On the cover: Interaction sites of REGA-3G12 in the domain structure of gelatinase B (see also Figure 1 in the section of Discussion and Perspectives).


**Katholieke Universiteit Leuven**

Faculty of Medicine

Rega Institute for Medical Research

Laboratory of Immunobiology

# Inhibition of gelatinase B/Matrix metalloproteinase-9

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Promotor: Prof. Dr. Ghislain Opdenakker

Thesis submitted in fulfillment of the requirement to obtain the degree of
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as mass spectrometry, detection of fluorogenic peptide conversion, enzyme kinetics and others. Now and then, he explained to me some interesting background knowledge.

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## Table of Contents

# Curriculum Vitae

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**Publications:**

1. Van Damme, E.J., **J.Hu**, A.Barre, B.Hause, G.Baggerman, P.Rouge, W.J.Peumans. 2001. Purification, characterization, immunolocalization and structural analysis of the abundant cytoplasmic beta-amylase from Calystegia sepium (hedge bindweed) rhizomes. *Eur.J.Biochem.* 268:6263-6273.

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3. **Hu,J.**, P.E.Van den Steen, M.Houde, T.T.Ilenchuk and G.Opdenakker.
2004. Inhibitors of gelatinase B/matrix metalloproteinase-9 activity Comparison of a peptidomimetic and polyhistidine with single-chain derivatives of a neutralizing monoclonal antibody. *Biochem.Pharmacol.* 67:1001-1009.

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8. **Hu,J.**, V.Dubois, P.Chaltin, P.Fiten, C.Dillen, P.E.Van den Steen and G.Opdenakker. 2006. Inhibition of lethal endotoxin shock with an L-pyridylalanine containing metalloproteinase inhibitor selected by high-throughput screening of a new peptide library. (*Submitted for publication*)

**Oral Presentations**

1. Hepatocellular Carcinoma (HCC). 11:00 AM, Dec. 3, 2004, Seminar Room (2nd floor), Rega Institute, Minderbroedersstaat 10, B-3000 Leuven.

2. Peptide Inhibitors of Gelatinase B – generation, kinetics and application. 11:00 AM, April 7, 2006, Seminar Room (2nd floor), Rega Institute, Minderbroedersstraat 10, B-3000 Leuven.

**Published Abstracts:**

1. **Jialiang Hu**, Philippe E. Van den Steen and Ghislain Opdenakker. A microbead assay for the analysis of enzymes and enzyme inhibitors.
"Seventh National BVC-ABC Symposium" Nov. 15, 2002. Namur, Belgium.

2. **Jialiang Hu**, Philippe E. Van den Steen, Francis J. Descamps, Helene Piccard, Ilse Van Aelst, Chris Dillen, Eric Martens, Pierre Fiten and Ghislain Opdenakker.
"1st European Conference on Chemistry for Life Sciences" October 4-8, 2005. Rimini, Italy.

## Abbreviations

| | |
|---|---|
| ADAM | A disintegrin and metalloproteinase |
| ADAMTS | A disintegrin and metalloproteinase with thrombospodin motifs |
| bFGF | Basic fibroblast growth factor |
| Bip | Biphenylalanine |
| CMT | Chemical modified tetracycline |
| CNS | Central nerve system |
| DIEA | Diisopropylethylamine |
| EAE | Experimental autoimmune encephalomyelitis |
| ECM | Extracellular matrix |
| ELISA | Enzyme linked immuno-sorbant assay |
| FACS | Fluorescently activated cell sorter |
| HAMA | Human anti-mouse antibody |
| HATU | N-[(1H-azabenzotriazol-1-yl) (dimethylamino) methylene]-N-methylmethanaminium hexafluophosphate N-oxide |
| HBTU | N-[(1H-benzotriazol-1-yl) (dimethylamino) methylene]-N-methylmethanaminium hexafluophosphate N-oxide |
| HOAt | 1-hydroxyazabenzotriazole |
| HOBt | 1-hydroxybenzotriazole |
| $IC_{50}$ | The concentration of inhibitor that reduces enzyme activity by half |
| IFN-ß | Interferon-beta |
| IgG | Immunoglobulin G |
| IGFBP-3 | Insulin-like growth factor-binding protein-3 |
| IL-2 | Interleukin-2 |
| ip | Intraperitoneal |
| iv | Intravenous |
| $K_m$ | Michaelis constant |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MMP | Matrix metalloproteinase |
| MS | Multiple sclerosis |
| MT1-MMP | Membrane type 1-matrix metalloproteinase |
| NMR | Nuclear magnetic resonance |
| ORF | Open reading frame |
| $PO_4$·Ser | Phosphoserine |
| Pyr·Ala | Pyridylalanine |
| RA | Rheumatoid arthritis |
| RP-HPLC | Reverse phase-high performance liquid chromatography |
| scFv | Single chain variable fragment |
| TACE | Tumor necrosis factor-alpha converting enzyme |
| TC | Tetracycline |
| TGF-ß | Transforming growth factor-beta |
| TIMP | Tissue inhibitor of metalloproteinases |
| TLR | Toll-like receptor |
| TNF-a | Tumor necrosis factor-alpha |
| ZBG | Zinc binding group |

# Introduction

## 1. Matrix metalloproteinases and matrix metalloproteinase-9/gelatinase B

Matrix metalloproteinases (MMPs) form a group of more than twenty enzymes that are involved in the remodeling of extracellular matrix components and thus play a role in physiological processes such as embryo implantation, bone remodeling, organogenesis, and tissue repair and in pathological conditions, including wounds, inflammation, and invasion of cancer cells (1-3).

MMPs are multidomain enzymes (see Figure 1). They all possess a zinc ion, coordinated by three histidines, in their active site. Another signature of MMPs is activation by the so-called cysteine switch (4). When cells produce MMPs, most of the enzymes are secreted in an inactive pro-form with a cysteine sulfhydryl group from the aminoterminal prodomain blocking the active site. Removal of the propeptide of about 10 kDa from the active site, e.g. by proteolysis, leads to enzyme activation. In addition to regulation by gene expression and activation processes, the activities of MMPs are further controlled by the natural tissue inhibitors of metalloproteinases (TIMPs). MMPs act together with serine proteinases and amplify proteolysis in an enzyme cascade.

