Published in final edited form as:

J Drug Target. 2007 January ; 15(1): 1–20. doi:10.1080/10611860600968967.

Matrix metalloproteases: Underutilized targets for drug delivery

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Abstract

Pathophysiological molecules in the extracellular environment offer excellent targets that can be exploited for designing drug targeting systems. Matrix metalloproteases (MMPs) are a family of extracellular proteolytic enzymes that are characterized by their overexpression or overactivity in several pathologies. Over the last two decades, the MMP literature reveals heightened interest in the research involving MMP biology, pathology, and targeting. This review describes various strategies that have been designed to utilize MMPs for targeting therapeutic entities. Key factors that need to be considered in the successful design of such systems have been identified based on the analyses of these strategies. Development of targeted drug delivery using MMPs has been steadily pursued; however, drug delivery efforts using these targets need to be intensified and focused to realize the clinical application of the fast developing fundamental MMP research.

Keywords

drug delivery; drug targeting; matrix metalloprotease (MMP); prodrug; drug activation

Introduction

The search for a “magic bullet” has been the major focus of drug delivery and drug discovery research for many years (Ehrlich and Bolduan, 1906). If active agents or delivery systems can be devised that selectively treat a disease while having no affect on healthy cells, tissues and organs, that particular disease will no longer plague humankind. However, for most diseases, the search for a drug targeting system (DTS) has not been successful to date. The major reason that the DTS search has failed is that most biologic processes are active in many tissues at varying levels. Diseased tissues tend to exhibit normal biology that only exists at either an elevated level or decreased level compared to the equivalent normal tissue or other normal tissues in the body. This fact has driven researchers to identify targets that have the largest difference between normal and diseased tissue or to find novel ways of utilizing multiple properties of the disease state to amplify the difference between normal and diseased tissue. In this way, we continue to investigate novel targets for designing and delivering active chemotherapeutic agents that are similar to the proposed “magic bullet”. Matrix metalloproteases (MMP) are one of such promising targets being explored for designing an array of DTS.

The drug targeting field has attempted to classify the various methods used for DTS design. The classification schemes utilize the physical, chemical, and biologic cues of the disease that are exploited to release or activate a drug to describe the DTS (Lubbe *et al.*, 2001). A

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DTS that utilizes a specific biochemical signal at the cellular level to target delivery would easily be classified as site-directed, 2nd order, cellular and active delivery. This is because the targeting would deliver the agent to the disease location in response to a specific biochemical extracellular event at the cellular level. Such a targeting mechanism would easily and clearly describe the topic of current review, matrix metalloprotease (MMP) targeting.

Matrix metalloproteases (MMPs)

It has been more than four decades since the first collagen degrading activity was observed during metamorphosis in tadpoles (Gross and Lapiere, 1962). This observation marked the beginning of discovery of a family of enzymes recently categorized into the matrix metalloproteases family. Much has been discovered about MMP structure, activation and inhibition, regulation of gene expression, and role in normal physiology and in diseases in this span and much is left to be determined (Brinckerhoff and Matrisian, 2002).

MMPs, also referred to as matrixins, are a family of approximately 24 human zinc-containing endopeptidases that are together capable of degrading all components of the extracellular matrix (ECM) and many other proteins. MMPs are produced as latent zymogens which must be proteolytically activated in the body to form the functional enzyme (Nagase and Woessner, 1999). MMPs have been subdivided into at least five classes based on their structure (Borkakoti, 1998; Rundhaug, 2005), Table 1. Matrilysins (MMP-7, 26) consist of a propeptide domain and a catalytic domain with the zinc binding site. The collagenases (MMP-1, 8, 13), in addition to the minimal matrilysin domain structure, contain a hemopexin-like domain connected to the catalytic domain via a proline-rich hinge region. The stromelysins (MMP-3, 10, 11) have similar structural domains as the collagenases but broader substrate specificity. The gelatinases (MMP-2, 9) contain an additional region of three fibronectin type II repeats within their catalytic domains. The fifth subclass of MMPs is the membrane-type MMPs (MT-MMPs) which are bound to the cell surface via a C-terminal transmembrane domain (MMP- 14, 15, 16, 24) or glycosylphosphatidylinositol anchor (MMP-17, 25). Other MMPs not included in above classification are metalloelastase (MMP-12), enamelysin (MMP-20), epilysin (MMP-28), MMP-19, MMP-27 and MMP-23. These MMPs with an exception of MMP-23 have domain structure similar to collagenases but have not been categorized in that subclass for various reasons. MMP-23 is unique in that it lacks the hinge and hemopexin domains and instead contains a short carboxy-terminal domain containing cystein array (Parks *et al.*, 2004).

Variations in the residues forming the catalytic domain play a major role in binding and catalysis, but discrete MMP substrate binding domains exist in non-catalytic regions that further increase the substrate specificity of different MMPs (Overall, 2002). Various approaches, including proteomics, affinity based methods and genetic knockout, have been used to identify MMP substrates (Guo *et al.*, 2002; McQuibban *et al.*, 2000; Tam *et al.*, 2004). In general, significant overlap is observed in specificity of different MMPs for natural physiological matrix and non-matrix substrates. For example, collagen I is degraded by MMP-1, 2, 8 and 13; fibronectin by MMP-2, 3, 7, 10 and 11; and ProMMP-2 by MMP-14 and 16 (Nagase and Fields, 1996). Among the non-ECM substrates, MMP-2 and 9 can activate latent TGF β 1 and TGF β 2 while MMP-1, 3 and 7 release TNF α from the cell surface (McCawley and Matrisian, 2001; Stamenkovic, 2003). In addition to natural protein cleavage, cleavage site specificity of several MMPs, mainly MMP-1, 2, 3, 7, 8, 9, 13 and 14, have been studied using large series of synthetic peptides based on natural substrates, phage display libraries and mixture-based libraries (Deng *et al.*, 2000; Nagase and Fields, 1996; Netzelarnett *et al.*, 1991; Turk *et al.*, 2001). If the cleavage site of a protease is defined as ... P3-P2-P1~P1'-P2'-P3' ... where P# is any specific amino acid, cleavage occurs between

P1 and P1 amino acids. MMPs generally require hydrophobic amino acids at P1 and P2, prefer hydrophobic or basic amino acids at P2, proline at P3, and small residues particularly serine at P1. Although a number of features have been identified for the cleavage site motif that are common to all MMPs examined, certain differences have also been observed that can distinguish MMPs. Whereas MMP-1, 2 and 9 prefer small residues, MMP-3, 7 and 14 select for methionine at P3. MMPs are also different in their relative tolerance for aromatic amino acids at P1. Although P2 is generally hydrophobic, the particular residues vary from enzyme to enzyme. Only, MMP-2, 3 and 7 select glutamic acid at P1. Thus, even though there is large similarity in the optimal sequences for cleavage by MMPs, it is possible to design substrate peptides that are selectively (greater than 1000 fold difference in activity) by specific MMPs, an important aspect for utilizing MMPs as targets for drug delivery (Turk *et al.*, 2001). The overlapping cleavage specificity is controlled *in vivo* by specific binding sequences to natural substrates and through exquisite regulation of the temporal and spatial control of MMP activity.

As part of the conserved structure, all MMPs contain a 'cysteine switch' that maintains the latency of the enzyme prior to activation. The sulfhydryl cysteine in the highly conserved PRCGXPD sequence in the propeptide domain coordinates with the catalytic zinc ion to prevent activity (Van Wart and Birkedal-Hansen, 1990). MMPs are activated *in vivo* by proteolytic removal of the propeptide domain. MMP activation is regulated by one of several proteolytic cascades. One such cascade is initiated by plasmin generated from plasminogen through the action of urokinase-type plasminogen activator (uPA). Plasmin can activate proMMP-1 and proMMP-3. Activated MMP-3 can in turn activate proMMP-1 and proMMP-9 (DeClerck and Laug, 1996; He *et al.*, 1989; Ramos-DeSimone *et al.*, 1999). Another cascade utilizes MT1-MMP to activate ProMMP-2 and 13 and focuses the activity at the cell surface (Knauper *et al.*, 2002; Sato *et al.*, 1994). Activated MMP-2 and 13 can both activate proMMP-9 (Fridman *et al.*, 1995; Knauper *et al.*, 1997). Thus, there are many cross-talk events between MMP activation cascades due to several MMPs having the capability to activate other MMPs and self-activate.

