zinc in the active site of PCP.

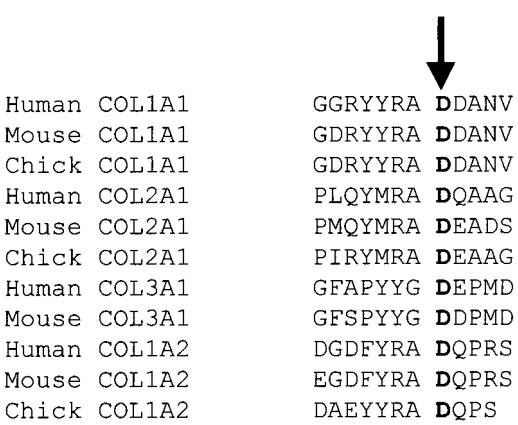


Figure 3 Sequence alignment of fibrillar procollagens in the telopeptide region. The initial alignment was performed with Multalin using the BLOSUM62 matrix and default parameters. It was then adjusted by hand to align the putative cleavage sites. P3-P2-P1-P1′-P2′-P3′ residues are shown in bold face. HUMAN COL1A1, human type I procollagen α1; MOUSE COL1A1, mouse type I procollagen α1; CHICK COL1A1, chick type I procollagen α1; HUMAN COL2A1, human type II procollagen α1; MOUSE COL2A1, mouse type II procollagen α1; CHICK COL2A1, chick type II procollagen α1; HUMAN COL3A1, human type III procollagen α1; MOUSE COL3A1, mouse type III procollagen α1; HUMAN COL1A2, human type I procollagen α2; MOUSE COL1A2, mouse type I procollagen α2; CHICK COL1A2, chick type I procollagen α2. In bold is the aspartic acid in the P1′ position of the cleavage site by PCP.

These results indicated that the presence of a hydroxamate group at the 'left-hand end' of the molecule (the amino end) and the presence of an aspartic acid residue in the P1′ position, were a good combination to produce an effective inhibitor of PCP. In the next series of experiments, we tested the relative contributions of the P1′ and P2′ positions to the efficacy of the compounds. To investigate the importance of the P1′ position, we generated a daughter of compound 37 having a glutamic acid side chain at the P1′ position. This compound, number 38, was a more potent inhibitor of PCP with an IC_{50} of 0.1 μM. We tested the possibility that compounds containing even larger side chains at P1′ might be better inhibitors of PCP. Compound 49 had an IC_{50} of 1 μM and was intermediate in potency between compound 37 and compound 38. This showed that the optimal mimic of the side chain at P1′ was glutamic acid. Using compound 49 as a reference molecule, we synthesized compound 58 in which the proton of the hydroxamate group was made a methyl group. This compound was not effective at inhibiting PCP at an inhibitor concentration of 500 mM. Presumably, the small size and precise electrophilic properties of the hydroxamate group are essential determinates of zinc binding.

In a final experiment, we examined the requirement of an acidic side chain at position P2′. The resultant compound, number 53, contained an alanyl group in place of an aspartic acid. This new compound had an IC_{50} of 10 μM, showing that an acidic side chain at P2′ was not necessary for PCP inhibitor activity.