MMPs have been grouped on the basis of their major substrates and protein domains into collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). Collagenases are capable of attacking a specific scissile bond in triple-helical collagens. Gelatinases typically cleave denatured collagens, whereas stromelysins, also called proteoglycanases, degrade extracellular matrix (ECM) proteoglycan core proteins. MT-MMPs provide cell-bound proteolysis. Functionally, MMPs are divided into constitutive and induced enzymes. In general, the constitutively expressed enzymes fulfill more homeostatic functions, whereas the induced members are linked to regulatory processes and pathological conditions. The best example of this functional dichotomy is observed with the gelatinases, where gelatinase A is mainly constitutive and gelatinase B highly regulated by inflammatory mediators (5).

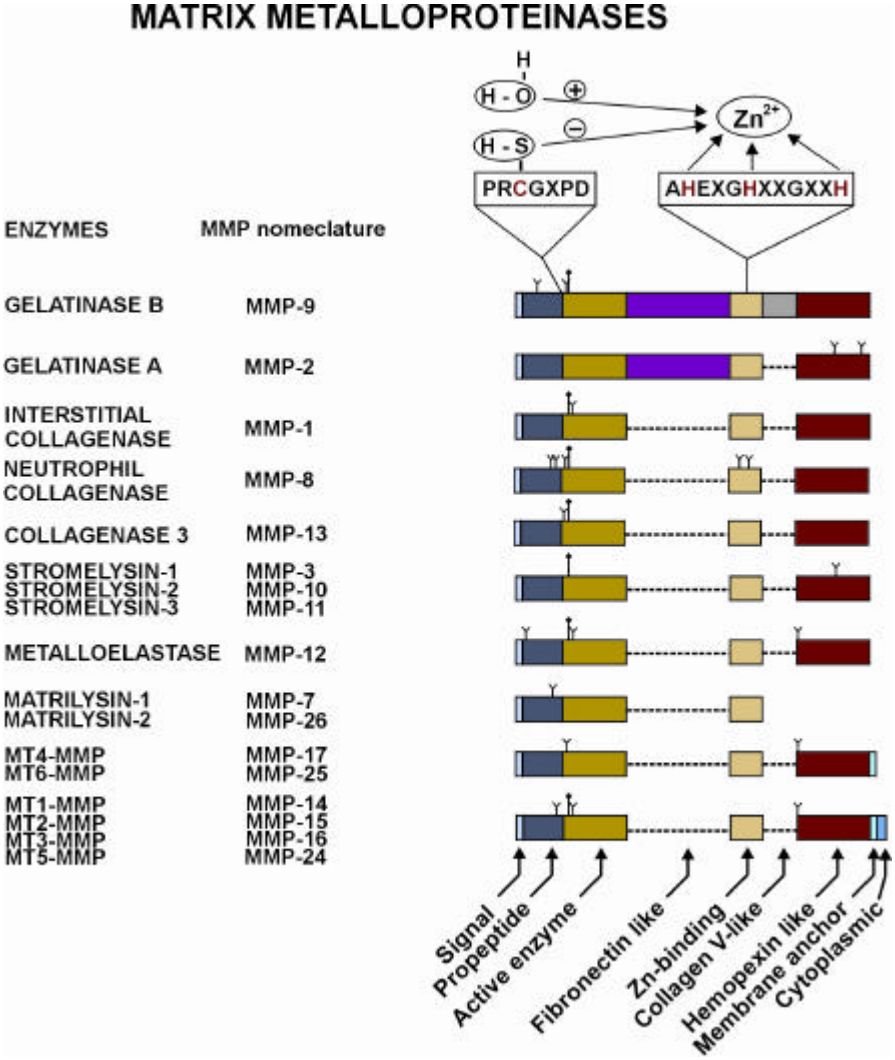



**Figure 1. Domain structures of MMPs.** MMPs are multidomain enzymes with a prodomain, an active domain, a $Zn^{2+}$-binding domain and a hemopexin domain (except MMP-7 and MMP-26). Additionally, membrane-bound MMPs (MT-MMPs) contain a membrane anchor and some MT-MMPs also have a cytoplasmic domain at the carboxyterminus. Gelatinases contain a gelatin-binding fibronectin-like domain and gelatinase B contains also a serine-, threonine-, and proline-rich collagen type V-like domain, which is in theory an attachment site for multiple O-linked glycans. N-glycosylation sites, one of which is conserved among most MMPs (♦), are indicated with a Y-symbol. Part of the propeptide, containing the chelating cysteine, and part of the $Zn^{2+}$-binding domain with three histidines are indicated in one-letter code for amino acids at the top of the figure.

Gelatinase B is a multidomain enzyme. The protein domains which are conserved among MMP family are a prodomain, which is 91 amino acids long for human neutrophil gelatinase B, an active domain and a $Zn^{2+}$-binding domain. Inserted between the active-domain and the $Zn^{2+}$-binding domain is a fibronectin domain composed of three fibronectin-repeats. Fibronectin domains bind to denatured collagen/gelatin with a high affinity. A carboxyterminal hemopexin-domain is present in gelatinase B which is important for the enzyme to bind with TIMP-1. Gelatinase B contains an additional Ser/Thr/Pro-rich collagen V domain between the $Zn^{2+}$-binding domain and hemopexin-domain, which is heavily glycosylated in neutrophil gelatinase B. Structurally, gelatinase B is one of the most complex members of the MMP family (6).

Gelatinase B activity can be regulated at different levels. The transcription of the unique gelatinase B gene depends on various *cis*-elements in its gene promoter and is induced or repressed by a large variety of soluble factors (e.g. cytokines, growth factors etc.) or by cell contacts. Secretion is a specific way of regulation for gelatinase B molecules stored in granules. After secretion, the progelatinase B must be activated through an activation network. The enzyme activity is further regulated by inhibition (e.g. by TIMPs) and by other mechanisms (e.g. stabilization by glycosylation) (6).

Gelatinase B is important for the migration of different cell types such as leukocytes and cancer cells. It contributes to the degradation of basement membranes and components of the extracellular matrix (e.g. gelatin, elastin and aggrecan). Gelatinase B has been shown to be involved in the migration of newly formed leukocytes from the bone marrow to the blood vessels and from the blood vessels to sites of infection in tissues (7, 8). Notably, gelatinase B is present in considerable amounts in the secondary and tertiary granules of neutrophils, which are the first-line defense cells of the innate immune system in inflammatory reactions. After appropriate stimulation, neutrophils degranulate within 20 minutes and release many molecules including the preformed gelatinase B. This makes gelatinase B an excellent pharmaceutical target in inflammatory diseases with a major involvement of neutrophils (5, 6).