Regardless of the cascade utilized to activate a particular MMP, extracellular activation of MMPs results from a two-step process. The first step is the cleavage by an activator protease in the exposed loop in the propeptide region thus destabilizing its zinc coordination. This is followed by a second cleavage, usually by a MMP, releasing the mature enzyme (Nagase, 1997). Specific MMP family members (MMP-11, MT-MMPs, MMP-23 and MMP-28) can also be activated by intracellular furin convertase enzymes (Pei and Weiss, 1995; Sato *et al.*, 1997). The multitude of activation mechanisms allows very precise control of MMP activity even when the expression levels of the proteases are constant. However, inhibition mechanisms exist that aid in further controlling MMP activity.

MMPs can be inhibited by general protease inhibitors, e.g. 2-macroglobulin, angiogenesis inhibitor thrombospondin-1 (TSP-1) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) (Egeblad and Werb, 2002), much as these inhibitors act on other proteases. More specifically, MMP activity is regulated by endogenous inhibitors, primarily the tissue inhibitors of metalloproteases (TIMPs). TIMPs act by forming 1:1 complexes between their N-terminal domain and the activated catalytic zinc in MMPs (Bode and Maskos, 2003). The TIMPs exhibit differential inhibitory activity against different MMPs based primarily on binding efficiency to the MMPs. Since the discovery of TIMPs, contradicting functions have been identified, specifically association of ProMMP-2 with TIMP-2 is necessary for MMP-2 activation by MT1-MMP (Cao *et al.*, 1998; Goldberg *et al.*, 1989). TIMPs also have other biological functions that are not related to MMP inhibition and are not entirely understood (Brew *et al.*, 2000). Thus, the MMP-TIMP inhibition system is quite complex and caution is warranted in interpreting observations due to these conflicting roles of TIMPs. Further

complicating the regulation of MMP activity, MMPs are not only regulated by activation and natural inhibition, but also at the transcriptional and post-transcriptional levels.

Most of the MMPs are not constitutively expressed but are induced by specific stimuli, such as cytokines, growth factors, activated oncogenes and cell-ECM interactions (Westermarck and Kahari, 1999). Several MMP promoter regions contain multiple cis-acting elements including activator protein-1 (AP-1) and erythroblastosis twenty six (ETS) binding sites (Overall and Lopez-Otin, 2002; Rivat *et al.*, 2003). These sites in cooperation with several other upstream elements regulate MMP gene expression. These various promoters also confer the tissue specificity for MMP expression (Crawford and Matrisian, 1996). Single nucleotide polymorphisms (SNPs) in these promoter regions alter the transcriptional regulation of the MMP gene and have been associated with specific diseases (Ye, 2000). MMP levels are also controlled at the post-transcriptional stage. MMP-9 levels have been shown to be controlled by translational efficiency in murine prostate carcinoma cells (Jiang and Muschel, 2002) and translational repression is responsible for the discordance observed between mRNA levels and protein expression of human MMP-13 following interleukin-1 or transforming growth factor- β stimulation (Yu *et al.*, 2003). Beyond these four levels of MMP regulation, further regulation is exerted by localization of MMP activity in the pericellular space.

Many MMPs are typically anchored to the cell membrane after secretion and hence act only on their specific substrates in the immediate environment of the cell. In addition to MT-MMPs which contain membrane anchoring regions, several other interactions of MMPs with cell surface proteins have been reported that facilitate localized cell migration and invasion. MMP-2 can bind to $\alpha_3\beta_1$ -integrin in a proteolytically active form on the surface of invasive cells (Brooks *et al.*, 1996). Also, a ternary complex of $\alpha_2\beta_1$ -integrin, proMMP-1 and collagen function together to drive and regulate keratinocyte migration indicating that several MMPs are directly localized by cell-surface receptors (Dumin *et al.*, 2001). In addition to integrins, other cell surface receptors are involved in MMP focalization. Localization of MMP-9 to the cell surface by hyaluronan receptor CD44 is required to promote tumor invasion and angiogenesis by activating latent transforming growth factor- β (Yu and Woessner, 2000). Heparan sulfate proteoglycans on or around epithelial cells and in the underlying basement membrane have been found to be the docking molecules for MMP-7 (Yu and Woessner, 2000). As suggested for MMP-1 and MMP-9, these MMP anchors might serve dual function of proenzyme activation and assisting enzyme-substrate interaction (Parks *et al.*, 2004). Through these specific docking mechanisms, MMPs are spatially regulated allowing precise control of their proteolytic activity.

As a final level of regulation, cellular catabolism of MMPs is mediated by the low density lipoprotein receptor-related protein (Hahn-Dantona *et al.*, 2001). It is clear that MMPs are highly regulated by every known mechanism of protein regulation. This is due to the control needed in normal physiology to precisely activate specific molecules and degrade other molecules.

Normal Physiologic MMP Function

Though MMPs were discovered as ECM degrading enzymes, an emerging view is that matrix degradation is not the only (or even primary) function of these enzymes (Mott and Werb, 2004; Woessner, 2002). MMPs act on many non-matrix proteins including cytokines, chemokines, receptors, cell-cell adhesion molecules, latent growth factors and antimicrobial peptides often potentiating activity of these proteins. Through these nonconventional substrates, MMP activity has been extended to cell growth, signaling, migration, differentiation, and apoptosis (McCawley and Matrisian, 2001; Vu and Werb, 2000). MMPs

are involved in normal physiological processes requiring ECM modification, such as ovulation (Zhang and Nothnick, 2005), muscle homeostasis (Carmeli *et al.*, 2004), rapid modeling and remodeling of connective tissues (Hulboy *et al.*, 1997) and wound healing (Parks, 1999).

Specific MMP gene knockout mice have enabled researchers to aim at detecting functions of various MMPs in normal developmental physiology. Surprisingly, mice that lacked MMP-2, -3, -7, -11 and -12 showed little or no phenotypic alterations under normal conditions. Inflammatory and immune-related differences were observed in all *mmp*-null mice described (Parks *et al.*, 2004). Redundancy in MMP function might have hindered the phenotype appearance in these cases (Lee and Murphy, 2004), but this hypothesis needs further confirmation. Despite several MMPs having no visible role in development, the role of MMPs during development has been observed in MMP-9 and MT1-MMP deficient mice. Deletion of MMP-9 caused a slight delay in bone growth-plate development (Vu *et al.*, 1998). More convincingly, MT1-MMP knock-out mice have severe phenotypic dwarfism, osteopaenia, and arthritis and the mice survive only a short time after birth (Holmbeck *et al.*, 1999). Mice lacking MMP-2 and MT1-MMP die immediately after birth with respiratory failure, abnormal blood vessels, and immature muscle fibers reminiscent of central core disease (Oh *et al.*, 2004). More studies with multiple MMP gene deletions are needed to elucidate the role of individual MMPs in normal physiology particularly due to the interdependence of many MMP activation events and activation cascades. The roles of MMPs in normal physiology and development are still poorly understood, but the presence and overactivity of MMPs in many diseases have become quite apparent despite the lack of clear understanding of normal function of these proteases.

MMPs in Pathologic Conditions

Considering the variety of effects of MMPs on cells and their microenvironment it is not surprising that MMPs have been linked with a number of diseases and the list has only been increasing in recent years. As such, a brief review of MMPs in various pathologic conditions will be highlighted with attention drawn to specific cases while citing recent reviews. The list below is by no means complete, but the listing is intended to exemplify the wide impact of various MMPs in several pathologies.

