Novel Strategies to Delineate Matrix Metalloproteinase (MMP)-Substrate Relationships and Identify Targets to Block MMP Activity

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Abstract: Adverse extracellular matrix (ECM) remodeling contributes to fibrotic disorders in the kidney, lung, and heart. Matrix metalloproteinases (MMPs) are key enzymes regulating ECM turnover, and MMP inhibition attenuates remodeling. Recent technological developments allow MMP-substrate relationships to be identified and explored as novel therapeutic targets. This review summarizes current and novel strategies to block MMP activity.

Key Words: Matrix metalloproteinases, extracellular matrix, MMP inhibitors, mechanism-based inhibition, proteomics, remodeling, fibrosis.

INTRODUCTION

Extracellular Matrix (ECM) Remodeling

Comprised of collagens, laminin, fibronectin, and proteoglycans, the ECM provides an environment for cells to migrate, grow, and differentiate [1]. As such, the ECM regulates cell and tissue function. ECM homeostasis is controlled at three levels: synthesis, post-translational modifications, and degradation. Under normal conditions in a young adult, ECM turnover occurs at a rate that allows normal replacement and maintains tissue structure and function. In response to injury, accelerated ECM turnover drives the remodeling process to generate a scar. Fibrosis is the excessive accumulation of ECM, particularly collagen.

Matrix Metalloproteinases (MMPs)

MMPs are a family of zinc-dependent enzymes that regulate ECM turnover. The MMP family is comprised of more than 25 individual members divided into specific classes based on *in vitro* substrate specificity for various ECM components. MMP activity is inhibited nonspecifically in the plasma by α_2 macroglobulin and specifically in the tissue by the tissue inhibitors of metalloproteinases (TIMPs), a family currently composed of four members. MMPs have been implicated in normal physiological processes, including ovulation, wound healing, and angiogenesis [2-4]. In addition, MMPs have also been identified in pathophysiological processes, including cancer, arthritis, periodontitis, multiple sclerosis, and several cardiovascular diseases (e.g. atherosclerosis, aneurysms, cardiomyopathy, myocardial infarction, and congestive heart failure) [5-10]. MMP-9, in particular, has been documented in all of the cardiovascular diseases mentioned above [11-13]. MMP-9 is a 92 kD MMP ubiquitously expressed by many cell types, including inflammatory cells (macrophages, neutrophils, and lymphocytes), vessel cells (endothelial and smooth muscle cells), and resident tissue cells (myocytes, fibroblasts, and hepatocytes) [14]. Additionally, the absence of MMP-9 ameliorates aspects of the remodeling phenotype [15, 16], which suggests that targeted inhibition of MMP-9 may prove to be a successful therapeutic strategy. To date, selective MMP-9 inhibitors have not been developed or extensively studied.

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CURRENT STRATEGIES IN MMP INHIBITOR DESIGN

Global Inhibitors

First generation MMP inhibitors were broad spectrum molecules designed as substrate mimics with functional groups to chelate the active site zinc ion. These inhibitors included hydroxamates, carbamoylphosphonates, thiols, and tetracycline analogs [17, 18]. Hydroxamates are the most abundant class, and several hydroxamates inhibit metalloproteinases with nM affinity. The dominant features that guided design of hydroxamates were the ability to chelate zinc and modulation of the P1' substituents in an attempt to achieve selectivity [19]. Marimastat (BB-2516) is one such hydroxamate inibitor, and its chemical structure is provided in Table **1**. When used in clinical trials, however, hydroxamates proved ineffective and produced poorly tolerated side effects, including joint and muscle-related pain [7].

The failure of first generation MMP inhibitors was due to several reasons. For one, only MMP-1, MMP-2, and MMP-3 had been identified and studied in detail when many of these inhibitors were first conceived, and the number and diversity of MMPs had not been fully realized [20]. In addition, the number and diversity within the zinc peptidase superfamily of metalloproteinases was also not fully understood. To date, over 20 members of the disintegrin and metalloproteinase (ADAM) family and 19 members of the disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs) family have been identified [21-23]. The active sites of many metalloproteinases (MMPs, ADAMs, and ADAMTs) are similar and are modulated similarly by broad spectrum inhibitors [24].