Gelatinase B seems to play a role in typical inflammatory and autoimmune diseases. For instance, gelatinase B levels and activity are elevated in the synovial fluid of patients with rheumatoid arthritis (RA), whereas gelatinase A levels remain constant (9). From genetic

knock-out experiments in mice, it has been deduced that gelatinase B has a disease-promoting role (10). Furthermore, gelatinase B potentiates human interleukin-8 at least tenfold and thus fuels acute inflammation (11). Similar findings have been observed in multiple sclerosis (MS): gelatinase B levels are higher in cerebrospinal fluid of MS patients compared with neurological controls, and young gelatinase B-deficient mice are protected against experimental autoimmune encephalomyelitis (12). In addition, gelatinase B cleaves interferon-beta and thereby destroys its disease-limiting activity in MS (13). Targeting gelatinase B in RA or MS may thus be beneficial for several reasons. A prototypic example of the role of gelatinases in acute inflammatory diseases is endotoxinemia and endotoxin shock. Endotoxin induces production of gelatinase B in many cell types *in vitro* and *in vivo* (6), and endotoxinemia may then result in vascular effects (14).

Gelatinase B is a highly regulated inflammatory proteinase that cleaves extracellular structural proteins, proteinase inhibitors, such as a1-antitrypsin, cell surface molecules, and, last but not least, signaling molecules, including hormones, cytokines, and chemokines (2, 5, 15-29).

## 2. Inhibition as a control mechanism of MMP activity

Both, macromolecular inhibitors (natural inhibitors such as tissue inhibitors of MMPs or TIMPs and monoclonal antibodies) and small molecules (synthetic and natural products) have been considered as potential therapies for diseases in which excess MMP activity has been implicated (30). However, technical difficulties with the production of macromolecular proteins have often been invoked as limitation for their development. Nevertheless, monoclonal antibody derivatives (31) which are specific for MMP-9 are promising drugs to be used as therapeutics. Small molecular weight inhibitors of gelatinase B have been discovered and evaluated for clinical use, but in general these seem to be unselective and to cause side-effects (32). The lack of selectivity of these drug candidates is likely due to their small molecular size. As no systematic review about MMP-9 inhibitors appeared yet, we classified the known inhibitors as follows.

### 2.1 Small molecular inhibitors

The structures of MMPs, in particular the catalytic sites, have been extensively studied with high-resolution X-ray crystallography and NMR analysis (33). The members of the family differ substantially in domain structure, but all members whose structures (of the catalytic domains) have been solved, possess similar core elements in their catalytic domains. A schematic representation of a hexapeptide substrate bound into an MMP active site is given in Figure 2. As different MMPs have similar core structures in the active sites, and since the insertion of the fibronectin domain does not change the active domain structure (34), it is reasonable to apply this information in the design of MMP-9 inhibitors.

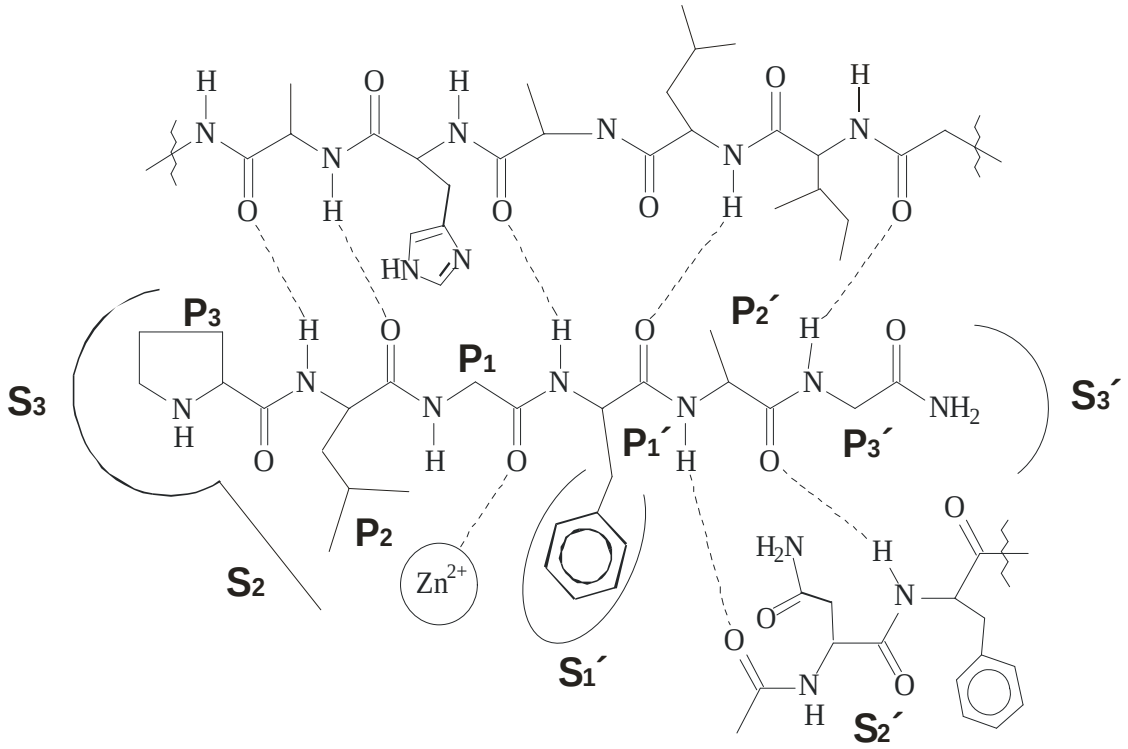



**Figure 2. Schematic representation of the interaction of a hexapeptide substrate with an MMP active-site.** Numbering follows that for MMP-8. The $P_1´$, $P_2´$ and $P_3´$ amino acids located on the right hand site of the cleavage site are also referred to as primed residues and are part of the peptide substrate. The residues at the top of the figure as well as the $Zn^{2+}$ and the residues which form the subsites ($S_3'$, $S_2'$, $S_1'$, $S_2$ and $S_3$) are from the enzyme (35).

The principal approach taken for the development of synthetic MMP inhibitors is the substrate-based design of peptides and analogues derived from the information of the sequence around the cleavage site. Later on, non-peptide-based inhibitors have been identified. However, most MMP inhibitors discovered by the process of structure-based design (based on enzyme structure) are analogues of compounds obtained by the substrate-based approach. For a general overview of inhibitors of all MMPs, the reader is referred to an excellent overview by Kontogiorges and colleagues (36). In our review, we will emphasize inhibition of one specific enzyme, MMP-9 or gelatinase B.

