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REVIEW

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Structural studies of matrix metalloproteinases

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Abstract The zinc- and calcium-dependent family of proteins called the matrix metalloproteinases are collectively responsible for the degradation of the extracellular matrix. Members of this family such as the collagenases, stromelysins and the gelatinases are involved in the routine tissue remodelling processes such as wound healing, embryonic growth and angiogenesis. Under normal circumstances the proteolytic activity of these proteins are precisely controlled at the transcriptional level, the production of the proteins in their inactive zymogen forms and also by the co-secretion of endogenous inhibitors. Imbalance between the active enzymes and their natural inhibitors leads to the accelerated destruction of connective tissue associated with the pathology of diseases such as rheumatoid and oesteoarthirtis. The potential for using specific enzyme inhibitors as therapeutic agents to redress this balance has led to intensive research focused on the design, syntheses and molecular structural analyses of low molecular weight inhibitors of this family of proteins. This review describes the essential structural principles and molecular interactions implicated in the innovation of matrix metalloproteinase inhibitors and discusses the features necessary for the specific inhibition of the collagenases.

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Abbreviations *MMP:* Matrix metalloproteinase · *RHS:* Right-hand side inhibitor · *TIMP:* Tissue inhibitors of metalloprotease

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent enzymes which are collectively responsible for remodelling of connective tissue [1, 2]. This group of homologous proteins, which include the collagenases, stromelysins and the gelatinases, possess distinctive substrate specificity directed towards extracellular matrix macromolecules [3, 4, 5]. MMPs are regulated at the transcriptional level by cytokines and growth factors which stimulate the syntheses and secretion of pro-MMPs and also their endogenous inhibitors tissue inhibitors of metalloproteases (TIMPs) [6, 7, 8]. Functionally competent forms of these modular proteins are released via the "cysteine switch" mechanism, whereby a protein residue of the latency domain is dissociated from the essential zinc of the catalytic module, through the proteolytic cleavage of loops necessary for propeptide stability [9, 10]. Excess MMP activation and expression have been implicated in the accelerated breakdown of connective tissue seen in pathological conditions such as arthritis, tumour metastasis, multiple sclerosis and Alzheimer's disease [11, 12, 13, 14], making MMPs viable targets for drug design. The progress in the discovery of clinically suitable low molecular weight inhibitors the collagenases, in the context of the molecular structural analyses of MMP-inhibitor complexes, is the subject of this review.

