Supplement Review

Engineering of tissue inhibitor of metalloproteinases mutants as potential therapeutics

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http://www.med.ic.ac.uk/divisions/template_divisions_general

Received: 2 April 2002
Accepted: 4 April 2002
Published: 9 May 2002

Arthritis Res 2002, 4 (suppl 3):S51-S61
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(Print ISSN 1465-9905; Online ISSN 1465-9913)

Chapter summary

Matrix metalloproteinases (MMPs) play a central role in many biological processes such as development, morphogenesis and wound healing, but their unbalanced activities are implicated in numerous disease processes such as arthritis, cancer metastasis, atherosclerosis, nephritis and fibrosis. One of the key mechanisms to control MMP activities is inhibition by endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs). This review highlights the structures and inhibition mechanism of TIMPs, the biological activities of TIMPs, the unique properties of TIMP-3, and the altered specificity towards MMPs achieved by mutagenesis. A potential therapeutic use of TIMP variants is discussed.

Keywords: aggrecanase, collagenase, extracellular matrix, matrix metalloproteinases, proteinase inhibitor

Introduction

The extracellular matrix (ECM) holds cells and tissues together, forms organized lattices for cell migration and interaction, and creates correct cellular environments. Timely degradation of the ECM is therefore crucial for controlling cellular behaviour that is required during the development, morphogenesis, and tissue remodelling that are associated with cell differentiation, migration, growth and apoptosis. The major enzymes that are involved in these processes are the members of the MMP family, also called matrixins. Recent studies have also indicated that members of the family called a disintegrin and metalloproteinase (ADAM) also participate.

The activities of these metalloproteinases must therefore be precisely controlled under normal physiological conditions. The disruption of this control results in many diseases, such as arthritis, cancer, atherosclerosis, nephritis, encephalomyelitis, fibrosis, etc., as a consequence of aberrant turnover of the ECM. While the regulation of the activities of ADAM metalloproteinases are less well understood at the present time, the activities of MMPs are controlled by endogenous inhibitors called TIMPs that are synthesized in a variety of tissues and by a plasma protein α₂-macroglobulin and related molecules. α₂-Macroglobulin, a protein of 725,000 Da, inhibits MMPs and most endopeptidases by entrapment of the enzymes, but its action is thought to be primarily in the fluid phase.

In the tissue, TIMPs are considered to be key inhibitors of MMPs. They form 1:1 enzyme–inhibitor complexes. Four TIMPs are currently identified in humans; they are homologous proteins of 21–29 kDa consisting of two domains, an N-terminal inhibitory domain and a C-terminal domain. The C-terminal domain mediates specific interactions with
some MMP zymogens. In particular, the binding of TIMP-2 to progelatinase A (proMMP-2) through their C-terminal domains is critical in proMMP-2 activation on the cell surface by membrane-bound membrane type 1 matrix metalloproteinase (MT1-MMP).

TIMP gene expression is regulated by growth factors and cytokines but their levels of modulation are less than those of MMPs. Therefore, elevated levels of MMPs over those of TIMPs are observed in diseases associated with enhanced proteolysis of the ECM. In addition to the inhibitory actions on MMPs, TIMPs have a number of other biological functions that are not attributed to MMP inhibition.

In general, TIMPs inhibit only the members of the MMP family, but recent studies indicate that TIMP-3 is an exception, since it also inhibits the members of the ADAM family, including tumour-necrosis-factor(TNF)-α-converting enzyme (TACE/ADAM-17) and aggrecanase (ADAM with thrombospondin type I domain [ADAMTS]-4 and ADAMTS-5). This suggests a broader importance for TIMPs, particularly TIMP-3 in regulating extracellular metalloproteinases. Mutagenesis of TIMPs at specific sites has been shown to modulate their specificity for MMPs. This suggests that the expression of TIMP variants directed to specific metalloproteinases in a targeted tissue may be a potential therapeutic.

**Background: TIMPs and arthritis**

Articular cartilage consists of a relatively small number of cells and an abundant ECM. The major components of the ECM are collagen fibrils and aggregating proteoglycan aggrecan. Collagen fibrils, mainly type II collagen together with minor types IX and XI, form a meshwork that provides the tensile strength of the tissue. Aggrecan forms a large aggregated complex interacting with hyaluronan via link proteins and fills the interstitium of the collagen meshwork. Aggrecan provides a hydrated gel that gives cartilage its ability to withstand compression.