A second factor that may have contributed to the failure of the first generation inhibitors is the poor selectivity of the

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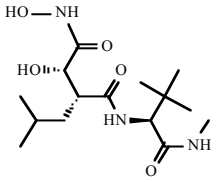
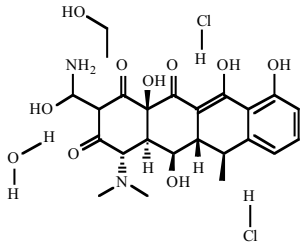
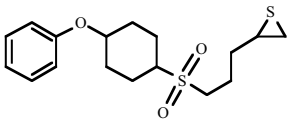
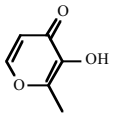
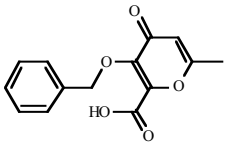
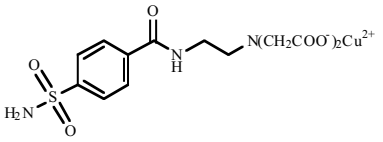
Table 1. Structures of the MMP Inhibitors Discussed in this Review

Inhibitor	Structure	Comments
Marimastat (BB-2516)		1st generation hydroxamate inhibitor
Periostat® (doxycycline hyclate)		Only MMP inhibitor approved for clinical use; functions by reducing MMP synthesis and decreasing MMP stability, not by directly inhibiting MMP activity
SB-3CT		Competitively inhibits MMP-2 without undergoing conformational change
Maltol		Pyrone zinc binding group
Pyrone backbone		Offers superior potency over hydroxamate zinc binding analogues; also displays novel selectivity
Conjugate 1		Multiprong mechanism, blocks active site accessibility without fitting into active site

metal chelating group. Hydroxamates are also very powerful iron-sequestering agents, and the lack of metal binding selectivity (for zinc, over iron, calcium, magnesium, or copper) may have played a role in the toxic side effects seen with early MMP inhibitors [25]. Nonselectivity of inhibitors that target both MMPs as well as other protease families is the major issue to be surpassed in future inhibitors [26]. After more than 40 years of MMP research, thousands of compounds synthesized and tested, and billions spent on clinical studies, only one MMP inhibitor has been successfully brought to market [7, 27]. That inhibitor is doxycycline hyclate (which does not contain a hydroxamate group), marketed as Periostat® by CollaGenex Pharmaceuticals, Inc. The structure of doxycycline hyclate is shown in Table **1**. Doxycycline inhibits MMPs by reducing enzyme stability, reducing RNA stability, and inhibiting transcription [20].

Selective Inhibitors

A major lesson learned from global MMP inhibitor trials is that the net biological effect of MMP inhibition on a disease process depends on the disease stage as well as the balance between disease-promoting and disease-suppressing activities between individual MMPs and even within the same MMP type [27]. Current drug discovery strategies involve the use of rational or combinatorial approaches to syn-

thesize small molecular weight compounds that block the active site and inhibit specific MMP enzymatic activity on particular substrates [26]. While MMPs exhibit high sequence similarity (56-64% in the catalytic domains), functional specificity still exists [28]. The failures of past studies highlight the necessity for suitable biomarkers to adequately assess inhibitor efficacy and refine the design of mechanism-based MMP inhibitors [29]. Additionally, crystal structures of MMPs and MMPs bound to substrates or inhibitors provide important structural information about the active site as well as the mode of interaction [25]. Structure-based approaches that take into account fine structural, chemical, and electronic differences are currently being used to design potent and selective inhibitors [30].

The current design process incorporates specificity into small molecule structures to eliminate the toxic side effects observed with broad spectrum MMP inhibition. The size, shape, and nature of the S1' pocket can be used to design specificity. For example MMP-13 has an unusually large S1' pocket [30]. After specificity has been achieved, potency can be altered to improve efficacy. Fine structural and electronic differences between the catalytic zinc ions within the active sites of MMP-2 and ADAM-17 (TNFα converting enzyme or TACE) have been used to design the inhibitor SB-3CT, which takes advantage of the increased polarity of the TACE active site compared to MMP-2 to derive an inhibitor that is two orders of magnitude more selective for MMP-2 compared to TACE [24]. SB-3CT is commercially available from Biomol, and its chemical structure is shown in Table **1**. While SB-3CT directly binds the metal ion of both TACE and MMP-2, the binding to MMP-2 is competitive and occurs without a conformational change. Binding to TACE, on the other hand, non-competitively induces a significant conformational change in the TACE structure. Regardless of the high structural similarity between MMP-2 and TACE, the electronic and chemical properties within the active sites are significantly diverse enough to achieve specificity [24].