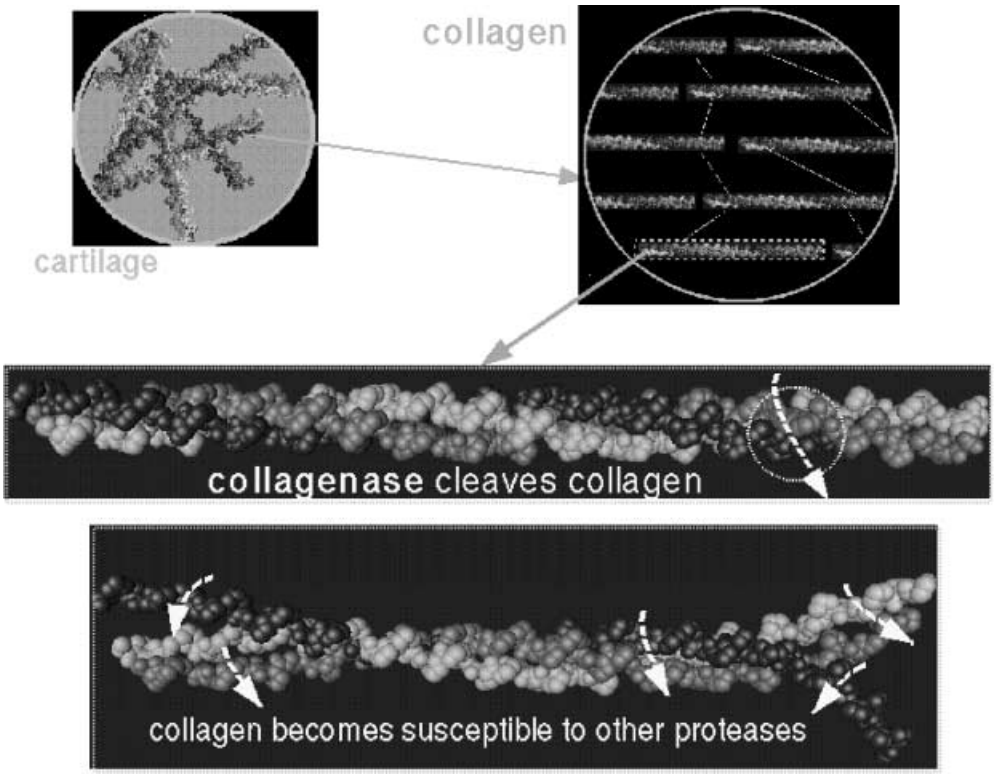

Fig. 1 Cartilage, collagen, collagenase and collagen breakdown. See text for details

Unique property of collagenase

Cartilage is a highly specialised connective tissue made up of two extracellular matrix components, collagen and proteoglycan [15, 16, 17, 18]. The biomechanical properties of cartilage depends on the integrity of collagen and the solvated proteoglycan molecules which are trapped in the collagen network. Loss of proteoglycan precedes the breakdown of the collagen matrix, the former being readily replaced. In contrast, damage to the collagen fibrillar network is irreversible. Collagen, which gives connective tissue its strength and rigidity, is resistant to proteases due to its unique structure. Three polypeptide chains of collagen supercoil around a common axis to form a right-hand triple helix, resulting in a fold that is supremely resistant to proteolytic attack. Collagenase is the only protein which is able to cleave all three chains of triple helical collagen at a specific locus, producing three-quarter and one-quarter fragments of the native molecule [19]. At physiological temperatures these products spontaneously denature and become susceptible to other proteases, ultimately causing the complete degradation of fibrillar collagen (Fig. 1). The crucial step in this irreversible process leading to cartilage destruction is the initial specific cleavage of the triple helix by collagenase. In diseases such as osteoarthritis and rheumatoid arthritis, cartilage damage due to excessive uncontrolled collagen degradation prevents joints from functioning normally [6, 15, 20, 21, 22, 23]. The modulation of collagenase activity using synthetic chemical moieties with appropriate selectivity and pharmacokinetics could therefore provide a new form of therapy for these degenerative diseases.

Characteristics of the catalytic domain

On the basis of substrate specificity and primary sequence similarities, three MMPs, namely MMP-1 (fibroblast collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3), belong to the collagenase family. Although all three collagenases cleave fibrillar collagen, the proteins have different affinities towards types I, II and III collagen [19]. For instance, while MMP-8 preferentially cleaves type I collagen, fibroblast collagenase (MMP-1) shows a 15-fold preference towards type II collagen. Further MMP-8 is stored within specific granules, and MMP-1 and MMP-3 are constitutively produced under the action of cytokines and inflammatory mediators [5, 6]. The differences in pattern of expression and substrate partiality between the collagenases allows these proteins to act on the same matrix component (collagen) at different times and locations during connective tissue turnover.

Primary sequences of the collagenases show that the proteins are homologous and modular, each possessing a signal peptide of 20 residues, a propeptide domain (80 amino acids) conferring latency, a zinc-containing catalytic domain of around 180 residues and a substrate specificity domain (210 amino acids) [3, 4, 24]. The enzymatic activity and inhibitor profile of the recombinant catalytic domain of fibroblast collagenase [25] is similar