Arthritis

Arthritis was the first disease to be linked with MMPs after the observation that rheumatoid synovial tissue degraded collagen (Harris and Krane, 1972). Studies over the past 40 years have confirmed that MMPs, especially the collagenases, and TIMPs play an important role in connective-tissue destruction in rheumatoid arthritis and osteoarthritis (Burrage *et al.*, 2006; Celiker *et al.*, 2002; Martel-Pelletier *et al.*, 2001; Mengshol *et al.*, 2002). Specifically, transcription-region SNPs in tissue type plasminogen activator and MMP-2 have been implicated as being related to development of rheumatoid arthritis while transcription-region SNPs in MMP-1, MMP-3, MMP-7, MMP-9, and MMP-13 are not correlated with arthritis development (Rodriguez-Lopez *et al.*, 2006). However, overactivity of these MMPs has been implicated in arthritis and as potential therapeutic targets in arthritis (Martel-Pelletier *et al.*, 2001; Mengshol *et al.*, 2002).

Inflammation and innate immunity

MMP activity in inflammation and immunity has recently been thoroughly reviewed and the many actions of MMPs in immunity clearly defined (Parks *et al.*, 2004). Increased or misregulated levels of many MMPs are observed in many diseases that are associated with inflammation. Injury induces expression of several MMPs by epithelial cells, the first line of

innate defense, e.g. MMP-1 for repair of skin wounds and MMP-7 for reepithelialization in lung and gut (Dunsmore *et al.*, 1998; Pilcher *et al.*, 1997). MMP-7 is also expressed by healthy mucosal epithelium in most adult tissues that are exposed to external environment. MMP-7 provides defense against microorganisms and enables apoptosis in these tissues (Saarialhokere *et al.*, 1995).

Not only are MMPs defensive as proteolytic enzymes, but MMPs take part in the maturation of the inflammatory process. MMPs also help to establish chemokine gradients by acting on the macromolecules in ECM to which chemokines are usually bound. Thus, MMPs indirectly regulate the influx of inflammatory cells in the tissue showing an inflammatory response. Several studies have indicated that MMPs can affect the activity of various cytokines including interleukin-1, interferon-, vascular endothelial growth factor, epidermal growth factors, fibroblast growth factors, transforming growth factor- β and tumor necrosis factor. MMPs are involved in both activation and inactivation of these inflammatory mediators and thus can control or support inflammation. MMP-1, 2, 3, 13 and 14 cleave chemokines thus converting the chemokines into antagonistic derivatives, thereby disrupting further recruitment of immune effector cells (McQuibban *et al.*, 2002). MMPs have been suggested to regulate the expression of chemokine receptors that may slow immune response (Khandaker *et al.*, 1999).

Cancer

As can be seen in many excellent recent reviews (Egeblad and Werb, 2002; Foda and Zucker, 2001; Lynch and Matrisian, 2002; McCawley and Matrisian, 2001; Zucker *et al.*, 2000) more may be known about the association between MMPs and cancer than is known about the role of MMPs in normal physiology. MMPs are known to break down the extracellular matrix to allow tumor growth, microenvironment development, metastasis and invasion (Sternlicht *et al.*, 1999). MMPs break down the matrix to simply allow growth, but they also are known to release angiogenic and proliferation promoting growth factors from the ECM (McCawley and Matrisian, 2001; Overall *et al.*, 2004).

Association of MMPs, primarily MMP-2 and 9, with tumor invasion and metastasis has now been well established through numerous *in vivo* studies (Liotta *et al.*, 1980; Stetler-Stevenson, 1990). There is ongoing debate concerning whether MMPs in certain cancers are expressed by the stromal cells surrounding the tumor rather than the tumor cells themselves (Hofmann *et al.*, 2005), but there is little debate that these MMPs are associated with cancer. In addition to MMP-2 and 9, MT1-MMP (MMP-14) has been proposed as being present in nearly every form of cancer if not the cause of many of the malignant transformations (Golubkov *et al.*, 2005a; Golubkov *et al.*, 2005b). MMP-1, 2, 3, 7, 9, 13 and 14 are overexpressed in colorectal cancers and degree of overexpression can be correlated with the stage of the disease and the prognosis. In contrast, overexpression of MMP-12 is associated with increased survival in colorectal cancer (Zucker and Vacirca, 2004). MMPs and TIMPs are widely expressed in both small cell lung cancer and non-small cell lung cancer (Michael *et al.*, 1999; Thomas *et al.*, 2000). In some cases, circulating MMP levels have been greatly increased when lung cancer is present (Hrabec *et al.*, 2001).

Due to these associations and many others, MMPs have attracted significant attention as therapeutic target for cancer. However, MMPs also have anti-metastatic and anti-proliferative actions (Overall *et al.*, 2004). Since several MMPs degrade ECM proteins that reveal hidden (cryptic) adhesion sequences that may deter migration or metastasis (Pirila *et al.*, 2003; Schenk and Quaranta, 2003) or release anti-angiogenic protein fragments (Bergers *et al.*, 2000), MMPs are now known to be both pro-, counter-and anti-cancer targets (Overall and Kleinfeld, 2006).

Angiogenesis

In addition to cancer-related angiogenesis, a definite link between MMPs and angiogenesis has been observed in various diseases characterized by neovascularization (Kvanta *et al.*, 2000; Rundhaug, 2005). MMPs are localized to invasive blood vessels and vascular endothelial cells have been observed to make a variety of MMPs in culture. Many growth factors including vascular endothelial growth factor, fibroblast growth factor-2 and tumor necrosis factor- α that regulate angiogenesis also induce MMPs expression (Stetler-Stevenson, 1999). MT1-MMP and MMP-2 and 9 seem to be predominant MMPs involved with angiogenesis (Bergers *et al.*, 2000; Fang *et al.*, 2000; Galvez *et al.*, 2001; Oh *et al.*, 2004), but many other MMPs may also be associated.

Cardiovascular diseases

As previously described for angiogenic diseases, MMPs play an important role in cardiovascular diseases (Newby *et al.*, 2006). MMP-1, 2, 3, and 9 found in the atherosclerotic plaque might be responsible for plaque progression and rupture (Galis *et al.*, 1994). MMPs may be present to degrade the plaque, as part of the inflammatory cascade, or in an attempt to remodel the disease.

MMP-2 overexpression and activity has been shown to induce lower contractility in cardiac tissue (Wang *et al.*, 2006). Increased MMP levels have been demonstrated to cause adverse left ventricular (LV) remodeling after myocardial infarction. Inflammatory processes occurring during the infarction play a role in induction of MMPs. Several distinct signaling pathways interact in myocardial interstitium and have a combined effect on MMP induction and activation. Hence, it is proposed that pharmacological intervention at the level of these signaling molecules may provide potential therapeutic strategies for the adverse ventricular remodeling associated with the progression of heart failure (Ahmed *et al.*, 2006; Janicki *et al.*, 2004; Tsuruda *et al.*, 2004).

Cerebrovascular diseases

As MMPs allow breakdown of basement membrane, MMPs are expected to be present in many ischemic conditions. Not surprisingly, levels of active MMP-2 and 9 are markedly increased during cerebral ischemia (Clark *et al.*, 1997). It has been hypothesized that activation of MMP-2 causes blood-brain barrier breakdown following focal cerebral stroke (Mun-Bryce and Rosenberg, 1998) and MT-1 MMP, MMP-2 and 9 have been found to be focally present in cerebral aneurysms (Bruno *et al.*, 1998). MMP upregulation, especially of MMP-2, 3 and 9, has been observed in intracranial hemorrhage and MMP-9 is correlated with cerebral edema (Fatar *et al.*, 2005). MMP-2 and 9 seem to be involved in different types of dementia (Adair *et al.*, 2004; Rosenberg *et al.*, 2001) although the mechanism of involvement is unclear. Increased levels of MMP-9 and TIMPs have been found in arterio-venous malformations of the brain (Hashimoto *et al.*, 2003).