The requirement for a small molecule to be an effective MMP inhibitor is a $Zn^{2+}$-binding group (ZBG, e.g. carboxylic acid, hydroxamic acid, sulfhydryl, etc.) and one or more non-covalent interactions between the inhibitor and the enzyme backbone such as hydrogen bonds or van der Waals interactions. By comparison of different ZBGs without changes in the rest of the inhibitor structure, the question is “which ZBG is optimal?”. In terms of MMP-1 inhibition, the orders are as follows: hydroxamate > sulfhydryl > phosphinate > carboxylate. Based on different ZBGs and the backbone structures, MMP inhibitors may be classified into a few broad classes. For the different ZBGs that have been explored, the hydroxamate function provides the best potency, followed by reverse hydroxamates, while thiols are typically 20-50-fold less potent and carboxylates are 100-2000-fold less potent (37). Certain large substituents at $P_1'$ lead to more potent inhibitors for most MMPs, including MMP-9 (Figure 2). In general, small effects on potency are observed by substitution at $P_2'$ and $P_3'$, although particular substituents have been found to provide useful levels of selectivity for specific MMPs.

Until now, many effective small molecule MMPIs have been discovered. Some structure-activity relationships are exemplified in the following sections. This overview is centered on the inhibition of MMP-9.

2.1.1 Hydroxamate based MMP/MMP-9 inhibitors

Most successful small molecule MMP inhibitors use hydroxamate as ZBG. The hydroxamate acts as a bidentate ligand with the active-site zinc ion to form slightly distorted trigonal-bipyramidal coordination geometry. In MMP-1 inhibition (Figure 3), the hydroxamate oxyanion forms a strong, short H bond to the carboxylate oxygen of Glu-219 that is oriented toward the unprimed binding regions. An H bond between the hydroxamate NH and the carbonyl oxygen of Ala-182 also contributes to binding. Thus a set of interactions are achieved at the site, without any significant unfavorable contact (33). The hydroxamate MMP inhibitors (MMPIs) can be further grouped as substrate-analogue peptide, succinyl hydroxamate, sulfonamide hydroxamate and derivatives.

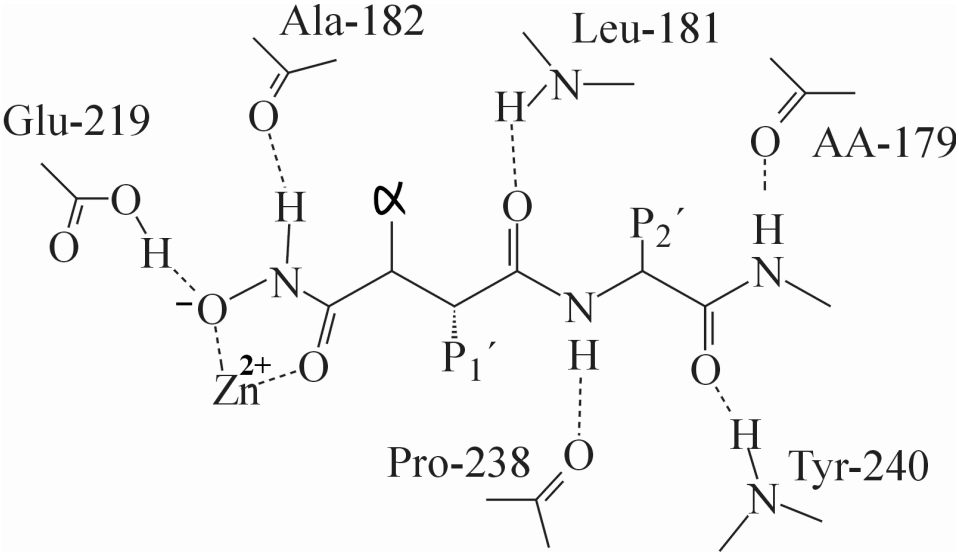



**Figure 3. Interactions of a right-hand side hydroxamate inhibitor with MMP-1 active site.**

*2.1.1.1 Succinyl hydroxamates*

Succinyl hydroxamate MMP inhibitors are among the early successful examples. The structure-activity relationships (SAR) for succinyl hydroxamate have been extensively explored (Figure 4) (33).

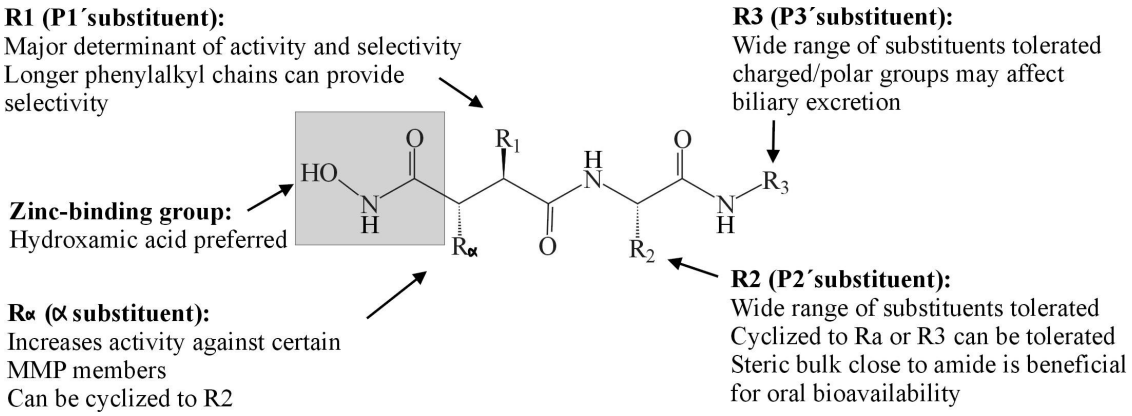



**Figure 4. Summary of structure-activity relationships for right hand side MMP inhibitors (33).**

The three best known examples of succinyl hydroxamate MMPIs are Batimastat, BB-1101 and marimastat (Figure 5). They are all broad-spectrum inhibitors which have displayed efficacy in animal models of human diseases. Marimastat was found to be orally available, partially due to the increased aqueous solubility achieved by the introduction of the a-hydroxyl group. The a-substituent such as allyl (in BB-1101) had a beneficial effect on the inhibition of TACE/ADAM-17 (TNF converting enzyme/a disintegrin and metalloproteinase -17). This results in a benefit in diseases which involve both inflammation and matrix remodeling (38) and hence involve MMPs and TACE.

Cyclization of $P_1$ and $P_2'$ is another possibility of modifying the inhibitor. $P_1$ and $P_2'$ substituents were observed to be directed away from the active site into the solvent (39). Similar strategies lead to the discovery of SE205 and SC903, cyclic inhibitors possessing similar potency to uncyclized analogues. Interestingly, this cyclization strategy resulted in a substantial increase in aqueous solubility (39) (Figure 5).