Additional MMP inhibitors are entering the pipeline. Puerta and colleagues recently described 11 new, potent MMP inhibitors, all of which more effectively inhibited MMP-3 than the gold standard acetohydroxamic acid [31]. This group has also designed and synthesized pyrone-based MMP inhibitors [32] as well as examined the use of nitrogenous ligands to inhibit MMPs [33]. Pyrones are synthetically versatile, biocompatible, and water-soluble—all of which are attractive attributes for clinical application. The pyrone based MMP inhibitors displayed superior potency when compared to their hydroxamate analogues. Maltol is the first generation pyrone inhibitor (Table **1**). Using the pyrone backbone, Seth Cohen's laboratory has made improvements by attaching simple aryl groups to the 2-position of maltol to achieve potent and selective MMP inhibitors [32]. Interestingly, several of the 6 inhibitors tested showed selectivity for MMP-3 over MMP-1 and MMP-2. MMP-3 is a good upstream MMP target, as MMP-3 activates additional members of the MMP family and can stimulate the MMP cascade [34]. Nitrogenous ligands may enhance the affinity of metalloproteinase inhibitors for MMPs and may increase selectivity as nitrogen chelators show specificity for metalloproteinases with zinc in their active sites. Incorporating knowledge gained from previous inhibitors continues to yield improvements that are potentially clinically viable.

In addition to inhibiting the active site, additional residues on the MMP surface can be targeted to achieve increased selectivity. Banerjee and colleagues recently described a multi-prong inhibitor that was synthesized based on differently branched benzenesulfonamide derivatives to obtain a specific and selective MMP-9 inhibitor (their conjugate 1 is shown in Table **1**) [26]. Unfortunately, binding to MMP-9 was only compared with binding to MMP-10, so selectivity over other MMPs (particularly MMP-2, another member of the gelatinase subfamily) was not examined.

NOVEL PROTEOMIC STRATEGIES

Many MMP inhibitors across many indications have failed clinical trials, in part due to inappropriate animal models, poor pharmacokinetics, unavoidable toxicology, poor clinical trial design, and lack of human efficacy (either because MMP inhibition was not achieved or MMP inhibition was not a suitable target) [27, 45]. As discussed above, improving selectivity and specificity are the two avenues currently being explored for optimization. Two additional issues will need to be addressed before inhibition can be targeted to a particular MMP. The first is that inhibition of a particular MMP does not mean that another MMP will not serve in its place to yield a negative result. Many MMPs share overlapping substrate lists (Table **2**), and the preference of a substrate for a particular MMP may shift when the dominant MMP is absent. The second issue is that inhibiting proteolysis of the entire substrate repertoire for a particular MMP may not yield a net benefit. In addition to issues of selectivity and specificity, inhibiting pertinent MMP substrates should also be included in drug design rationale. Matrix degrading activities account for only a small portion of the biologic response diversity regulated by MMPs [27]. In addition to cleavage of gelatin, collagen IV, collagen V, fibronectin, and elastin, MMP-9 also processes a wide range of non-matrix proteins including interleukin-1β [44] interleukin-8 [37], and plasminogen [46] (Table **3**). Targeting the substrate rather than the MMP may offer optimal specificity. Cleavage sites within these proteins are variable and not entirely predictable based on consensus sequence analyses, suggesting that inhibitors to prevent specific substrate proteolysis could be designed.

Table 2. Overlapping MMP Substrate List

ECM Substrate	MMPs capable of processing substrate
Fibrillar collagens (full length or denatured)	MMPs-1, -2, -3, -7, -8, -9, -10, -11, -13, -14, -15, -16, -17, -18
Fibronectin	MMPs-1, -2, -3, -7, -9, -10, -11, -12, -13, -14, -15
Collagen IV	MMPs-2, -3, -7, -9, -10, -11, -14
Laminin	MMPs-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -14, -15

Table 3. Non-Matrix MMP-9 Substrates

Substrate	Activity	Ref
α 1 proteinase inhibitor	↓	[24]
α 2 macroglobulin	↓	[34]
Connective Tissue Activating Peptide- III (C-X-C chemokine)	↓	[46]
Endothelin-1	↑	[18]
Fibrin	↑	[23]
Galectin-3	↓	[29]
Growth Related Oncogene-α (C-X-C chemokine)	↓	[46]
Heparan Sulfate Proteoglycans	↑	[50, 51]
Intercellular Adhesion Molecule 1	↓	[19]
Interleukin-1β	↑	[38]
Interleukin-8	↑	[46]
Plasminogen (to Angiostatin)	↑	[30]
Platelet Factor 4 (C-X-C chemokine)	↓	[46]
Stromal Cell-derived Factor-1 (C-X-C chemokine)	↓	[26]
Substance P	↓	[2, 14, 28]
Tissue Factor Pathway Inhibitor	↑	[5]
Transforming Growth Factor β_1	↑	[49]

Recent advances in proteomic technologies, particularly those based on mass spectrometry approaches, may allow the compilation of complete MMP substrate catalogues. Abersold and Mann have written an excellent review that summarizes recent advances in mass spectrometry-based proteomic technologies [47]. In addition to the now relative straightforward ability of mass spectrometry to obtain protein identities, mass spectrometry-based protocols also provide information on protein quantitation, protein interactions, and protein modifications. For example, isotope-coded affinity tag (ICAT) analysis allows two mixtures of proteins to be separately labeled and combined for quantitative mass spectrometric analysis [48]. Ratios are derived for all proteins containing a cysteine, and identifications can be made for proteins with ratios that are not equal to 1. The newer generation iTRAQ labeling reagents allow analysis of up to 4 groups and remove the need for a protein to contain a cysteine [49]. Improvements in fractionation techniques, solubility, labeling reagents, and mass spectrometry technologies will continue to improve our ability to examine the proteome.