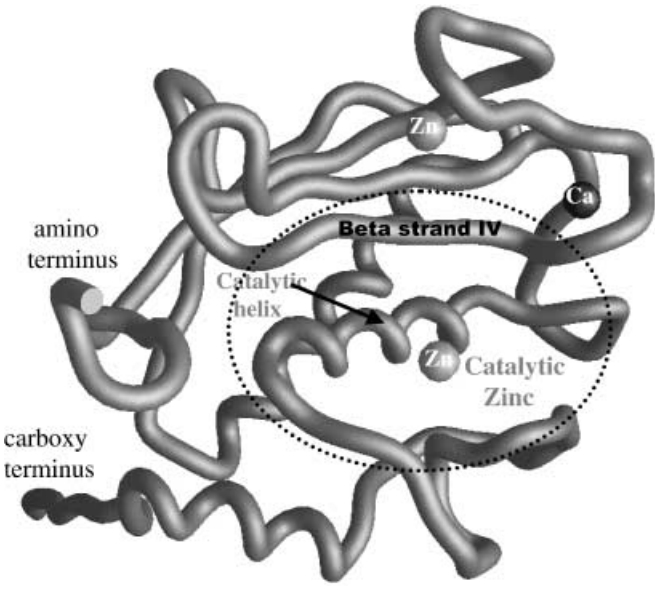

Fig. 2 The catalytic domain of ligand-free fibroblast collagenase, MMP-1, as observed in a solution study [33], shown in a simplified form. *Dark spheres* Calcium atoms; *lighter spheres* zinc atoms. The area shown in Figs. 3, 4, 5, 6, and 7, together with the labels of the secondary structural elements, is also identified. (From [63]

to that of corresponding full-length proteins so that the truncated domain is a suitable surrogate for the structure based inhibitor design of MMP-1.

The X-ray structures of the catalytic fragment of inhibited fibroblast [25, 26, 27, 28, 29] and neutrophil [30, 31, 32] collagenase and the solution study of ligand-free fibroblast collagenase [33] have shown that the three-dimensional scaffold consists of a five-stranded β-sheet and three helices (Fig. 2), similar to the architecture of all zinc-dependent endopeptidases [34]. A deep cleft around the catalytic zinc located towards the C-terminus of the domain signifying the active site and substrate binding region dominates the fold. Three histidine residues of the protein and atoms of the bound inhibitor chelate the catalytic zinc (Fig. 3). Additionally, there is a second, structural, zinc and several calcium atoms which stabilise the structure (Fig. 2). The solution study underscores the flexibility of the active site of the MMP-1, suggesting that enzyme inhibition is achieved by stabilising the mobile active site. The X-ray structure of activated full-length MMP-1 [46] reveals that the architecture of the active site observed for the isolated catalytic domain is maintained in the context of the full-length protein.

Protein-inhibitor interactions

Physiological inhibition of collagenase by pro-peptide (during latency) and by the endogenous inhibitors TIMPs after activation occurs through the interaction of inhibitor atoms with the catalytic zinc [10, 35, 36]. The binding mode of low molecular weight peptidic inhibitors of collagenase (Fig. 3) is similar to the inhibition of MMPs by TIMP in that the direction of the peptide chain in both cases spans the catalytic site in the same direction as the substrate [24]. Molecular structural studies on collagenase [25, 26, 27, 28, 29, 30, 31, 32] have confirmed the biochemical findings [37] that six subsites, three on either side of the cleavage site, are mandatory for the proteolytic activity of the protein. Synthetic inhibitors, which mimic subsites C-terminal to the scissile bond (Fig. 3) bind to collagenase in an extended conformation, with metal chelating moieties binding to the catalytic zinc. A deep, mainly hydrophobic specificity sub-