Structural data of MMPs revealed that the $S_1'$ subsite is a deep pocket for several of the enzymes (e.g. MMP-2, -3, -8 and -9), but is occluded for a few of the MMPs (e.g. MMP-1, -7). A bulky $P_1'$ side group may confer selective inhibition for MMP-2, -8 and -9. This has been confirmed in our own library screening work in which peptides with a biphenyl group at $P_1'$ showed, in general, higher inhibitory activity against MMP-9 (*vide infra*).

X-ray crystallographic analysis of MMP-inhibitor complexes revealed that the $P_2'$ group of peptidyl succinyl hydroxamic acid-based MMP inhibitors points away from the enzyme and makes few contacts with the $S_2'$ cleft. Modification of the $P_2'$ side-group has

a modest effect on *in vitro* inhibitory activity. However, the group at $P_2'$ can have an effect on the pharmacokinetic properties of the inhibitors. The oral activity of marimastat and Ro31-9790 (Figure 5) results from the beneficial effect of a sterically bulky tert-butyl group which may assist the adjacent amide bond during absorption from an aqueous environment to the lipid environment of the cell membrane (40). KB-R7785 is orally active and the beneficial effect of the $P_2'$phenyl group on absorption is also attributed to its amide shielding and lipophilicity, which may assists transepithelial resorption (41).

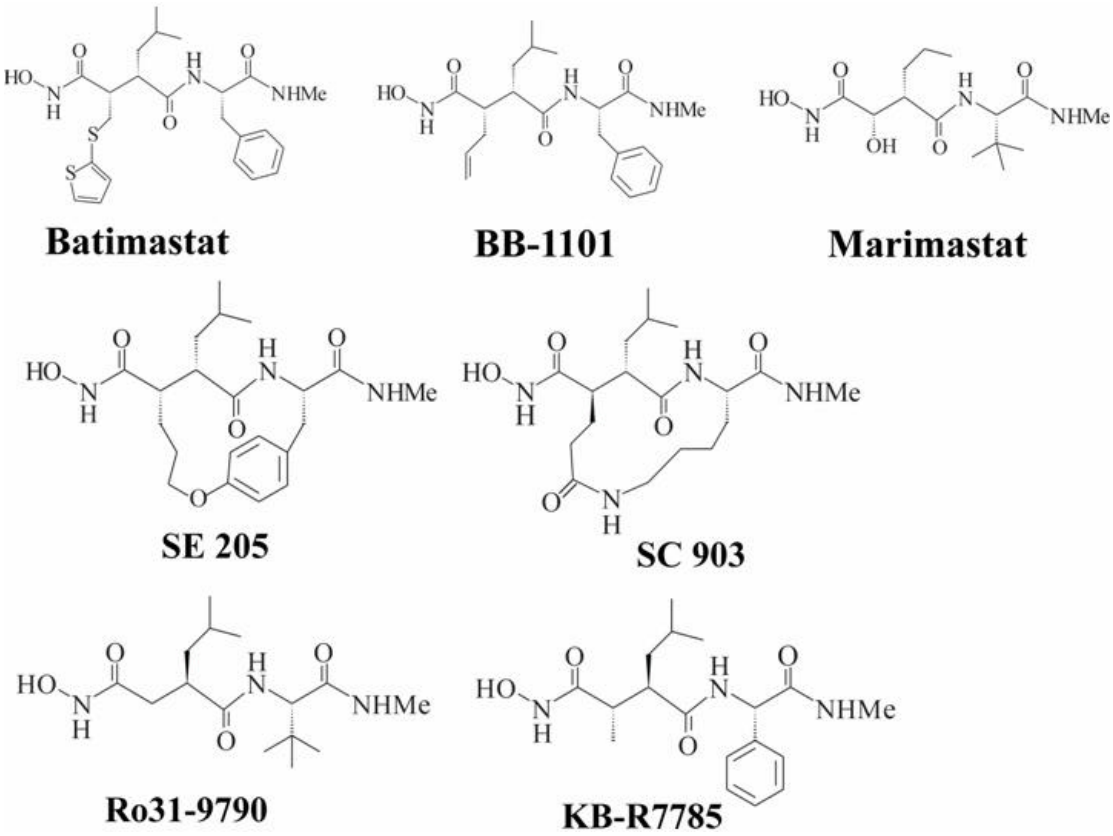



**Figure 5. Succinyl hydroxamate MMP inhibitors.** Batimastat, BB-1101 and marimastat are the three best known succinyl hydroxamates inhibitors. In SE 205 and SC 903, cyclization makes the inhibitors more soluble than their uncyclized analogues. Sterically bulky $P_2'$ side-groups make Ro31-9790 and KB-R7785 orally active.

*2.1.1.2 Sulfonamide hydroxamates and derivatives*

CGS 27023A (Figure 6) is an N-sulfonyl amino acid hydroxamate. It is an orally available broad-spectrum inhibitor (42). Key structural features of CGS 27023A are said to be the isopropyl substituent which slows down metabolism of the adjacent hydroxamic acid group and the basic 3-pyridyl substituent which may aid partitioning into the hydrated negatively charged environment of the cartilage (42). The binding mode of CGS 27023A to MMP-3 has been investigated by NMR spectroscopy. The p-methoxy phenyl substituent of CGS 27023A occupies the $S_1'$ specificity pocket, while the pyridylmethyl and isobutyl substituents occupy the $S_2'$ and $S_1$ subsites, respectively (43).

Modification of the substituent a to the hydroxamic acid in CGS 270323A leads to increases in the inhibition of the deep pocket MMPs, e.g. by the thioester derivative CGS 27023 (44). (Figure 6)

By incorporation of a cyclic quaternary center a to the sulfonyl moiety, RS-113,456 (Figure 6) was identified with a strong inhibitory effect against MMP-1, -2, -3, -8, -9, -12, -13 ($IC_{50}$ for MMP-9 is 0.065 nM). Oral availability and half-life were improved in this series by shifting the cyclic group to be a to the hydroxamic acid as in the compound RS-130,830 (Figure 6). Both RS-113,456 and RS-130,830 lack any stereocenters, yet retain potent inhibitory activity for the deep pocket MMPs, which suggests induced fit of large $P_1'$ substituents with a larger open $S_1'$ pocket (45).