Pulmonary diseases

Increased MMP-9 mRNA and protein levels have been detected in bronchial tissues of patients with bronchial asthma. MMP-9 has been thought to be expressed by inflammatory cells in this case (Ohno *et al.*, 1997). MMP-1 and 2 have been suggested to play an important role in airway smooth muscle hyperplasia and airway obstruction in asthma (Johnson and Knox, 1999; Rajah *et al.*, 1999). Elevated levels of various MMPs have been reported in chronic obstructive pulmonary diseases including pulmonary emphysema and chronic bronchitis (Segura-Valdez *et al.*, 2000; Zheng *et al.*, 2000). MMP-2 and 9 have been implicated in acute respiratory distress syndrome based on experimental animal studies (Torii *et al.*, 1997). Increased MMPs have also been linked with pulmonary hypertension,

acute lung injury and interstitial lung diseases (Ohbayashi, 2002). The increased MMP levels in many of these diseases may be due to the increased turnover of lung epithelium, inflammatory response, or attempts to regulate the ECM mechanics, but as with many other pathologies, the presence and activity of MMPs is clear in many pulmonary diseases.

Ocular diseases

MMP-2 and MMP-9 are expressed during corneal wound healing as long term and short term response, respectively (Fini *et al.*, 1992). MMPs are also involved in corneal neovascularization that occurs after extensive corneal damage (Mohan *et al.*, 2000). MMP-2 and 9 have been found to be constitutively expressed in normal human vitreous and retina, respectively. Various MMPs have been found to be upregulated in pathologies of posterior segment of the eye, including age-related macular degeneration, proliferative diabetic retinopathy, glaucomatous optic nerve head damage, vitreal liquefaction and vitreoretinopathy (Sivak and Fini, 2002). Also, a degenerative genetic eye disease, Sorsby's fundus dystrophy has been linked to a mutation in the *timp-3* gene (Weber *et al.*, 1994).

Gastrointestinal diseases

High expression of MMPs has been recently implicated in the pathogenesis of many diseases of the gut characterized by ulceration. High expression of MMP-1 and 3 has been shown in Crohn's disease (Kirkegaard *et al.*, 2004). MMP-1, 3, 7, 9, 10, 12 and 14 are overexpressed by different cell types in inflammatory bowel disease (Pender and MacDonald, 2004). MMP-3 is overexpressed in necrotizing enterocolitis, a disease characterized by extensive hemorrhagic inflammatory necrosis of the terminal ileum and ascending colon of premature infants (Pender *et al.*, 2003). MMP-1 and 3 are increased in peptic and duodenal ulcers (Saarialho-Kere *et al.*, 1996) while MMP-7 is overexpressed in gastric epithelial cells exposed to *Helicobacter pylori* (Wroblewski *et al.*, 2003). Certain gastrointestinal diseases, e.g. collagenase colitis and diverticular disease, are characterized by increased synthesis of ECM and by the absence of certain MMPs and higher levels of TIMPs (Gunther *et al.*, 1999; Mimura *et al.*, 2004).

Oral Diseases

As a final example of MMP-pathology relation, oral diseases are an excellent example of MMP as a drug target. Increased presence and activity of collagenases, especially MMP-8, has been known to cause substantial connective tissue destruction leading to periodontitis (Sorsa *et al.*, 2004). The general MMP inhibitor, doxycycline, has been FDA approved for the treatment of periodontitis (Ingman *et al.*, 1996). MMPs may also be involved in other oral diseases, such as dental caries and oral cancer (Chaussain-Miller *et al.*, 2006). This one case where MMP targeting has yielded clinical acceptance should be heralded and promote further MMP targeting research in other areas.

This extensive list of MMP-related diseases indicates that MMPs are involved in various conditions and pathologies. Specific MMPs have been shown to be over or under active in the diseases. If appropriate methods can be identified, targeting MMPs for therapy can clearly be advantageous for disease treatment.

Targeting MMPs with small molecules

After MMPs were linked to tumor invasion and metastasis, they were considered as prime targets for anti-cancer drug discovery programs by almost all major pharmaceutical companies. Matrix metalloprotease inhibitors (MMPIs) developed under these programs included pseudopeptides mimicking MMP substrates as well as small molecules that bind the catalytic zinc. Non-peptidic small molecules were mostly hydroxamate derivatives

(Whittaker *et al.*, 1999). Following the intense competition in developing hydroxamate compounds and their failure in clinical trials, non-hydroxamate small molecules binding zinc are being developed as MMPi (Breuer *et al.*, 2005). Many MMPi had reached clinical trials by the early 2000s with the majority of MMPi being broad spectrum because of their non-selective binding mechanism and structural similarity in active sites of MMPs. The first MMPi to enter clinical development was British Biotech's batimastat, a broad-spectrum hydroxamate derivative. It was soon replaced by the orally bioavailable analog, marimastat, in clinical trials. Marimastat failed due to patients experiencing joint pain after long-term administration and lack of efficacy at the tolerated doses (Bramhall *et al.*, 2001; Evans *et al.*, 2001). The lack of efficacy was measured by survival of end-stage patients, but this may not have been the appropriate patient population or therapeutic endpoint. A relatively selective inhibitor of MMP-2, 3 and 9, Bayer's BAY12-9566 (Tanomastat), proved to be worse than the standard treatment in a similar trial to marimastat. Several other phase III clinical trials failed to reach their end points of increased survival (Fingleton, 2003; Zucker *et al.*, 2000). In spite of failure as anti-cancer drugs so far, MMPi have been considered as therapies for various other diseases. Animal models have shown their effectiveness in inflammatory diseases including multiple sclerosis, emphysema, aortic aneurism and atherosclerosis (Whittaker *et al.*, 1999).

Several factors, such as lack of selectivity, mechanism of MMPi activity, trial design, and side effects, may have caused the failure of MMPi clinical trials. Redundancy and overlap of different MMPs in substrate specificities, expression patterns and functions along with complex interplay of various factors that regulate their induction, activation and inhibition also pose significant challenge in development of safe and effective MMP inhibitors. It is very important to determine which MMPs are potential drug targets (pro-tumorigenic) and which are clearly anti-targets (anti-tumorigenic) (Overall and Kleifeld, 2006). Blocking MMP anti-targets counterbalances the beneficial effects of target inhibition thus leading to the failure of MMPi. It is possible that the main cause of clinical failure of broad spectrum MMPi is their non-selectivity and hence loss of protective actions of MMP anti-targets in cancer (Overall and Kleifeld, 2006).

Therefore, identification and validation of specific MMPs as targets and anti-targets in different types and stages of cancer should be the first step in the drug development of MMPi followed by discovery of new chemical leads for the specific inhibition of target MMPs that spare anti-targets. To satisfy the later requirement, new MMPi need to be designed that bind specific features of individual MMP targets instead of relying on Zn^{2+} binding which is common to all MMPs. Efforts are now underway to explore such new generation of selective MMPi. Novel Zn^{2+} binding groups that also interact with active site side groups have been designed (Puerta and Cohen, 2003). Specific dynamic features offered by MMP active site flexibility can also be exploited to obtain selectivity (Bertini *et al.*, 2005; Cuniasse *et al.*, 2005; Morales *et al.*, 2004). It is also important to optimize design of such MMPi to avoid MMP anti-targets specifically MMP-3 and 8 (Overall and Kleifeld, 2006).

In addition to careful MMPi design, *in vivo* assays such as imaging with surrogate markers are required to prove the inhibition of MMPs by these inhibitors during clinical trials (Coussens *et al.*, 2002; Overall and Lopez-Otin, 2002) to fully understand the mechanisms and locations of action of these agents. As research in basic biology, molecular biology and physiological and pathological functions of MMPs continues, careful clinical trial design may lead to clinical use of MMPi for treatment of MMP-linked diseases in the future. Despite the disappointment of MMPi as therapeutic molecules, other approaches that utilize MMPs to activate drugs have become heavily investigated in recent years.

Matrix metalloprotease activated drug delivery

As is clear from the action of MMPs in natural and pathologic states, MMPs cleave active agents from the extracellular matrix by degrading proteins. When specific MMPs are implicated as pharmacologic targets for a particular disease, MMPs can either be inhibited or be used to cleave prodrugs and thus release the active drug selectively in the diseased tissue overexpressing MMPs. Drug activation would lead to increased local concentration of the active drug at the disease site versus the surrounding tissue and other parts of the body where MMP activity is very low. Attempts to exploit MMP activity for targeting purposes does not readily appear in the literature until the late 1990s, making this a recently exploited target for DTS. This attractive idea of drug delivery has been pursued steadily since. Here, recent DTS research exploiting the activity of MMPs for targeting therapeutic entities has been grouped and briefly described. The major factors and limitations are identified.