In normal cartilage, the turnover and synthesis of ECM macromolecules is at equilibrium, but in rheumatoid arthritis (RA) and osteoarthritis (OA) the loss of ECM components exceeds new synthesis. The primary cause of this imbalance is elevated activity of the proteinase that degrades aggrecan and collagen. Aggrecan loss initially occurs most markedly just beneath the joint surface, which is followed by mechanical failure of the tissue and collagen degradation [1,2].

MMPs are a family of extracellular zinc metalloendopeptidases that function in the turnover of components of the ECM [3,4]. They are produced by many types of cells, but their synthesis is regulated by many factors such as inflammatory cytokines, growth factors, cellular transformation and physical stimuli [3,4].

Certain members of the MMP family have been considered to be the major enzymes that participate in the degradation of aggrecan and collagen in cartilage. Collagenases (MMP-1, MMP-8 and MMP-13), gelatinase A (MMP-2) and gelatinase B (MMP-9), stromelysin 1 (MMP-3), matrilysin 1 (MMP-7) and membrane-type MT1-MMP (MMP-14) are found in cartilage, and most are elevated in the synovium and in the cartilage from patients with RA and OA [5,6].

All of these MMPs cleave the aggrecan core protein at various sites, but the critical site is the Asn341–Phe342 bond located in the interglobular domain located between the two N-terminal globular domains G1 and G2, as this cleavage can release aggrecan molecules from the cartilage [7,8]. The N-terminal fragments with the C-terminal sequence Val-Asp-Ile-Pro-Glu-Asn341 are found in both OA and RA cartilage as well as in normal cartilage [9]. On the contrary, Sandy et al. [10] found that the core protein was cleaved at the Glu373–Ala374 bond, but not at the Asn341–Phe342 bond, when bovine cartilage in culture was stimulated by IL-1. This activity was called ‘aggrecanase’. The products resulting from this cleavage accumulate in the synovial fluids of patients with OA or inflammatory joints [11,12].

Two enzymes responsible for this cleavage have been purified and cloned. They are referred to as aggrecanase 1 and aggrecanase 2 (also ADAMTS-4 and ADAMTS-5, members of the ADAM protein family, respectively) [13,14]. Later, it was also found that ADAMTS-1 has aggrecanase activity [15]. The degradation of type II collagen occurs slower than aggrecan degradations in arthritis. This is all due to the action of MMPs, and potential collagenolytic enzymes are MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14.

MMP activities in the tissue are regulated by endogenous inhibitor TIMPs [16]. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, TIMP-4) are found in humans. They are homologous with each other and consist of two domains, an N-terminal inhibitory domain of about 125 amino acids and a C-terminal domain of about 65 amino acids. Each domain is stabilized by three conserved disulfide bonds. While the N-terminal domains of TIMPs (N-TIMPs) are primarily responsible for the inhibition of MMPs [17], the C-terminal domains can also influence their binding affinity. The balance between the metalloproteinases and their endogenous inhibitors is critical for the appropriate maintenance of tissues.

Early work by Dean et al. [18] showed that both MMP levels and TIMP levels were elevated in OA cartilage compared with unaffected cartilage, but that the total amount of MMP was slightly higher than that of TIMP, whereas this balance was reverse in the unaffected cartilage. This
subtle difference in the ratio of MMPs and TIMPs is considered to be a cause of the gradual degradation of the cartilage matrix.

TIMP-1, TIMP-2 and TIMP-3 are present in the joint tissue. Some elevated levels of TIMP-1 were reported in synovial fluids [19] and in serum [20,21] of RA patients, but not in the serum of OA patients [22]. However, the changes of TIMP-1 levels are not very large compared with the overexpression of MMPs. Overexpression of TIMP-1 using systemic adeno-virus-based gene delivery reduced destruction of the joints of TNF-α transgenic mice [23]. On the contrary, the overexpression of TIMP-1 did not prevent osteochondral injury in the mouse model of collagen-induced arthritis [24]. Since there are differences in specificity among TIMPs, further investigation is clearly needed to elucidate the biological and pathological significance of TIMPs.