The sulfonamide moiety of CGS 27023A can be replaced by a phosphinamide group as in the compound shown in Figure 7 (46). This compound is a potent inhibitor of MMP-3, the collagenases (MMP-1, -8, -13) and the gelatinases (MMP-2, -9). An X-ray crystal structure of phosphinamide bound to the MMP-3 catalytic domain reveals that the phosphinamide phenyl group is accommodated into the $S_1'$ pocket and that the phosphinamide oxygen is within hydrogen bonding distance to the N-H of Leu-164 and Ala-165 (MMP-3 numbering) (46). However, hydrolysis of the phosphinamide bond which occurs at low pH may limit the potential of these compounds to be developed into orally available drugs. These compounds might be very well useful as enzyme targets after parental administration.

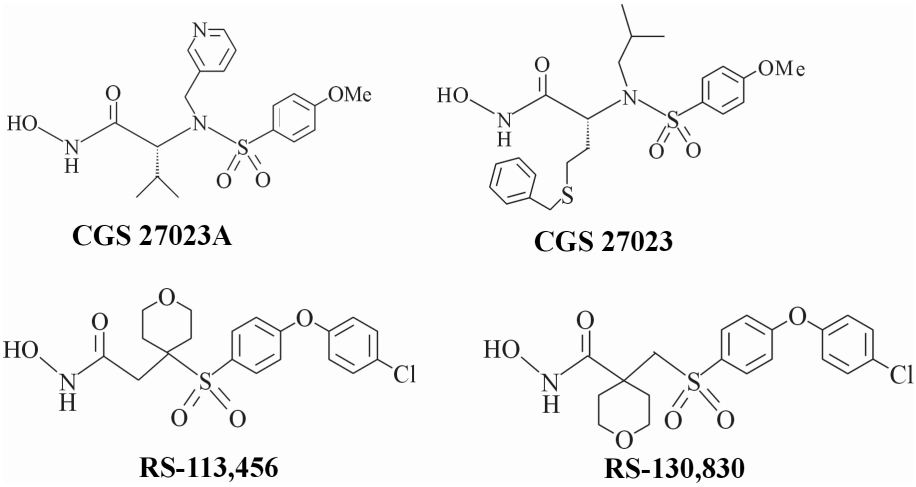



**Figure 6. N-sulfonyl amino acid hydroxamates.** CGS 27023A is an N-sulfonyl amino acid hydroxamate and its a-thioester derivative (CGS 27023) increases the inhibition of deep pocket MMPs. In RS-113,456 and RS-130,830, cyclic quaternary center shift improve their oral availability and half-life.

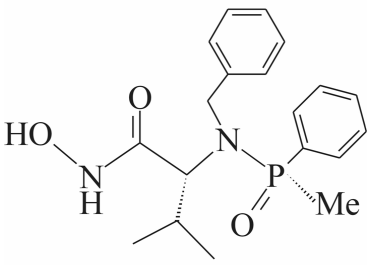


**Figure 7. A phosphinamide-based hydroxamate MMP inhibitor.**

2.1.2 Non-hydroxamate

Successful MMP inhibitors with hydroxamate as zinc binding group were developed. However, most hydroxamate inhibitors lack specificity and inhibit non-MMP zinc based enzymes. New compounds were discovered which made use of alternative $Zn^{2+}$-binding groups, such as carboxylic acid, thiol ZBGs, phosphorus-based ZBGs and some novel ZBGs.

*2.1.2.1 Carboxylates*

Several factors appear to contribute to the advantage in hydroxamate binding over carboxylate binding (Figure 8) and the most important factor is the difference in acidity. Carboxylate inhibitors bind more tightly, about 1 log unit per pH unit, to MMPs as the

pH is lowered, whereas hydroxamate binding is essentially pH independent (pH range 5-8). The neutral carboxylic acid form is binding to the enzyme (i.e. I to III-c in Figure 8). Thus, the 2-3 log unit difference in binding affinity is similar to the 3 pH units between the $pK_a$ of the carboxylates and the neutral pH of 7.4. Thus, at neutral pH, the carboxylate inhibitors are much weaker than hydroxamic acid inhibitors.

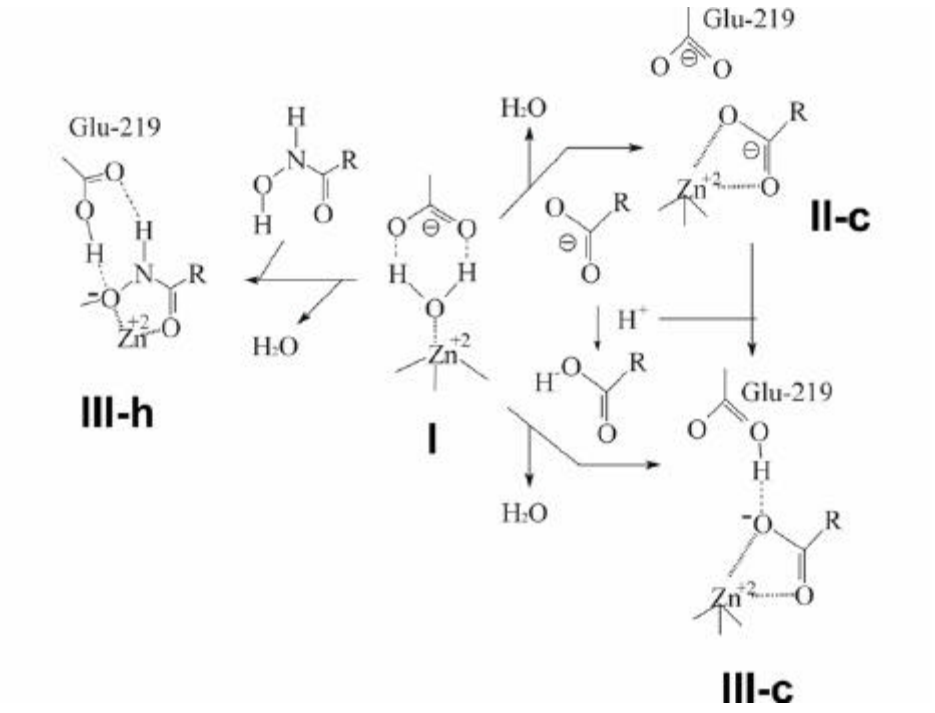



**Figure 8. Interactions of a hydroxamate or a carboxylate group with the active site of a metalloproteinase.** Two hydrogen bonds were formed upon binding of a neutral hydroxamate (III-h) while a proton was needed to transfer from binding state II-c (anionic carboxylate, at neutral pH) to the binding state of III-c (neutral carboxylate, at pH 4.0). In I state, the intact MMP has a water molecule in the enzyme active site.