MMP activated peptide prodrugs

The first class of MMP-activated DTS includes simple enzyme prodrugs, Figure 1, activated by MMPs. In the prodrugs, therapeutic drugs are attached to a MMP substrate peptide. When the peptide is cleaved, the drug becomes active. The prodrug can be orally available or parenterally administered and targeting depends only on the peptide sequence specificity. Many such prodrugs have been designed for various anti-cancer agents (Copeland *et al.*, 2005; Firestone and Telan, 2005; Glazier, 1997; Mincher *et al.*, 2002; Timar *et al.*, 1998). MMP specific conjugates of doxorubicin (Dox) were shown to preferentially release leucine-modified Dox and Dox in a targeted fashion in MMP-expressing xenografts. It was found that peptide substrates with three or four prime side (-COOH terminal side of the cleavage site) residues like PLG~LYL and PLG~LYAL were cleaved efficiently by MMP-2 and 9. These conjugates also showed much higher therapeutic index than Dox in this preclinical model (Albright *et al.*, 2005).

Peptidomimetic analogues of such peptide conjugates were designed for doxorubicin, auristatins and CBI-TMI. These peptidomimetic analogues were designed to release the free drug after MMP cleavage. However, these peptidomimetics were found to be non-substrates of MMPs. The peptide conjugates of Dox (PLG~LDox) and auristatin that were substrates of MMPs did not show selective activity against MMP-2 and-9 expressing cells versus nonexpressing cells during a 4 hour exposure. This study indicates the need for careful evaluation of substrate selectivity for these types of prodrugs (Kline *et al.*, 2004).

Similarly, a topoisomerase inhibitor anthracene-9, 10-dione was attached to the C-terminus of MMP-9 substrate heptapeptide (ala-AALG~NVA-P) to produce a prodrug, EV1. Fluoresceine isothiocyanate (FITC) was conjugated to EV1 at the N-terminus to form EV1-FITC. Intrinsic fluorescence of the FITC label was quenched internally by the anthraquinone chromophore when the heptapeptide was intact and fluorescence could be detected when the prodrug was cleaved by MMP-9. Selective activation of EV1-FITC by MMP-9 in diseased bone marrow and spleen cells was identified in the 5T33 MM (multiple myeloma) mouse model (Van Valckenborgh *et al.*, 2005). Incorporating such fluorescent markers aides in following the fate of the conjugate and directly correlates the activation with the observed pharmacological effect without any ambiguity.

MMP activated carrier-peptide drug conjugates

Since peptide-prodrugs suffer from clearance and non-specific accumulation in non-target tissues, systems that would protect the drug from clearance, protect the drug from non-specific activation, and allow increased accumulation in the diseased tissue were developed, carrier-peptide drug conjugates, Figure 2. To allow biologic acceptance, the carrier molecule

can be of natural origin, such as albumin which is the most abundant protein in the circulation. Using this scheme, a maleimide doxorubicin derivative incorporating a MMP-2 specific peptide sequence (GPLG~IAGQ) was synthesized that binds rapidly and selectively to cysteine of albumin. Thus, circulating or purified albumin were used as a macromolecular carrier that had the potential to accumulate in tumor tissue by the EPR effect. In addition, the peptide was used for further targeting the delivery of Dox to tumor cells expressing MMP-2. Efficient and specific cleavage of the conjugate by MMP-2 to produce peptidyl-Dox was observed in the presence of purified MMP-2, A375 melanoma cell culture supernatants and A375 melanoma tissue homogenates. When 100 μ M albumin conjugate was treated with 2mU activated MMP-2 for 1.5 hrs, the initial peak of the conjugate completely disappeared during reverse-phase HPLC analysis and a new peak for Dox-tetrapeptide was observed. In the presence of A375 melanoma tissue homogenates Dox-tetrapeptide was cleaved to an additional Dox-peptide and then to free Dox within minutes. The potency of the conjugate to inhibit A375 cell proliferation was lower than that of doxorubicin; however, this was expected due to remaining amino acids attached to the doxorubicin molecule after MMP activation. In A375 melanoma xenograft-bearing mice, equivalent intravenous doses of doxorubicin and the conjugate did not show significant difference in antitumor response, but the maximum tolerated dose of the conjugate was substantially increased thus allowing administration of high doses (Kratz *et al.*, 2001; Mansour *et al.*, 2003).

In addition to natural polymers, the strategy of MMP cleavable peptide prodrug has been combined with polymer therapeutics concept to design polymer-peptide-drug conjugates (Benjamin *et al.*, 2004; Suarato *et al.*, 2002). The targeting ability of simple prodrugs was further improved by conjugating peptide prodrug to a polymeric carrier that exploited passive targeting mechanism (Duncan, 2003). MMP activated methotrexate-peptide-dextran conjugates were studied *in vitro* and *in vivo*. Dextran was modified to contain carboxymethyl groups used for conjugation to the peptide (PVG~LIG) as well as changing the polymer backbone to a negative overall charge. For comparison, MMP-insensitive conjugate containing scrambled peptide linker, GIVGPL, was prepared. The methotrexate-PVG~LIG-dextran conjugate released 89 and 61% peptidyl methotrexate after 24 h in the presence of MMP-2 and -9, respectively, whereas the scrambled control peptide did not show any significant release. In initial experiments, MMP-2 did not exhibit the expected activation of the conjugate, but the activation of the conjugate was improved by blocking the negative charge on the dextran backbone, thus confirming the authors' previous suspicion that there was electrostatic repulsion between MMP-2 and the conjugate (Chau and Langer, 2003; Chau *et al.*, 2004). The conjugate was relatively stable in presence of serum, an important prerequisite for systemic delivery. Peptidyl-drug was released by MMP-2 and -9 positive cells while no cleavage was detected in the MMP-2 and -9 negative cells. Released peptidyl-methotrexate was effective in inhibiting the proliferation of tumor cells but with a lower potency than the free drug (Chau *et al.*, 2004). The conjugate demonstrated enhanced anti-tumor efficacy compared to free drug and saline control in human tumor xenograft models expressing MMP-2 and -9 versus an alternate (RT-112) model with significantly lower MMP expression. However, the role of MMP-targeting mechanism in improving anti-tumor efficacy over the free drug could not be confirmed as proliferation rate of RT-112 was also very slow and conjugate containing scrambled peptide, though acutely toxic, was also effective in controlling tumor size (Chau *et al.*, 2006a; Chau *et al.*, 2006b). By comparing the plasma pharmacokinetics of the conjugates with free methotrexate, passive targeting was confirmed and may account for activity. Slightly more accumulation was found in the tumor tissue after treatment with MMP-sensitive conjugate than the MMP insensitive conjugate. However, since this difference in the tumor accumulation was not statistically significant, it was speculated that high dosage may have saturated the MMP mediated drug release and caused the endocytotic uptake to dominate. Also, most of the methotrexate in the tumor tissue was uncleaved thus downplaying the role of MMPs in tumor-specific delivery of the

macromolecular peptidyl-methotrexate *in vivo*. Also, the cleavage of the conjugates in the small intestine and subsequent toxicity was surprising and did not correlate with MMP activation. The MMP-sensitive conjugates exhibited higher intestinal cleavage than the MMP-insensitive conjugate, but the MMP-insensitive conjugate was significantly more toxic. This confirmed that enzymes other than MMP were involved in the activity of the conjugates in normal tissues and particular attention must be paid to peptide selection (Chau *et al.*, 2006a; Chau *et al.*, 2006b). This study showcased the complexity of factors that should be considered in interpreting *in vivo* data obtained with the drug-peptide conjugates designed to be cleaved specifically by MMPs.

Others have developed higher order structures utilizing the same carrier system type, Figure 2B. The polymer-peptide-drug conjugates can be formulated as micelles if the drug is hydrophobic and a proper architecture is maintained. When micelles of poly(ethylene glycol) methyl ether (mPEG)-GPLG-V-Dox conjugates were injected intravenously in Lewis lung carcinoma xenograft bearing mice, the life span increased significantly compared to the free doxorubicin group. However, a higher dose of the conjugate than doxorubicin was required to show similar therapeutic effect (Bae *et al.*, 2003). It was proposed that these systems took advantage of both the active targeting by the MMP-activation as well as the passive accumulation at the tumors based upon the EPR effect (Maeda *et al.*, 2003).