**Selectivity of TIMPs**

Important features of the interaction of TIMPs with MMPs are their high binding affinities and differences in specificity despite their high levels of sequence similarity. TIMP-1 inhibits most MMPs with $K_i$ levels of 0.1–2.8 nM [25]. TIMP-1 has a higher affinity for full-length MMP-1 [26] as compared with MMP-1 that lacks the C-terminal hemopexin domain (see the MMP domain structure composition in the chapter by Murphy et al., this issue). The removal of the hemopexin domain from MMPs often results in an approximately 5-fold to 20-fold increase of the $K_i$ value, indicating that the hemopexin domain assists the interaction of TIMP-1 with MMP. Interestingly, Olson et al. [27] reported that the C-terminal hemopexin-domain-deleted MMP-2 does not bind to TIMP-1. However, N-TIMP-1 is an effective inhibitor of full-length MMP-2 with a $K_i$ value comparable with that of MMP-1. Both the hemopexin and the catalytic domains of MMP-2 are therefore necessary for binding to TIMP-1, or the catalytic domain of MMP-2 may have a significantly different structure from that of the corresponding domain in the full-length enzyme. TIMP-1, however, has little inhibitory activity for MT1-MMP [28,29].

TIMP-2, TIMP-3 and TIMP-4 inhibit all MMPs so far tested. TIMP-2 binds to MMP-2 most tightly. Studies by Hutton et al. [30] indicated that binding was via a two-step mechanism, with a $K_i$ value of 1 µM for the initial step and an association rate for the final step of 33 s$^{-1}$. The overall dissociation constant was estimated to be 0.6 fM, essentially irreversible. This tight interaction is largely due to the C-terminal domain of TIMP-2 and the C-terminal hemopexin domain of MMP-2 [31]. Removal of the hemopexin domain increases the dissociation constant to 33 pM. TIMP-3 exhibits a relatively low affinity for MMP-3 with $K_i = 67$ nM, but the affinities towards MMP-1 and MMP-2 are 1.2 and 4.3 nM, respectively [32]. TIMP-4 has similar inhibition constants to TIMP-2 for MMP-2 and MT1-MMP [29].

In addition to the inhibitory activity of TIMPs, some TIMPs bind to the zymogen forms of gelatinases. For example, proMMP-2 binds to TIMP-2, TIMP-3 or TIMP-4 through the C-terminal domain of each molecule [33–35], and proMMP-9 (progelatinase B) binds to TIMP-1 and TIMP-3 through C-terminal domain interaction [35,36]. These complexes are potential inhibitors of MMPs. To activate the proMMP-9 of the proMMP-9–TIMP-1 complex by MMP-3, TIMP-1 must be saturated by MMP-3 or other MMPs [37]. Alternatively, TIMP-1 needs to be inactivated by proteolysis [38]. These mechanisms provide precise regulation of MMP activation and the activities of activated MMPs.

**Importance of TIMP-2 for the activation of proMMP-2 by MT1-MMP**

MT1-MMP was cloned and identified as an activator of proMMP-2 by Sato et al. [39]. This finding is important since proMMP-2 is not readily activated by other tissue proteinases. The activation of proMMP-2 by MT1-MMP, however, requires TIMP-2 [40,41]. In the current model, proMMP-2 secreted from the cell is recruited to the cell surface through the interaction of its C-terminal hemopexin domain and the C-terminal domain of TIMP-2 that is bound to MT1-MMP on the cell surface. The interaction of TIMP-2 and MT1-MMP is via the N-terminal domain of TIMP-2, and therefore the MT1-MMP is inhibited. To activate the cell surface-bound proMMP-2, another molecule of MT1-MMP, free of TIMP-2, needs to be present close to proMMP-2.

The association of two or more molecules of MT1-MMP was recently shown to be through interactions of their hemopexin domains [42]. Disruption of this hemopexin domain association by the overexpression of the MT1-MMP hemopexin domain together with a transmembrane sequence and a cytoplasmic tail prevented proMMP-2 activation. An excess of TIMP-2 also inhibits proMMP-2 activation as it inhibits all MT1-MMP. Activation of MMP-2 and MT1-MMP activity are implicated in tumour cell invasion and neovascularization of endothelial cells [43,44]. This system is therefore likely to be involved in angiogenic processes in rheumatoid synovium.