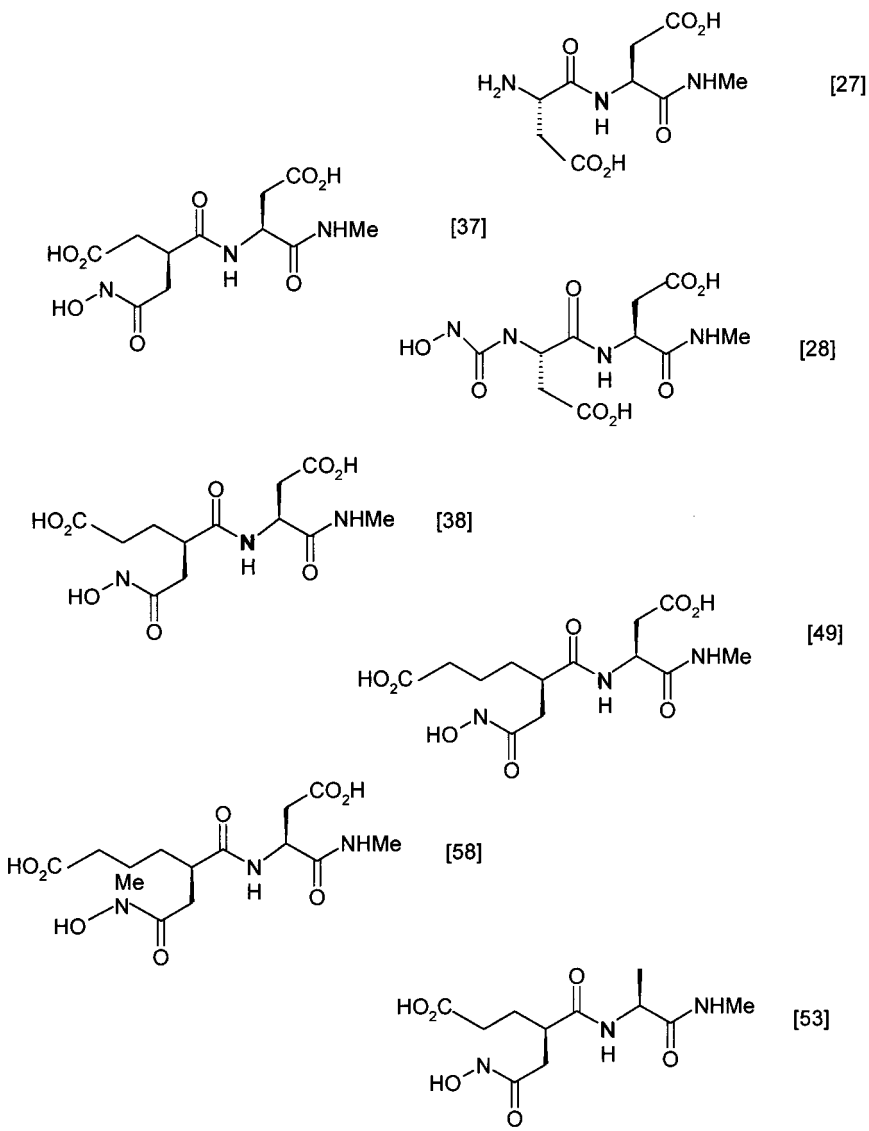


Figure 4 Chemical structures of the compounds synthesized.

DISCUSSION

Cleavage of the *C*-propeptides of procollagen by PCP is both necessary and sufficient to initiate the self-assembly of collagen fibrils in the extracellular matrix of animal tissues. For this reason, PCP is a prime target for anti-fibrotic research aimed at controlling the excess and ectopic deposition of collagen that occurs during a fibrotic injury response. PCP is identical to BMP-1, which is part of an extended family of metzincin proteinases that includes the MMPs. Extensive study has shown that MMPs are inhibited by peptidomimetics that both resemble the cleavage site of MMPs in the triple

Table 1

Compound number	IC_{50} (μM)
[27]	†
[28]	10^{-4}
[37]	10^{-5}
[38]	10^{-7}
[49]	10^{-6}
[53]	10^{-5}
[58]	†

† Does not inhibit cleavage of type I procollagen by PCP when incubated for 3 h at 37 °C at 300 μM.