In an attempt to circumvent systemic drug activation, locally implanted hydrogel-based devices have also been designed. Such implants placed at the site of the tumor after surgical resection can help prevent recurrence of the disease. A cytotoxic drug, cisplatin, was attached to MMP cleavable peptide (CG~LDD) pendant on the polymeric hydrogel network, Figure 2C. The drug was released in the presence of MMPs and MMPs expressed by the tumor cells. In the case of the hydrogel devices, drug release rate depends on the penetration of MMPs into the network of hydrogel in addition to peptide cleavage kinetics (Tauro and Gemeinhart, 2004; Tauro and Gemeinhart, 2005). Each of these carrier-peptide drug conjugates has only begun to be examined. Further research is ongoing to understand the properties that are necessary to control the drug activation process. These systems also can be expanded from small molecule delivery to biomacromolecule delivery.

MMP activated peptide and protein delivery

Much as small molecule therapeutics can be attached to carrier, biomacromolecules can be attached to carrier molecules for MMP activation. Decay accelerating factor (DAF), an anti-complement protein, is effective in reducing activity of complement in inflammatory diseases, including rheumatoid arthritis, but would have systemic effect unless inactivated as a prodrug. A MMP-3 and 8 sensitive polypeptide sequence derived from interglobular domain of aggrecan, a major proteoglycan in cartilage, was incorporated as a linker in a fusion protein of DAF with human immunoglobulin G (IgG). Fusion with IgG increased the circulation half-life of DAF while the MMP-sensitive linkers helped to target DAF to sites of inflammation and prevent systemic inhibition of the complement system. Specific cleavage was shown using purified MMPs, MMPs secreted from cytokine-stimulated chondrocytes and in the complex milieu of synovial fluid *ex vivo* in humans and rats and *in vivo* in rats. Initial *in vivo* studies demonstrating distribution of prodrug and efficacy in reducing inflammation are warranted (Harris *et al.*, 2003). This system allowed a natural inhibitor to be delivered in a targeted manner.

MMP can also be used to activate cytolytic protein toxins. Two mutated anthrax toxin protective antigen (PA) proteins were constructed in which furin recognition site was replaced by the MMP-sensitive sequences GPLG~MLSQ and GPLG~LWAQ. The mutated proteins were completely resistant to furin but were efficiently processed by MMP-2 and 9.

When combined with cytotoxin FP59, MMP activated PA proteins caused internalization of this cytotoxin leading to cell death. Thus, MMP-sensitive PA proteins selectively killed MMP-2 and 9 -overexpressing human tumor cells in coculture model with gelatinase nonexpressing cells (Vero cells). These proteins were also shown to be cleaved by MT1-MMP. This study indicated that PA activation occurred on the tumor cell surface and not in the supernatant and that the agents were selectively internalized within the activating cells (Liu *et al.*, 2000).

Similarly, a natural cytolytic peptide, melittin, can be incorporated into MMP-activated systems. Melittin is highly active and targeting is necessary to prevent side-effects. Biotinylated melittin-MMP-2 cleavable peptide, Figure 3, has been interacted with avidin, as a carrier, rendering melittin inactive until the peptide linker was cleaved by MMP-2. Unfortunately, the MMP-sensitive peptide sequence was not described by the authors. Specificity of the DTS was shown by strong cytolytic activity against cancer cells with high MMP-2 activity while being much less cytolytic against normal mouse L-cells. In mice, B16 tumors injected with the conjugate were significantly smaller compared to untreated tumors at the end of 25 days. Not only was there a difference in size, but the conjugate inhibited further growth of the tumor (Holle *et al.*, 2003).

In an attempt to reduce tumor proliferation, tumor necrosis factor (TNF) can be used as a prodrug. However, many TNF prodrugs have not exhibited adequate specificity. Multi-component prodrugs composed of a fusion protein of TNF with an N-terminal single chain antibody variable fragment (scFv) targeting abundant tumor markers and a C-terminal TNF receptor inhibitory fragment (TNFR1) were proposed for dual-level targeting. A MMP cleavable TNF prodrug (scFv-TNF-Linker-TNFR1) was designed by introducing distinct MMP-2 recognition motifs between the TNF and TNFR1 fragments. Two MMP-2-sensitive linkers described in this study were comprised of three consecutive repeats of the motif GPLG~VRGK or HPVG~LLAR. Processing of these TNF prodrugs by recombinant active MMP-2 was confirmed by western blot analyses. The prodrugs were designed to be specifically activated upon antigen binding. These prodrugs were found to be activated by MMP-expressing HT1080 cells after specific membrane binding of the prodrug via the targeting scFv (Gerspach *et al.*, 2006).

Overexpression of MMP in glioma has been used to target fusogenic membrane glycoprotein (FMG), potent therapeutic transgenes, which otherwise induce undesirable fusion in normal human astrocytes and fibroblasts. Expression constructs were made expressing the hyperfusogenic FMG, Gibbon Ape Leukemia Virus envelope glycoprotein (GALV), linked to a blocking ligand (the C-terminal extracellular domain of CD40 ligand) via a MMP cleavable linker (GALV M40). The MMP cleavable linker, GGPLG~LWAGG was based on the commercially available fluorogenic substrate of MMP-2 and 9 (Netzelarnett *et al.*, 1991). A control construct containing the non-MMP specific linker GGGGS was used for comparison. When tested in malignant glioma and normal human astrocytes cell lines expressing varying levels of MMPs, fusion occurred after transfection with GALV M40 to an extent dependent on expression of MMP in the specific cell line. The fusion observed was completely blocked by broad-spectrum MMP inhibitors. Transfection with vectors expressing MMP-insensitive peptides exhibited little fusion and cell death. Fusion was also observed in MMP-poor cell lines transfected with GALV M40 constructs after mixing with MMP overexpressing, untransfected glioma cells as well as when stable transfectants expressing MT-1 MMP and MT2-MMP were established in these MMP-poor cell lines. When pre-infected U87 cells were inoculated in nude mice, the GALV M40 infected group showed significant suppression of tumor growth compared to control groups (Johnson *et al.*, 2003). Therapeutic efficacy of this targeting approach was proved in glioma xenografts *in vivo* by using adenoviral vectors encoding the targeted constructs (Allen *et al.*,

2004). These biomacromolecule delivery systems exemplified how specific proteins and peptides can be released, and similar strategies can be designed for MMP-targeted activation of almost any biomacromolecule.

MMP activated DNA delivery

MMPs have been used for targeting viral vectors for gene delivery. Formation of noncovalent complexes between homotrimeric viral envelope glycoproteins and a larger extraviral glycoprotein component (SU) are required for the fusion of viral and cellular membranes. Inhibitory polypeptides have been fused with the N-terminus of the SU component of the viral membrane to block the infectivity of the retroviral vectors by inhibiting this complex formation. Protease-cleavable linkers were used for the attachment of inhibitory polypeptides such that exposure to relevant protease cleaves the polypeptide from the vector, thereby restoring the infectivity, Figure 4. Whether this protease targeting strategy can be used to target gene delivery *in vivo* using MMP was tested using retroviral vectors displaying two different inhibitory polypeptides, epidermal growth factor (EGF) residues 1-53 and CD40 ligand residues 116-261, fused through MMP cleavable linker (AAAPLG~LWA). The EGF domain was shown to be efficiently cleaved from the chimeric envelope by activated MMP-2, whereas control chimeric envelope containing MMP-insensitive linker (AAAGGGGS) was unaffected. MMP-activatable vectors could discriminate between MMP-rich and MMP-poor cells in a heterogeneous mixture of the two cell types whereas control vector containing non-cleavable linker did not show any significant selectivity. When the EGF-displaying vectors were administered by intratumoral inoculation of xenografts in nude mice, selective transduction of MMP-rich xenografts was apparent (Peng *et al.*, 1997; Peng *et al.*, 1999).