Itoh et al. [45] have reported that there are two binding modes of TIMP-2 on the cell surface of concanavalin-A-treated fibroblasts: about 50% of TIMP-2 binding is blocked by a peptidyl-hydroxamate inhibitor of MMPs, whereas the other 50% is not blocked by the inhibitor. The former interaction is through MT1-MMP as it is inhibited by a synthetic MMP inhibitor. TIMP-2 bound to the membrane in a hydroxamate inhibitor-insensitive manner specifically inhibits MMP-2 activated on the cell surface but does not inhibit other MMPs, and this inhibitory process is triggered by interaction of the C-terminal domains of the two molecules. This further emphasizes the intricacy of the roles of TIMP-2 in proMMP-2 activation and inhibition.
Unique properties of TIMP-3

Among the four TIMPs, TIMP-3 has a number of unique properties. TIMP-3 was originally found as a 21-kDa protein secreted from chick embryonic fibroblasts transformed with Rous sarcoma virus, but it was strongly bound to the ECM [46]. The protein was later shown to have MMP inhibitory activity [47]. The ECM binding property is due to the interaction of the N-terminal domain of TIMP-3 and the polyanionic components [48]. As well as inhibiting MMPs, TIMP-3 also prevents the shedding of TNF-α receptor [49], L-selectin [50], IL-6 receptor [51] and syndican-1 and syndican-4 [52] from the cell surface.

The enzymes responsible for these activities are yet to be identified, but they are thought to be membrane-bound metalloproteinases belonging to the ADAM family. ADAMs are multidomain proteins consisting of a N-terminal propeptide domain, a metalloproteinase domain, a disintegrin-like domain, an epidermal growth factor-like domain, a transmembrane domain and a cytoplasmic domain. The primary structures of the metalloproteinase domains of ADAMs and MMPs have little sequence similarity except around the catalytic zinc binding motif HEXXHXXGXXH [53]. Indeed, evidence for the unique ability of TIMP-3 to inhibit a member of the ADAM metalloproteinases was first reported for TACE (ADAM-17) [54], and subsequently for ADAMD-10 [55] and ADAM-12 [56]. The apparent $K_i$ value reported against TACE is 182 pM.

Using the N-terminal domain of TIMP-3 expressed in Escherichia coli, Kashwagi et al. [32] have shown that it inhibits two aggrecanases (ADAMTS-4 and ADAMTS-5), a subclass of the ADAM proteinases. The $K_i$ values for ADAMTS-4 and ADAMTS-5 were estimated to be less than 0.5 and 0.1 nM, respectively, whereas the $K_i$ values for MMP-1, MMP-2 and MMP-3 were 1.2, 4.3 and 66.7 nM, respectively. These data suggest that the primary target enzymes of TIMP-3 in cartilage are aggrecanases. TIMP-3 mRNA is expressed in cartilage and skeletal tissue during development of mouse embryo [57], in normal bovine and human articular chondrocytes, and in synoviocytes [58]. The expression of TIMP-3 in chondrocytes in culture is upregulated by transforming growth factor β [59] and by oncostatin M [60]. An antiarthritic agent, calcium pentosan polysulfate, increases the synthesis of TIMP-3 without altering its mRNA levels, and this effect is enhanced in the presence of IL-1 [61]. Elevated TIMP-3 production may be beneficial for the protection of cartilage from degradation not only by preventing the action of aggrecanases and MMPs in cartilage, but also by blocking the release of TNF-α by TACE from synovium.

Another important feature of TIMP-3 is that a point mutation in the C-terminal domain (S156C, G166C, G167C, Y168C or S181C) [62], a splice mutation [63] or a premature termination codon at Glu179 [64] is linked to Sorsby’s fundus dystrophy, an autosomal-dominant inherited macular disorder that causes irreversible loss of vision with onset in the third or fourth decade of life. Choroidal neovascularization is a feature of this disease that closely resembles the events seen in age-related macular degeneration. Qi et al. [65] reported that the S156C mutant expressed in human retinal pigment epithelial cell lines exhibited reduced MMP inhibitory activity and that the conditioned medium had angiogenic activity, suggesting that increased MMP activity may participate in neovascularization in Sorsby’s fundus dystrophy.