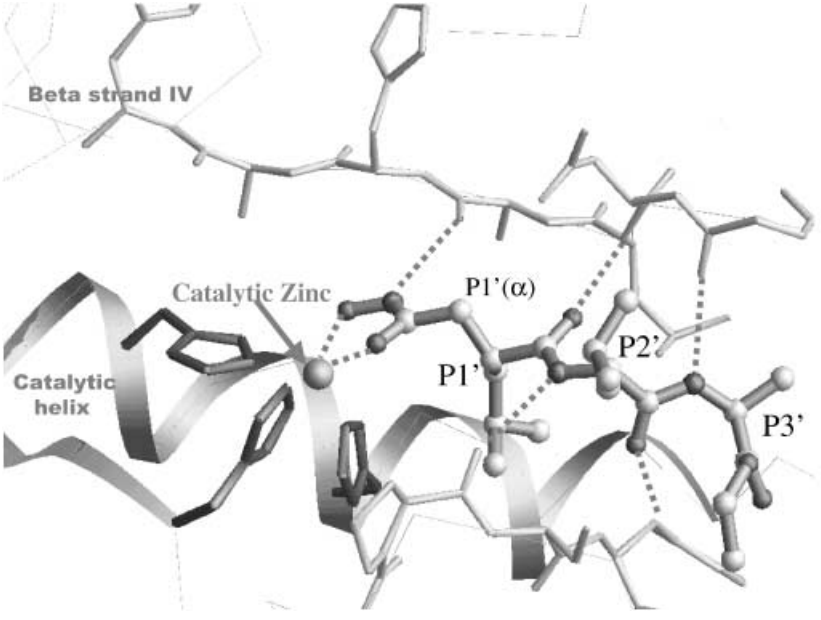

Fig. 3 The typical mode of binding of potent inhibitors to matrix metalloproteinases. The figure illustrates the interaction of the peptidic inhibitor (Ro 32-4724 [25], ball-and-stick representation) at the active site of fibroblast collagenase (area identified in Fig. 2). The zinc-chelating histidines and the enzyme subsites are marked. The catalytic helix is shown in a ribbon representation. *Dotted lines* Potential hydrogen bonds. (From [64])

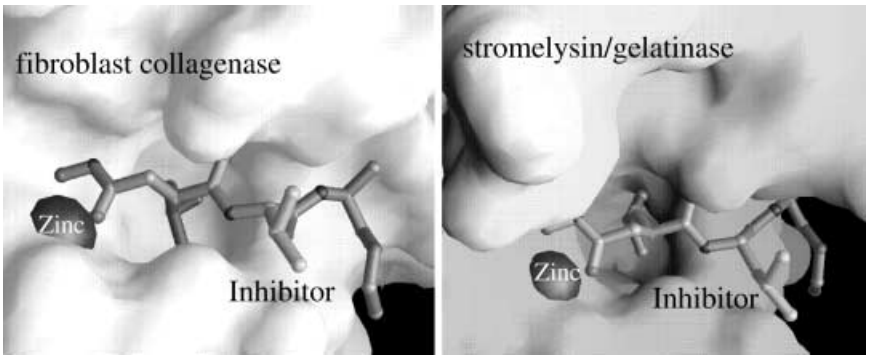

Fig. 4 Comparison of the specificity subsites of the MMPs. Surface representation around the P1′ pocket of MMP-1 and MMP-3 illustrat}es the differences in topology in this region the proteins. *Dark sphere* The catalytic zinc atom; *ball-and-stick* a peptidic inhibitor. (From [63])

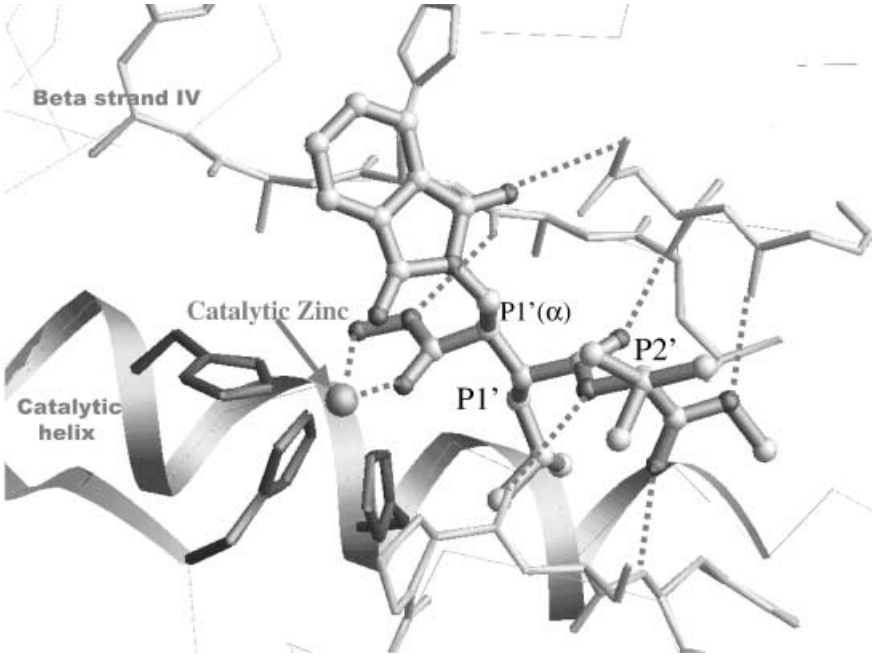

Fig. 5 The binding of a synthetic inhibitor to fibroblast collagenase [24]. The interaction of the P1(α) substituent with a side chain of the protein is specific for fibroblast collagenase. Other identifiers are the same as given for Fig. 3