Similarly, a recombinant Sendai virus (SeV) that is selectively allowed to propagate and produce progeny in response to human tumor cells expressing MMPs has also been generated. This is a non-virulent oncolytic SeV in which the tryptic cleavage site of fusion glycoprotein (GVPQSR) is changed to a site susceptible to MMP cleavage (PLG~MTS). Cleavage is required for activation of fusion protein and thus for the infectivity of the virus. This is considered “gene delivery” due to the fact that the virus cannot form progeny in a tissue unless the viral protein is activated. The recombinant virus with the MMP cleavage motif spread efficiently in cell lines expressing MMPs, but not in cells that do not express MMP. The virus spread extensively in tumor xenografts in mice without disseminating to the surrounding normal cells (Kinoh *et al.*, 2004). These two cases exemplify the creative ways that MMPs can be used to deliver specific agents to diseased tissue. MMPs have also been used to improve the targetability of drug-carrier systems to incorporate MMP-sensitivity.

MMP-deprotection of liposomes

Due to the tissue specific but not disease specific nature of some targeting ligands, combining tissue-specific ligand-targeting with disease-specific MMP-deprotection of the ligand can be used to allow selective disease targeting, Figure 5. Specifically, MMPs have been used to improve the targeting ability of galactosylated liposomes to hepatocellular carcinoma (HCC). These liposomes incorporated dioleoylphosphatidylethanolamine (DOPE) conjugated with poly(ethylene glycol) (PEG) bound to a MMP-2 substrate peptide (GPLG~IAGQ). The liposomes also contained a galactosylated cholesterol derivative (Gal-C4-Chol). These liposomes (Gal-PEG-PD-liposomes) were designed to target HCC by two mechanisms, MMP-deprotection and galactose-receptor binding. Due to galactose, the ligand, being buried within the PEG corona, liposomes would not be taken up by normal hepatocytes. In the local environment of HCC cells, the MMP-sensitive peptide can be cleaved by MMP-2, thus exposing the galactose moieties that can be selectively recognized

by asialoglycoprotein receptors on HCC cells. Release of PEG coating from the liposomes due to specific cleavage by MMP-2 was confirmed by time-dependent appearance of NH₂-IAGQ-DOPE after incubation of liposomes with purified hMMP-2. The PEG-PD cleavage was blocked by a known MMP inhibitor. The liposomes selectively delivered the drug load to HCC cells. The concept was confirmed by showing higher uptake of hMMP-2 preactivated Gal-PEG-PD-liposomes in HepG2 cells (Terada *et al.*, 2006). This dual targeting mechanism approach can be extended to other types of drug carriers.

MMPs used in ligand targeting

An indirect, but more traditional, way of using MMPs to target drug delivery is through use of specific MMP inhibitors, Figure 6. A novel cyclic peptide inhibitor (CTT) specific for gelatinases (MMP-2, 9) was complexed to liposomes to enhance their uptake by tumor cells expressing these MMPs. Thus, MMP-2 and 9 served as receptors of CTT ligand for liposomal targeting of invasive cells. CTT enhanced cellular uptake of liposome encapsulated, water-soluble fluorescent marker, rhodamine B, by 3- to 4- fold by gelatinase expressing cells. Augmented cytotoxicity in leukemia and sarcoma cells was obtained by enhanced delivery of adriamycin-containing CTT-liposomes compared to the liposomes without the peptide (Medina *et al.*, 2001). The same concept was applied to tumor imaging using liposomes coated with Technetium- 99m labeled CTT. These labeled liposomes showed tumor homing ability that could be detected by gamma imaging in live mice bearing KS1767 Kaposi's sarcoma xenografts (Medina *et al.*, 2005). As such, MMP proteolytic activity can be used to target different diseases by releasing an active agent, or simple ligand-binding can be used to bind an agent to or near the surface of a cell using MMPs.

Key considerations for MMP activated drug targeting

As is apparent from the multitude of diseases in which MMPs are implicated, MMPs are viewed by many as therapeutic targets. MMPs have become a major area of DTS research in the last several years, but much is still to be discovered about the potential of MMPs as targets for drug delivery. Several issues are recurring in the literature and need to be addressed by MMP-based DTS researchers.

1. **Validation of specific MMPs as targets-** It is important to realize that MMP-targeted delivery systems are bound to face many of the same difficulties as MMPis in the clinic. Hence, the clinical implication for the system must be clear so that specific MMPs overexpressed and overactive in the particular disease can be targeted by choosing specific and selective substrates as linkers. Similar MMPs may be targets in one disease and counter- or anti-targets in another, so choosing the appropriate MMPs for targeting is quite important. Also, choice of single MMP or multi-MMP targeting should be validated. MMP-2 and MMP-9 have very similar substrate specificity and are difficult to differentially target. These two MMPs have been identified as targets and anti-targets in cancer (Overall and Kleifeld, 2006). Identifying MMP-2 (validated target) but not MMP-9 (suggested anti-target) specific substrates or DTS would be beneficial. Similarly, substrates that specifically target MMP-1, 2, and 7 but not others would be beneficial since they have all been identified as targets (Overall and Kleifeld, 2006).
2. **Specificity of the peptide linker-** Peptides used as MMP-sensitive or MMP-inhibitory linkers should be specific substrates of the MMP intended to be exploited for targeting. It is desirable that the linker is rapidly cleaved once it encounters MMP in the diseased tissue. Enzyme kinetics studies should be conducted to ensure high catalytic efficiency of MMP towards the selected linkers. These peptide linkers can be rationally designed based on the literature available

for MMP substrate specificities (Massova *et al.*, 1997; Nagase and Fields, 1996; Turk *et al.*, 2001). Since much is already known about the specificity of many MMPs, growth in this area may be in the non-natural peptide sequences or peptidomimetic chemistries.

3. **Selectivity of the peptide linker-** Peptide selectivity for the specific MMP for which it is designed is very important for the success of this targeting approach. Cleavage of the linker by other enzymes, including serum proteases or other MMPs expressed in non-disease tissues, would lead to non-specific release of drug causing systemic side effects (Chau *et al.*, 2006a; Chau *et al.*, 2006b). Despite the depth of knowledge concerning substrate specificity, significantly less is known about MMP selectivity. Several reports have shown that most peptide substrates are cleaved by multiple MMPs with only 2–10 fold selectivity. In certain circumstances this may not be important, but when a target and counter-target both cleave a particular sequence, the MMP-based DTS will fail.
4. **Chemistry of the attachment of the peptide with the drug-** It is necessary that the bond introduced between the drug molecule and the peptide be stable so that nonspecific release of the drug is avoided. The exact chemistry used to create the MMP-sensitive DTS will be based upon the carrier (if present) and the active molecule. In the case of peptide and protein molecules, using fusion technologies to create pro-drugs is a wise choice. When the active agent is a small molecule, covalent attachment is typically preferred as non-covalent bonds can cause increased nonspecific release (Tauro and Gemeinhart, 2005). Caution should also be exercised in using ester linkages which may be hydrolyzed faster than the MMP cleavage. The final delivery platform may dictate the design of the linkage as well. For example, links between the drug and pendant peptide in a hydrogel device intended to be implanted for long term delivery should be very stable, whereas, such a link in a peptide-drug conjugate administered more frequently can be allowed to be degraded after the peptide is cleaved by the MMP. Finally, the chemistry of the attachment of drug to the peptide should be designed carefully such that the prodrug remains inactive until the peptide is cleaved while the peptidyl-drug moiety generated after cleavage regains its activity. To achieve this, it may be necessary to avoid involvement of functional groups of the drug molecule that are required for activity and exhibit less bulk tolerance during conjugation.
5. **Choice of the carrier-** In case a carrier macromolecule, e.g. polymer, protein or lipid, is used to prolong circulation of the peptide-drug conjugate, the carrier molecule should not hinder access of the enzyme to the linker. Such hindrance may be caused by the bulk of the carrier and/or electrostatic repulsion of negatively charged MMP catalytic site by the negative charge on the carrier (Chau *et al.*, 2004). These difficulties can be overcome by selecting carriers that are not or less negatively charged and using a flexible linker between the carrier and the peptide. Non-specific or deleterious interactions are also possible between the carrier and the drug. It is possible that aggregation or misfolding could occur with either natural or synthetic macromolecular carriers. As such, basic characterization of the system is needed to confirm that higher-order structures are not formed, and if they are formed that the higher-order structures maintain functionality.
6. **Activity of peptidyl-drug moiety-** The currently described MMP-activated systems, and most foreseeable systems, typically retain approximately 1–4 amino acid attached to the drug after MMP cleavage of the prodrug. It is necessary to test the activity of this peptidyl-drug compared to the free drug. This is due to the endopeptidase nature of the MMP family (Nagase and Fields, 1996; Turk *et al.*,

2001). It is likely that the potency of the drug will be reduced due to attachment of amino acids; however, the decrease in potency should not compromise the advantage of increase in dosage allowed by reduced side effects. The peptidyl-drug moiety may be designed to be further cleaved to amino acid derivative of the drug and/or the active drug molecule by other enzymes present at the disease site. Chemistries that allow the original parent drug to be released would be advantageous in most circumstances (Kline *et al.*, 2004).