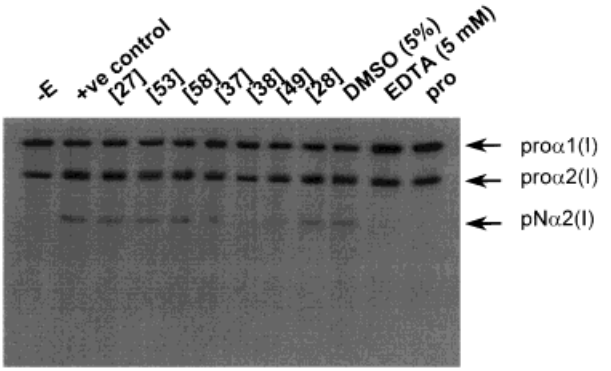


Figure 5 Assay of PCP inhibitors. Human type I ^{14}C-U-L-procollagen (12 μg/ml) was incubated with purified PCP (50 Units/ml) in 50 mM Tris-HCl buffer (pH 7.4 at 20 °C) containing 5 mM $CaCl_2$, 0.12 M NaCl, 0.01% Brij-35 and 0.01% NaN_3 at 37 °C for 30 min. Samples were examined by SDS-PAGE under reducing conditions and the procollagen cleavage products detected using a phosphorimager. PCP inhibitors were included in the assay at a concentration of 300 μM, as indicated with the code number above the lanes. −E, procollagen incubated for the duration of the experiment in the absence of PCP; +ve control, procollagen incubated with PCP; DMSO, procollagen and PCP incubated in the presence of 5% DMSO; EDTA, procollagen and PCP incubated in the presence of 5 mM EDTA; pro, procollagen prepared directly for SDS-PAGE.

helical domain of collagen and which contain a zinc-binding domain that strongly binds the catalytic zinc ion in the active site of the MMPs. The most potent inhibitors are 'right-hand side' inhibitors that mimic the amino acid sequence immediately carboxyl of the scissile bond and which have a hydroxamate group as the zinc-binding domain.

In this study we have shown that PCP can be inhibited by right-hand side hydroxamic acid peptidomimetics with aspartic acid residue mimics at the P1′ and P2′ positions. Our initial targets for potential collagenase *C*-proteinase inhibitors were based around compounds found to be effective at inhibiting MMPs. These included the anti-cancer drugs batimastat (BB-94) and marimastat (BB-2516) which were developed by British Biotechnology to combat tumour metastasis. Batimastat and marimastat are broad-spectrum MMP inhibitors. Batimastat is active against interstitial collagenase (IC_{50} 3 nM), stromelysin-1 (IC_{50} 20 nM), gelatinase-A (IC_{50} 4 nM), gelatinase-B (IC_{50} 4 nM) and matrilysin (IC_{50} 6 nM). This is useful in anticancer treatment where a range of MMPs can be involved and it can be difficult to determine precisely which enzyme should be targeted. A wide range of studies have been carried out using these compounds and have been reviewed [15]. Marimastat has a similar inhibitory spectrum to batimastat but has the advantage of being soluble in aqueous media. Studies in our laboratory showed that marimastat does not inhibit PCP. In general, right-hand side peptidomimetics are more effective than left-hand side peptidomimetics in inhibiting zinc-containing enzymes and are widely represented in the literature. A short peptide length is usually sufficient, mimicking two or three residues of the natural substrate. If the inhibitor mimics three residues the terminal, P_3^1, residue tends to be a simple amide and not an amino acid residue.

The basic structure decided upon for PCP inhibitors was an aspartic acid-aspartic acid dipeptide with an *N*-methyl amide occupying the P3′ position as the amino terminal. The principal zinc-binding functionality that was to be used was the hydroxamic acid. In right-hand side inhibitors this is attached to a modified amino acid residue where the amine has been substituted by a methylene group. There are other zinc-binding functionalities which are attached to the same, modified, peptide including carboxylic and phosphinic acids.

The hydroxamate compounds described here are perhaps the first compounds designed to inhibit PCP. Such inhibitors are a good starting point for the development of pharmaceutical agents for inhibiting excess deposition of collagen in the treatment of fibrosis, and for investigating the structure-function properties of PCP. The hydroxamate selectivity profile of zinc proteinases does not always directly correspond to their natural peptide preference [16]. However, it is clear from crystal structure studies of other metzincins, e.g. the MMPs, that the way in which the hydroxamate chelates to the zinc places the first amino acid in the S1′ pocket. Our studies are consistent with the positioning of the acidic side chain hydroxamates into the active site of PCP.

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