site (P1′, Fig. 3), proximal to the zinc chelating group is the predominant feature of the protein. This site is well defined and towards the body of the enzyme, so that changes in this location of the inhibitor effect protein selectivity. The orientation of the P2′ subsite (Fig. 3, Fig. 4) is directed towards solvent, allowing a wide range of substitutions to be tolerated in this position [20, 38, 39]. Since there is minimal protein-inhibitor contact at this subsite, modifications at the P2′ location of the inhibitor can be effective in manipulating the physicochemical and pharmacokinetic properties of the inhibitor, while maintaining the affinity of the inhibitor for the protein. Further, as shown in Fig. 3, the region of the inhibitor C-terminal to the P3′ subsite is at the periphery of the catalytic domain of the protein, suggesting that molecules truncated at this location would also have viable activity against the protein (Fig. 5). Similar analyses of X-ray data using inhibitors which mimic residues N-terminal to the cleaved bond [25, 40, 41] failed to reveal other well formed subsites, thus emphasising the importance of the zinc-binding group and the P1′ subsite as the principle recognition site for collagenase. Implementation of these design principles in order to effect new chemical moieties are elaborated below.

Inhibitor design and enzyme selectivity

Zinc binding

The X-ray crystallographic data of enzyme-inhibitor complexes mentioned above allow specific interactions of molecular recognition to be inferred. Inhibitor atoms provide ligands to the catalytic zinc (Fig. 3). The favourable contacts between the zinc atom and the hydroxamate moiety (bidendate zinc chelation) together with the additional interaction of the nitrogen atom of the hydroxamate with a main chain carbonyl atom of the protein contributes to the observed potency of all hydroxamate containing MMP inhibitors [25]. Crystal data [24] have shown that suphur- and phosphorous-containing inhibitors interact at the zinc in a monodentate manner. The

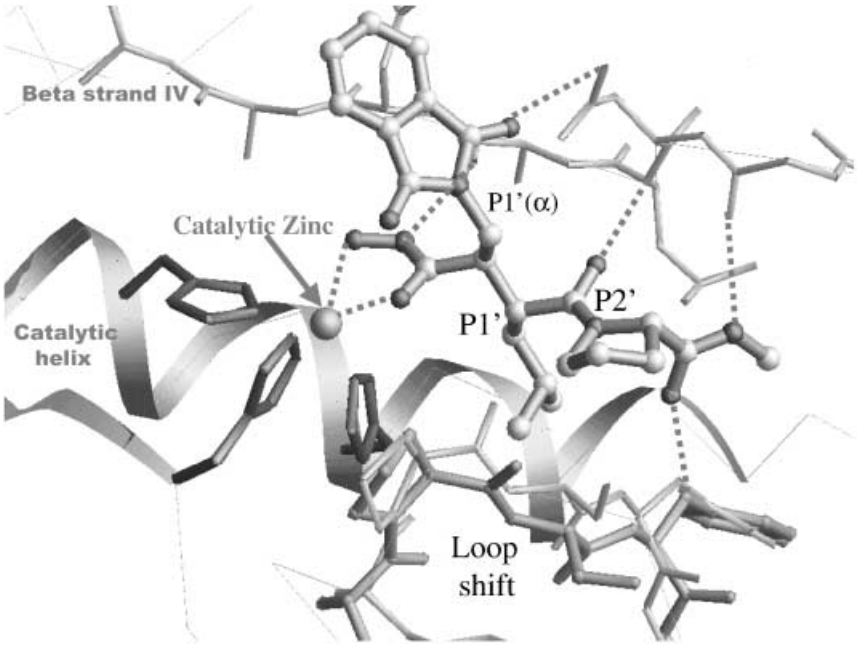

Fig. 6 Conformational change observed on inhibitor binding to fibroblast collagenase. Identifiers are the same as given for Fig. 3. Details discussed in text. (From [34])

carboxylic acids serve as bidentate ligands to the zinc, but are less potent than corresponding hydroxamates due to distorted coordination geometry around the catalytic metal [42, 43]. The zinc-binding group plays a decisive role in achieving inhibitor potency.