A proteomics based experimental approach to identify new MMP-9 substrates is illustrated in Fig. (**1**). In this example, tissue homogenates are incubated in the presence or absence of recombinant active MMP-9. Tissue culture based cell stimulation experiments have also been performed to assay for proteins shed from the cell surface and released into the media following MMP stimulation [50, 51]. Two-dimensional electrophoresis is used to identify proteins that are present or absent in the treated group. The loss of a particular spot or the addition of a spot suggests protein cleavage, since addition of the MMP is the only change between the two groups. Mass spectrometry is then used to identify proteins differentially expressed in the presence or absence of MMP-9. From the identifications, cleavage can then be confirmed by *in vitro* substrate assays using the recombinant protein and recombinant MMP-9. Confirmed substrates are then added to the known substrate list. The final step in the process is to determine cause and effect relationships between particular substrate products and deleterious effects on remodeling or tissue physiology. This can be accomplished in a number of ways, including using the substrate peptides as competitive inhibitors. Targeted strategies that would prevent proteolysis of particular substrates will provide an increased level of selectivity. For example, instead of inhibiting MMP-9 globally, perhaps MMP-9 proteolysis of plasminogen to generate angiostatin fragments should be inhibited without affecting the ability of MMP-9 to proteolyze additional substrates. Whether these inhibitors will be substrate peptide mimetics or other types of inhibitors remains to be explored.

CONCLUSION AND PERSPECTIVE

Inhibiting the MMP system may be a viable therapeutic option for several pathophysiological processes. Key issues that remain to be worked out include enhancing specificity, achieving selectivity, and determining optimal timing of inhibition. Some of these issues may not be fully reconciled

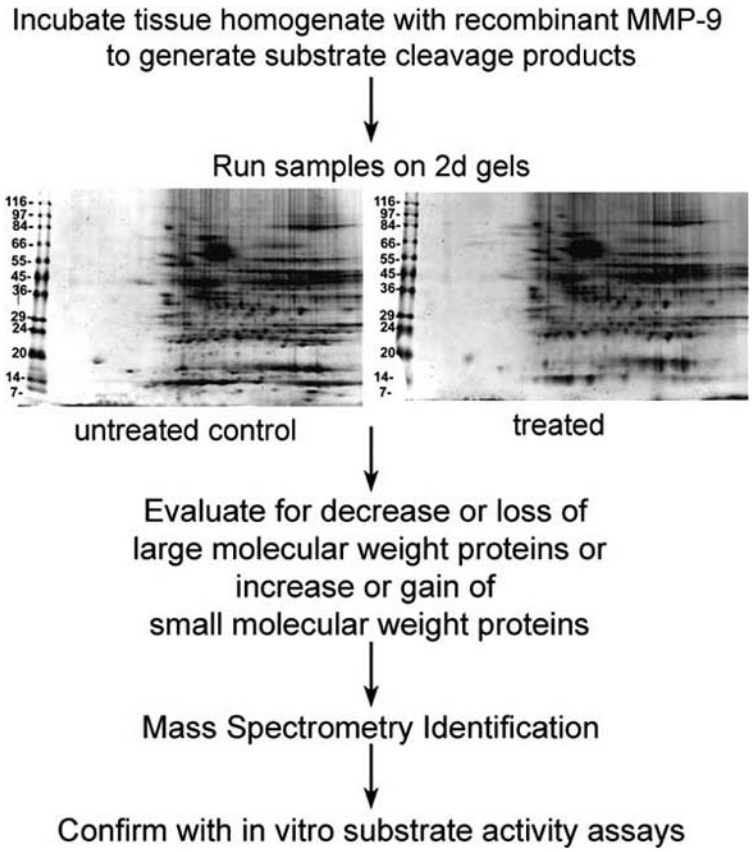


Fig. (1). A proteomics based experimental approach to identify novel MMP substrates. MMP-9 is used here as an example.

until a more complete catalog of MMP substrates is available, and novel proteomic strategies provide a mechanism to achieve this goal. Targeting the MMP substrate, rather than the MMP, may provide a more specific way to regulate MMP inhibition.

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ABBREVIATIONS

ECM = Extracellular matrix

MMP = Matrix metalloproteinase

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