Yeow et al. [66] also reported that S156C mutant protein slightly reduced MMP inhibitory activity, but this reduction is not considered significant. Their study showed that mutations (S156C and S181C) produced multiple higher-molecular-weight complexes due to aberrant protein–protein interactions, and increased cell adhesiveness to ECM, suggesting possible effects on normal function and turnover of Bruch’s membrane.

TIMPs are multifunctional proteins

TIMPs have a number of biological activities other than inhibiting MMPs, some of which are not attributed to inhibition of MMPs. When TIMP-1 was first cloned [67], it was found to be identical to a factor that has erythroid potentiating activity [68]. TIMP-1 also has cell growth-promoting activity on human keratinocytes and other cell types [69,70]. Similar cell growth-promoting activity is seen with TIMP-2 [71,72]. On the contrary, the overexpression of TIMP-1, TIMP-2 and TIMP-3 reduces tumour cell growth (see [73] for review). This may be partially due to the inhibition of MMPs.

TIMP-2, but not TIMP-1, inhibits fibroblast-growth-factor-2-induced human endothelial cell growth [74]. TIMP-2 has metanephrinic mesenchymal growth activity and promotes morphogenesis of the ureteric bud by inhibiting its branching and by altering the deposition of basement membrane [75]. The former activity is not due to MMP inhibitory activity, whereas the latter activity is mimicked by a synthetic MMP inhibitor.

The overexpression of TIMP-3 causes apoptotic cell death of a number of cancer cell lines and vascular smooth muscle cells [49,76–78]. Smith et al. [49] suggest that the induction of apoptosis is due to the stabilization of TNF-α receptors, perhaps by inhibiting receptor shedding. Studies by Bond et al. [79] also suggest that the inhibitory activity of TIMP-3 is required for induction of apoptosis. In contrast, TIMP-1 and TIMP-2 suppress the apoptosis of B cells [80] and BB16F10 mouse melanoma cells [81], respectively. Antiapoptotic activity of TIMP-1 is independent of MMP inhibition [80].
Inhibition mechanisms of MMPs by TIMPs

The NMR solution structure of the N-terminal domain of TIMP-2 (N-TIMP-2) revealed a five-stranded β-barrel with a Greek key topology and two α-helices, a structural form known as an OB fold [82]. This category of structure is found in a group of oligonucleotide-binding and oligosaccharide-binding proteins such as staphylococcal nuclease, bacterial entotoxins and some tRNA synthases [83]. This structure did not, however, identify the MMP interaction site in TIMP or clarify its mechanism of inhibition.

The inhibitory site of TIMP-1 was first proposed from a combination of differential proteinase susceptibility studies [84] and site-directed mutagenesis studies [85]. The former studies were based on the observation that human neutrophil elastase inactivated TIMP-1 by cleaving the inhibitor into 10 and 20 kDa fragments. This cleavage by the elastase was, however, prevented when TIMP-1 formed a complex with MMP-3. The full TIMP-1 activity was recovered from the elastase-treated TIMP-1–MMP-3 complex after dissociation of the complex [84].

Sequence analysis of the TIMP-1 fragments indicated that elastase cleaved the Val69–Cys70 bond of the free TIMP-1, suggesting that the MMP interaction site is located near this region. Based on this information and chemical modification studies, a series of mutagenesis studies were carried out with N-TIMP-1. The mutation of Thr2 to alanine resulted in a more than 100-fold decrease in affinity for MMP-3 and in about a 1000-fold decrease for MMP-1 [85]. Mutation of either Cys1 or Cys70, which are disulfide-bonded in native TIMP-1, decreased the affinity for MMP-3 by more than three orders of magnitude. These studies suggest that residues around the disulfide bond between Cys1 and Cys70, which are conserved among TIMPs, are critical for the interaction with MMPs. The NMR structure of N-TIMP-2 indicated that this region forms an exposed ridge structure on the inhibitor molecule [82].

The mechanism by which TIMP inhibits MMPs was revealed by the crystal structure of the complex of human TIMP-1 and the catalytic domain of MMP-3 [MMP-3(∆C)] [86], and of the complex of the bovine TIMP-2 with the catalytic domain of MT1-MMP [87], both determined by Bode and colleagues. The structure of the TIMP-1–MMP-3(∆C) complex shows that TIMP-1 is a ‘wedge-shaped’ molecule, and its edge corresponding to the aforementioned exposed ridge structure inserts into the catalytic site and substrate binding groove of MMP-3 (Fig. 1).