Many hydroxamic acid MMPIs were prepared by conversion from a carboxylic acid precursor. Although the carboxylate group is a less effective binding group toward $Zn^{2+}$, many carboxylates have been shown to be effective MMP inhibitors and some appear to have promise as therapeutics. The compound in Figure 9 panel A is an example of a good MMP inhibitor with an $IC_{50}$ against MMP-9 of 91 nM.

Interesting structure-activity relationship studies also appear in the development of carboxylate MMPIs. Replacing the nitrogen atom of the amino-carboxylate with a carbon provided a series of glutamic acid derivatives with similar *in vitro* potency and much improved oral bioavailability and *in vivo* pharmacological properties (47) (Figure 9B).

An N-sulfonyl amino acid hydroxamate MMP inhibitor with a biphenyl group is shown in Figure 9 panel C. It is a potent inhibitor of MMP-2 and -9, whereas the equivalent carboxylic acid showed a 40-fold lower potency (48). However, the hydroxamate was found to be unstable *in vivo* because it was metabolically converted to a hydroxylamine compound. The biphenyl group may contribute to the inhibitor binding.

The carboxylate BAY12-9566 (Figure 9D) was applied in trials for osteoarthritis and cancer. The compound exhibits modest *in vitro* activity against most MMPs. It inhibited the invasion of human HT1080 fibrosarcoma cells *in vitro* and was not toxic. Oral treatment of mice (50 mg/kg for 7 days) inhibited angiogenesis induced by Matrigel and bFGF in a subcutaneous pellet assay (49).

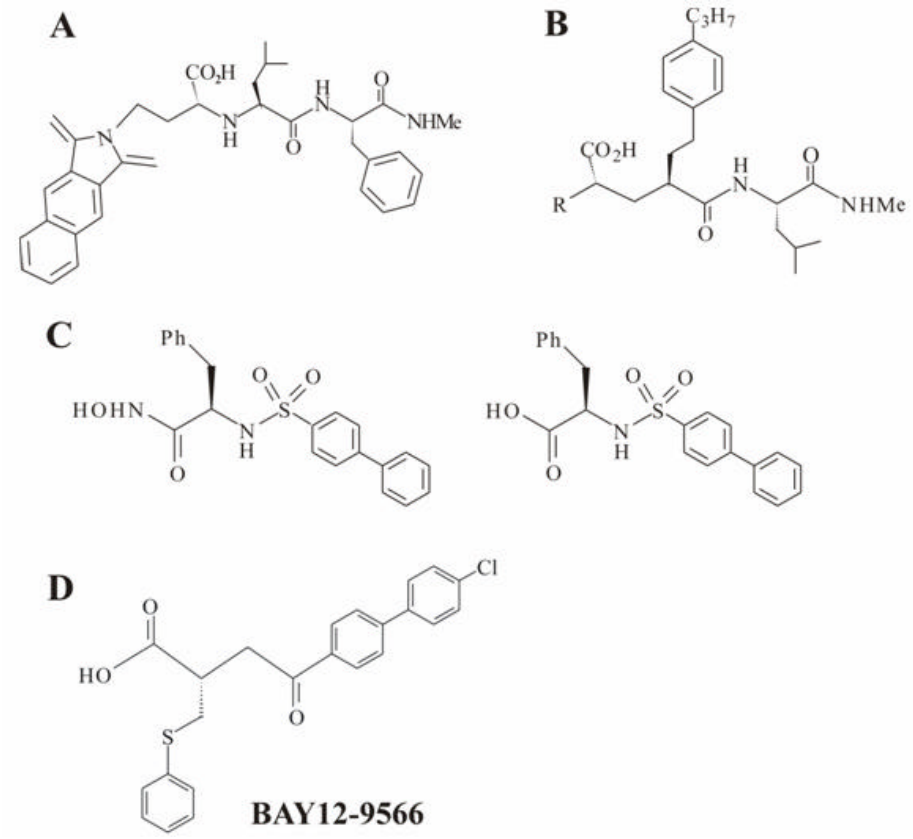



**Figure 9. Carboxylate MMP inhibitors and their derivatives.** (A) Example of a carboxylate inhibitor with an $IC_{50}$ of 91 µM for MMP-9. (B) Replacement of the nitrogen atom of the amino-carboxylate with a carbon provided a series of glutamic acid derivatives with improved *in vivo* kinetic properties. (C) Incorporation of a biphenyl group is also a strategy for an N-sulfonyl amino acid hydroxamate MMP inhibitor and its equivalent carboxylic acid to achieve inhibition of MMP-2 and -9. (D) BAY12-9566.

*2.1.2.2 Thiol ZBGs*

Although the intrinsic affinity of the monodentate thiolate ZBG for $Zn^{2+}$ is probably less than that of oxygen-based bidentate ZBGs, the low desolvation cost as well as the ease of ionization are strongly compensating factors. In general, the potency of class I thiol inhibitors (possessing two sp3 carbons between the ZBG and the first peptide bond) against MMPs is intermediate between that of hydroxamate- and carboxylate-based inhibitors.

The compound A with a mercaptoacyl ZBG shown in Figure 11 panel A is a moderate inhibitor against a range of deep pocket enzymes and is orally active in a rat adjuvant arthritis model (50). A modeling investigation on this compound docked into MMP-8 confirmed that the thiol and acyl carbonyl could cooperate in binding to the active site zinc (51). Modification of compound A in a similar way as that used for the modification of the succinyl hydroxamates leads to compound B (Figure 11A), which is 10 times more potent than compound A.

Campbell and Levin have also prepared a novel series of inhibitors developed from the initial observation on the amino acid a-mercaptoamides similar to compound A shown in Figure 10A (52). As they found that the mercaptoamide to be unstable in solution, probably owing to proteolysis of the acetamide bond, a series of mercaptoalcohols and mercaptoketones were prepared. The mercaptoalcohols (e.g. compound A in Figure 11B) exhibit modest activity against MMP-1, -3, -9, while the equivalent mercaptoketones (e.g. compound B in Figure 11B) could be optimized to active broad-spectrum inhibitors. A related series of succinyl mercaptoalcohols and mercaptoketones were also prepared (53). The series of compounds were broad-spectrum inhibitors. The mercaptoalcohols and mercaptoketones may act as bidendate zinc ligands.