Specificity Pocket P1′

Analysis of specificity pocket P1′ of fibroblast collagenase shows that the subsite is largely hydrophobic and is limited in size by a buried arginine (R214) side chain (Fig. 4). In silico optimisation of chemical moieties in the P1′ cavity, suggested that P1′ substituents with increased size and hydrophobicity (Fig. 4) as superior for the MMP-1 specificity subsite.

X-ray data on other MMPs, gelatinase (MMP-2) [10], stromelysin (MMP-3) [41, 42] and matrilysin (MMP-7) [43], show that these proteins have a much deeper P1′ pocket due to substitution at the R214 protein location by smaller amino acids (Fig. 4) [35]. This makes inhibitors with long alkyl chains effective for all MMPs except fibroblast collagenase. Recent X-ray structures [28] have shown that movement of R214 on inhibitor binding can increase the depth of the MMP-1 P1′ pocket. However, the energy penalty necessary for this change is reflected in the compromised potency of long chain P1′ inhibitors of fibroblast collagenase [38].

Subsite P1′(α)

The position spanning the zinc binding group and the P1′ subsite of the peptidic inhibitor [P1′(α) in Fig. 3] does not interact with the protein, and is therefore available for substitution. Due to steric clash with the enzyme the additions in this location prefer to be solvent exposed, similar to the P2′ subsite. Replacement by substituents bearing a suitably spaced imide moiety at this location is favoured by fibroblast collagenase because of an additional interaction between the oxygen atom of the imide group of an asparagine side chain unique to MMP-1 [34, 38] (Fig. 5).

Subsite P2′

Apart from the zinc binding moiety, atoms of the inhibitor on either side of the P2′ subsite interact with protein (Fig. 3). The carbonyl oxygens and amide nitrogens at these inhibitor locations are within hydrogen bonding distance form the main chain counterparts in the enzyme. The inhibitor forms an antiparallel β-strand with respect to the β-sheet of the protein, using several enzyme-inhibitor hydrogen bonds to stabilise the complex. These enzyme interactions are critical for proper substrate recognition and maintenance of latency [35, 39], although, as discussed below, not all the hydrogen bonds are mandatory for effective inhibition by synthetic inhibitors [42]. The solvent exposed shallow location of P2′ is reflected in the lack of binding differences among a wide range of substituents at this site [20, 38].

X-ray analyses of compounds from a series lacking the P2′ amide nitrogen (Fig. 6) confirmed a similar mode of inhibitor binding to the parent series. A movement of the inhibitor binding loop of the protein by around 3A amplifies the P2′/P3′ area such that steric overlap between inhibitior and protein are avoided. Thus the inherent flexibility of the enzyme in its substrate binding region allows structurally diverse series of inhibitors to interact at the same positions [28, 34, 44].