A schematic display of the secondary structure of TIMP-1 is shown in Fig. 2. Most (75%) of the protein–protein contacts in TIMP-1 are from a contiguous region composed of the N-terminal stretch of Cys1 to Val4 and residues Met66 to Val69 linked by the Cys1–Cys70 disulfide bond. The key feature of this interaction is the binding of residues 1–4 of TIMP-1 to the active site of the enzyme in an analogous fashion to the P1-P1′-P2′-P3′ residues of a peptide substrate (the P1 and P1′ residues become the new C-terminus and the new N-terminus, respectively, after hydrolysis), but cleavage does not take place. Residues Ser68 and Val69 fit into the substrate binding sites S2 and S3 in an arrangement that is nearly inverted from that of a substrate. A key feature of this interaction is the bidentate coordination of the catalytic Zn2+ of the enzyme by the α-amino and carbonyl groups of the N-terminal cysteine of TIMP-1 and the projection of the side chain of Thr2 into the S1′ specificity pocket of MMP-3 (Fig. 3a).

This mode of interaction is similar to that of a synthetic hydroxamate inhibitor of MMPs (Fig. 3b). The HO group of Thr2 interacts with Glu202 of MMP-3 and displaces a water molecule from the active site that is essential for hydrolysis of a peptide bond.

On binding to TIMP-1, a large conformational change occurs in the N-terminal region of MMP-3. This change involves the disruption of the salt bridge between the α-amino group of the N-terminal Phe83 and the carboxylate side chain of Asp237, and thus results in a movement of 15 Å by the N-terminal region and in an interaction with...
Met66 of TIMP-1. Other MMP interaction sites are the A–B loop, the E–F loop, and residues Leu133 and Ser134 of the C-terminal domain (see Fig. 2). The structure of the TIMP-2–MT1-MMP complex shows a similar inhibitor–enzyme interaction to that of the TIMP-1–MMP-3 complex.

**Generation of selective TIMP variants**

The interaction of residues 2 of TIMP-1 and TIMP-2 with the S1′ site of an MMP appears to be a conserved feature of the TIMP–MMP interaction. Because of the dominant role of the P1′ residue of a substrate in MMP specificity and because of the differences in size of the S1′ specificity pockets of different MMPs, TIMP variants with chemically different side chains at position 2 may be more selective for different MMPs.

Meng et al. [88] investigated this possibility by substituting position 2 in N-TIMP with 14 different amino acids and measuring the $K_i$ values of variants against MMP-1, MMP-2 and MMP-3. Table 1 shows that residue 2 has a major role in TIMP–MMP recognition. The absence of a side chain (glycine mutant) reduced the affinity for MMPs by three to five orders of magnitude, reflecting a loss of 33–55% of the free energy of interaction. Thus, although Thr2 is only a small part of the TIMP side of the interaction interface, it has a major role in the stability of the protein–protein interaction, and therefore represents a ‘hot spot’ for complex formation.

One striking feature of residue 2 in N-TIMP-1 is that mutation at this site significantly alters the affinity for different MMPs.

**Figure 2**

A schematic display of the secondary structure of tissue inhibitor of metalloproteinases 1 (TIMP-1). The crystal structure of TIMP-1 was determined as a complex with the catalytic domain of MMP-3 [86]. Strands (A–J) and helices (H1–H4) are shown. Two glycosylation sites are indicated by diamonds.

**Figure 3**

A schematic representation of (a) the N-terminal region of tissue inhibitor of metalloproteinases 1 (TIMP-1) and (b) a peptidyl-hydroxamate inhibitor. The scheme of TIMP-1 is based on the crystal structure of the TIMP-1–MMP-3(ΔC) complex [86].
MMPs. It is notable, however, that a comparison of the effects of a particular amino acid in the P1′ position of a peptide substrate on $k_{cat}/K_m$ [89] (Table 2) with its effects as residue 2 of TIMP on MMP binding ($1/K_i$) show a poor correlation [88] (Fig. 3). This indicates that there is a large difference between recognition of the P1′ residue of a substrate and residue 2 of TIMP for MMPs. This discrepancy is probably due to a greater loss of conformational entropy associated with peptide substrate–MMP interactions compared with TIMP–MMP interactions. The orientation of residue 2 of TIMP-1 may also be influenced by the rigid structure around the two disulfide bonds in this region. Several mutants show potentially useful changes in specificity (e.g. the Arg2 mutant, which discriminates strongly against MMP-1).