7. **Cleavage profile in native disease tissue-** Biology of any disease tissue, especially tumor tissue, is very complex. Regulation of MMP activity at the disease site is further complicated by interplay of various induction, activation and inhibition mechanisms. Hence, it is very important to extend the proof of concept of MMP cleavable drug targeting to *in vivo* models. For example, cells in culture may not express TIMPs at the levels observed in tumor tissue and hence results obtained in the cell culture studies may not be reflected in animal models. Furthermore, it is also important to take into consideration inter-species differences in MMP expression while making any extrapolation to human clinical application.
8. **Combination with other targeting strategies-** Delivery approaches can be designed that synergistically combine the MMP targeting mechanism with other active targeting strategies. For example, a cytotoxic protein that is targeted to a tumor cell surface protein and depends on MMPs for activation can be expected to have higher therapeutic index (Liu *et al.*, 2000).

Conclusions

The abundance of MMPs in different diseases makes them attractive targets to be exploited for drug delivery. Development of targeted drug delivery using MMPs has been steadily pursued since the birth of the concept in late 1990s, however, not at a pace expected based on the emergence of new knowledge about MMPs and their association with various diseases. As research in molecular biology and pathophysiology of MMPs continues, more information is being made available to decide which specific MMPs should be targeted in particular diseases. Research experience has helped us to understand the important considerations for the design of drug targeting strategies utilizing MMPs. Based on this groundwork, drug delivery efforts using this ubiquitous target need to be intensified to realize the clinical application of the fundamental MMP research.

Acknowledgments

Our original work in MMP-based drug targeting has been funded by the National Eye Institute (EY014357) and was conducted in facilities constructed with support from Research Facilities Improvement Program Grant Number C06 RR15482 from the National Center for Research Resources, NIH. The authors thank Ernie Gemeinhart for careful proofreading and critical review of this manuscript.

ABBREVIATIONS

AP-1	activator protein-1
DAF	decay accelerating factor
Dox	doxorubicin
DTS	drug targeting system
ECM	extracellular matrix
EGF	epidermal growth factor

EPR	enhanced permeability and retention
ETS	erythroblastosis twenty-six
FGF	fibroblast growth factor
FMG	fusogenic membrane glycoprotein
GALV	Gibbon Ape Leukemia Virus envelope glycoprotein
HCC	hepatocellular carcinoma
IgG	immunoglobulin G
IL	interleukin
LV	left ventricular
MMP	matrix metalloprotease
MMPIs	Matrix metalloprotease inhibitors
mPEG	poly(ethylene glycol) methyl ether
PA	protective antigen
PEG-PD	poly(ethylene glycol)-peptide
RECK	reversion-inducing cysteine-rich protein with Kazal motifs
scFv	single chain antibody variable fragment
SeV	Sendai virus
SNPs	single nucleotide polymorphisms
TGF	tumor growth factor
TIMPs	tissue inhibitors of metalloproteases
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TSP-1	thrombospondin-1
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
L-amino acids in the peptide sequences are designated by one-letter abbreviations used under standard convention	
D-amino acids in the peptide sequences are designated by three-letter abbreviations used under standard convention	
MMP cleavage sites within peptides are denoted by “~”	

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Figure 1.

Schematic depiction of MMP-activation of a peptide prodrug. To utilize MMP targeting, an inactive peptide (blue) is attached to a drug (magenta). When MMP is present, the MMP (yellow) cleaves the peptide (blue) releasing an inactive peptide (blue) and a peptide-modified (aqua) drug (magenta). The peptide-fragment that is retained as part of the chemotherapeutic agent must be appropriate that the agent is active following MMP-cleavage.

Figure 2.

Schematic depiction of MMP-activation of carrier-peptide prodrugs. Three general subtypes of this category exist. In the first (A), a macromolecule is modified to bear one or more peptide-prodrugs on the macromolecule chain. The peptide-prodrug may be randomly placed on the chain, specifically placed on the chain (as in a protein), or at the end(s) of the macromolecular carrier. In the second, (B), macromolecular carriers may assemble into higher order structures such as micelles. In the final carrier-peptide conjugate (C), the drug is held within a three-dimensional hydrogel network. In each, the macromolecular carrier (green), MMP-sensitive peptide (blue), and therapeutic agent (magenta) are presented. The activation mechanism is similar to the prodrug (Figure 1) except the therapeutic agent

(typically with an inactive portion of the MMP-sensitive peptide) is released from the inactive macromolecule.

Figure 3. Schematic structure of the avidin-biotin-peptidyl-melittin MMP activated drug targeting system. Cleavage of the MMP-sensitive peptide by MMP releases the cytolytic melittin. Avidin (green), biotin (light blue), MMP-sensitive peptide (dark blue), peptidyl (aqua)-melittin (magenta) are presented before and after MMP-cleavage.

Figure 4.

Schematic depiction of viral activation by MMPs. Blocking agents (orange star) are attached to the viral attachment and fusion proteins (green circles) via MMP-cleavable linkages (blue lines). The blocking agents prevent viral integration with the host cell (pink). Upon MMP cleavage, viral integration is possible as the blocking agents are freed and host integration is possible.

Figure 5. Schematic depiction of MMP-deprotection of liposomes. Poly(ethylene glycol) (light green) is released from MMP-sensitive peptide (dark blue line)-containing lipids (red) freeing cholesterol-bound galactose (orange star) to bind to liver cancer cells.

Figure 6.

Schematic depiction of MMP-inhibitor as a ligand for MMP targeting. In this case, an MMP-inhibitor or MMP-binding molecule (blue triangle) act as a ligand for surface-bound MMP (yellow). The MMP may be a MT-MMP, an integrin-bound MMP, or any MMP bound to the ECM.

Table 1**Mammalian Matrix Metalloproteinases (MMP) Family**

MMP subclass	MMP designation	Common Name	Domain Structure [†]
Matrilysins	MMP-7	Matrilysin
	MMP-26		
Collagenases	MMP-1	Interstitial collagenase
	MMP-8	Neutrophil collagenase	
Stromelysins	MMP-13	Collagenase-3
	MMP-3	Stromelysin-1	
	MMP-10	Stromelysin-2	
	MMP-11	Stromelysin-3	
Gelatinases	MMP-2	Gelatinase A
	MMP-9	Gelatinase B	
Membrane-type MMPs	MMP-14	MT1-MMP
	MMP-15	MT2-MMP	
	MMP-16	MT3-MMP	
	MMP-24	MT5-MMP	
	MMP-17	MT4-MMP	
	MMP-25	MT6-MMP	
Others	MMP-12	Metalloelastase
	MMP-20	Enamelysin	
	MMP-19		
	MMP-27		
	MMP-28	Epilysin	
	MMP-23

SP, signal peptide; Pro, pro-domain; F, furin-cleavage site; Fn, fibronectin repeat; C5, type-V-collagen-like domain; TM, transmembrane domain; Cs, cytosolic; GPI, glycosylphosphatidylinositol; Cys, cysteine array; Ig, immunoglobulin

[†] domain structures adapted from (Parks, 2004)