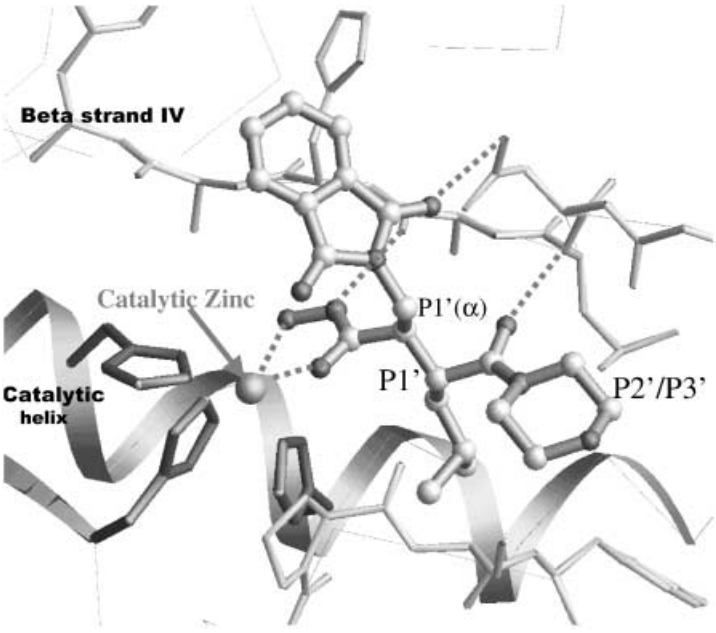

Fig. 7 The interaction of a "truncated" inhibitor to fibroblast collagenase. Identifiers are the same as given for Fig. 3. Details are given in the text. (From [34])

Subsite P3′/P4′

As noted above, the subsite P3′ is at the edge of the active site as defined by the catalytic domain, inhibitor segments in this location turning out into solvent. However, it is possible that in the full-length protein, the substrate binding domain, which is coupled to the catalytic module by a flexible linker [46], is able to influence the binding propensities of the P3′/P4′ subsites.

For all MMPs except the stromelysins there is little gain in inhibitor affinity on changing either P3′ or P4′ [38, 45]. Furthermore, the elimination of terminal methylamide moiety lead to a new "truncated" chemical series with effective collagenase inhibitors. The binding mode of these compounds (Fig. 7) are comparable to the longer inhibitors. Furthermore, their smaller size, enhanced selectivity, improved pharmacokinetic properties and low toxicity have culminated in a member of this series currently being evaluated as a cartilage protective agent for use in the treatment of rheumatoid and osteoarthritis [39, 47].

Additional details of inhibitor binding

Enzyme subsites at the N-terminal side of the scissile bond are shallow and ill defined [30, 48] so that inhibitors binding to this region of the MMPs have relatively modest potency [20]. MMP inhibitors currently undergoing clinical evaluation [49, 50, 51, 52, 53] belong to the more potent C-terminal [also called right-hand side inhibitor (RHS)] binding class of compounds, encompassing the critical subsites discussed above. Competent non-peptidic MMP inhibitors [28, 44, 54, 55], identified through mass screening of compound libraries against metalloprotease targets, also bind to the RHS of the catalytic site. Structural data [24] on a variety of non-peptidic analogues have revealed that these compounds exploit the P1′ subsite and also form hydrogen bonds with the same main chain enzyme atoms as the pseudopeptide inhibitors (Fig. 3) described above.

Conclusion

Since the early 1980s there has been rapid progress in resolving the role of metalloproteinases in tissue degradation and understanding the molecular basis of their action. The pursuit of appropriate synthetic MMP inhibitors for use as drugs to limit connective tissue breakdown has resulted in frenzied activity in the MMP field [49, 50, 51, 52, 52]. The role of individual MMPs in the breakdown of tissues in disease conditions (arthritis, cancer, Alzheimer's disease) has been resolved with various levels of certainty. An orally active, selective inhibitor for the collagenases which has been shown to prevent the degradation of articular cartilage in vitro and in vivo [56, 57, 58] is currently undergoing clinical evaluation. Conventional treatments for arthritis do not affect the underlying joint erosion. The control of collagenase activity as a means of protecting cartilage of arthritic joints presents the possibility for introducing a new class of disease modifying agents. Non-selective MMP inhibitors can interact with other zinc-dependent proteins such as tumour necrosis factor α convertase (a member of the A disintegrin and metalloproteinase family [59]) due to the overall structural similarity in the zinc-binding domain. Such interactions may partially explain the results of recent clinical evaluation of MMP-2 (gelatinase) inhibitors in various cancers [60, 61, 62] which have shown that although these agents are effective for delaying tumour growth, musculoskeletal contraindications of are seen at

higher doses. It is clear that in spite of the advances in the MMP field there are still open questions such as the relative merits of broad versus selective MMP inhibition as the appropriate approach for therapy. As research in this area continues and the MMP family enlarges through the discovery of new members, some answers and perhaps more questions will be forthcoming. In the meantime, the importance of the MMPs and their inhibitors in the biology of disease has already been firmly established.

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