Because the interaction between TIMP and MMP involves multiple sites, more specific mutants with multiple substitutions can be designed. Val4 and Ser68 were chosen because they are part of the core contact region with the MMP (Fig. 4). Substitutions for Val4 and Ser68 have significant effects on specificity (Wei et al., unpublished observations). The properties of the multisite mutants exhibit further enhancement in selectivity. The triple mutant T2L/V45/S68A exhibits high selectivity for MMP-2 (Table 1). Further experiments are necessary, but the unique structures around the reactive site of TIMPs provide new leads for designing selective MMP inhibitors.

**Future prospects**

The balance between MMPs and TIMPs is critical for the appropriate maintenance of tissues, and its disruption perturbs tissue homeostasis. A number of MMPs and ADAMTSs play major roles in cartilage matrix breakdown in arthritis. Several potent, orally available MMP inhibitors have been developed by a number of pharmaceutical companies and some were clinically tested for the treatment of arthritis or cancer, but none were found to be efficacious [90]. The reasons for this failure are not clear. It may be due to inhibition of nontargeted metalloproteinases or the inhibitor concentration may not have reached an effective level in the target tissue. In addition, there are general concerns about the safety of synthetic MMP inhibitors. For example, when the broad-spectrum MMP inhibitor Marimstat (British Biotech Pharmaceuticals, Oxford, UK) was used in cancer trials, it caused musculoskeletal problems manifested by tendonitis, joint pain, stiffness and reduced mobility. This may be due to nonselective inhibition of metalloproteinases that are biologically important.

Alternative approaches to preventing accelerated matrix breakdown may be to deliver natural inhibitors or natural inhibitor-derived selective inhibitors to the target tissue using gene transfer technologies.

**Concluding remarks**

The elucidation of the mode of interaction of TIMPs with MMPs and their inhibition mechanisms has introduced a new opportunity to engineer TIMP so that the variants selectively inhibit MMPs. In combination with gene transfer...
The surface structure of tissue inhibitor of metalloproteinases 1 (TIMP-1). The N-terminal domain and the C-terminal domain are shown in light red and green, respectively. The region within 4 Å contact with the matrix metalloproteinase (MMP) catalytic domain is shown in blue. Mutation sites coloured red modulate the selectivity of N-TIMP-1 against different MMPs. The image was prepared from the Brookhaven Protein Data Bank entry (1UEA) using the Swiss PDB viewer [91].

technologies, it is hopefully possible to deliver a selective TIMP variant to the target tissue. Mutagenesis studies conducted in our laboratories indicate that the rigid nature of the reactive site of TIMP provides a unique mode of interaction with MMPs that is significantly different from those of peptidomimetic synthetic inhibitors. The use of this type of interaction may allow us to design new types of inhibitors. This requires a thorough understanding of the interaction between the target enzyme and the inhibitor. Further investigations of the mode of interaction of TIMP-3 with aggrecanases and TACE are particularly important for the future development of selective inhibitors against these enzymes as potential therapeutics to prevent cartilage matrix breakdown.

Glossary of terms
ADAM = a disintegrin and a metalloproteinase; ADAMTS = ADAM with thrombospondin type I domain; ECM = extracellular matrix; MMP = matrix metalloproteinase; MMP-3 (ΔC) = catalytic domain of MMP-3; MT1-MMP = membrane-type 1 matrix metalloproteinase; N-TIMP = N-terminal domain of tissue inhibitor of metalloproteinases; proMMP =zymogen form of MMP; TACE = tumour-necrosis-factor-alpha-converting enzyme; TIMP = tissue inhibitor of metalloproteinases.

Acknowledgements
The authors thank Dr Rob Visse and Dr Eric Huet for preparation of the illustrations. This work was supported by NIH grant AR40994 and the Wellcome Trust Grant 097508.

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