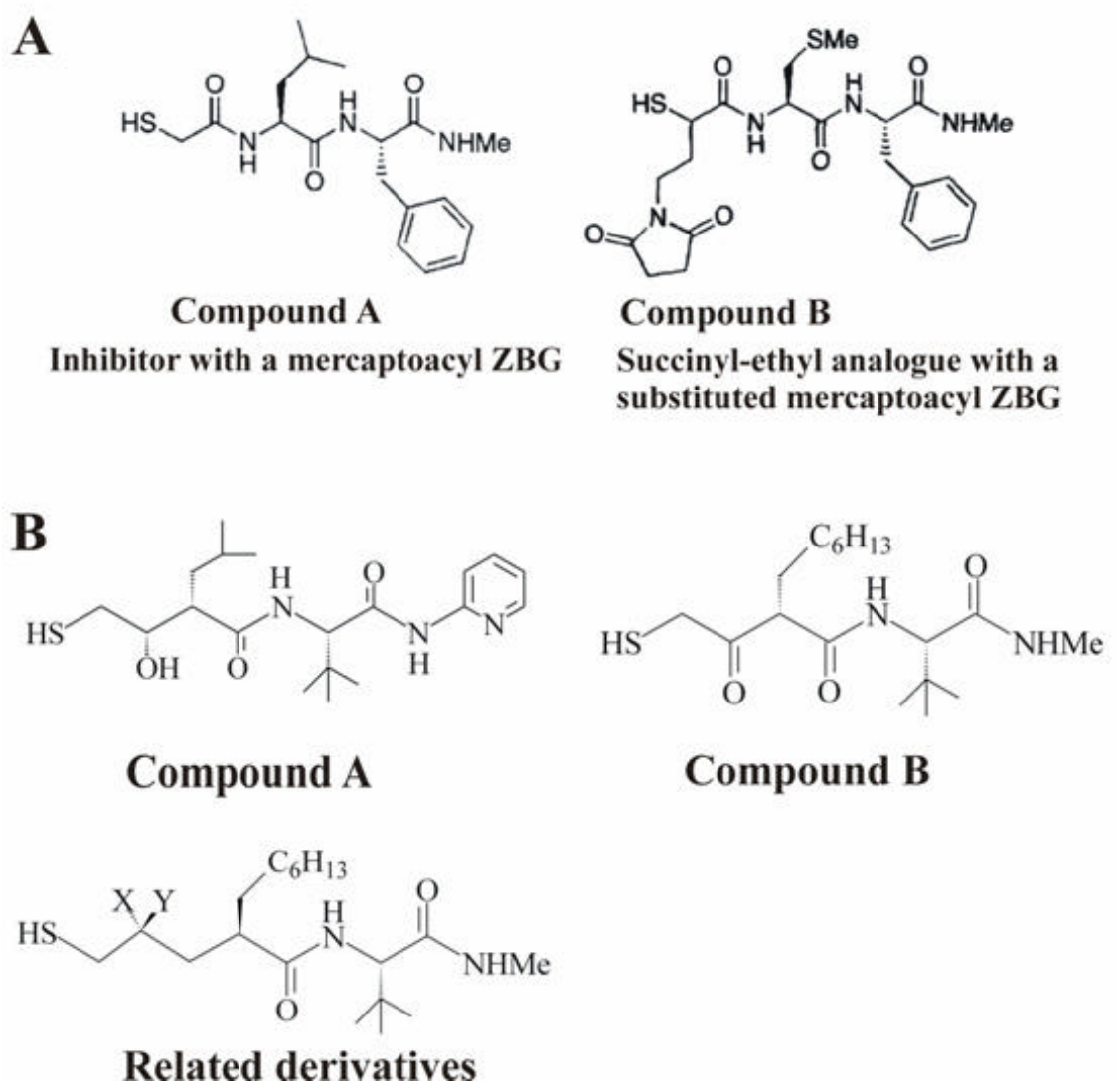



**Figure 11. Examples of MMPIs with a thiol ZBG.** (A) Modifying compound A in a similar way as that used for the modification of the succinyl hydroxamates leads to compound B. (B) A mercaptoalcohol, a mercaptoketone and a series of related derivatives are more stable than inhibitors with a-mercaptoamides.

*2.1.2.3 Novel MMPIs*

Potential new ZBGs are evaluated to obtain better and more selective MMPIs. Grams et al. used a high-throughput screening method to identify the pyrimidine-trione template of 5-[4-(2-hydroxyethyl)]piperidine-5-phenyl-pyrimidine-2,4,6-thione as a new zinc chelator for metalloproteinases (54). When evaluated *in vitro* in a chemoinvasion assay, this compound inhibited the invasiveness of human HT1080 cells. Structure-based drug design and combinatorial chemistry have been used to optimize the residues attached to the initial pyrimidine-trione core. This optimization strategy led to the selection of 5-biphenyl-4-yl-5-[4-(4-nitro-phenyl)-piperazine-1-yl]-pyrimidine-2,4,6-trione (Ro-28-2653) (Figure 12A). This compound is characterized by a high selectivity toward MMP-2, -9 and MT1-MMP. These three MMPs are consistently associated with tumor aggressiveness and poor prognosis (55). Crystallographic studies revealed that this new class of MMPI binds to the MMPs in a manner that saturates nearly all of the possible interactions of the pyrimidine core moiety to the protein (54).

Starting from a 6H-1,3,4-thiadiazine scaffold, a screening effort was utilized to identify some chiral 6-methyl-1,3,4-thiadiazines that are weak inhibitors of the catalytic domain of human neutrophil collagenases (MMP-8). Further optimization of this lead compound revealed general design principles that involve the modification of the thiadiazine ring and the side groups. These modifications can improve the primed and unprimed side affinity, chelating action on the catalytic $Zn^{2+}$ and selectivity. A unique combination of the above-described modifications produced the selective inhibitor (2R)-N-[5-(4-bromophenyl)-6H-1,3,4-thiadiazin-2-yl]-2-[(phenylsulfonyl)amino]propanamide with high affinity for MMP-9 ($K_i$ = 40 nM) (56) (Figure 12B).

A collection of potential zinc binders was assayed against murine gelatinase B. Among the compounds tested, aminomethyl benzimidazole showed promising activity. The aminomethyl benzimidazole is also advantageous for further optimization because diversity can be introduced at multiple positions on this scaffold using efficient synthetic chemistry. Evaluation of a series of aminomethyl benzimidazole analogues indicated that substitution on the aromatic ring (Figure 12C) results in compounds with improved activity. Specifically, aminomethyl benzimidazole binds to the enzyme active site rather

than chelating the $Zn^{2+}$. A completely nonpeptidic gelatinase B inhibitor (Figure 12C) was identified with modest inhibitory activity against MMP-9 ($IC_{50}$=13 µM) (57).

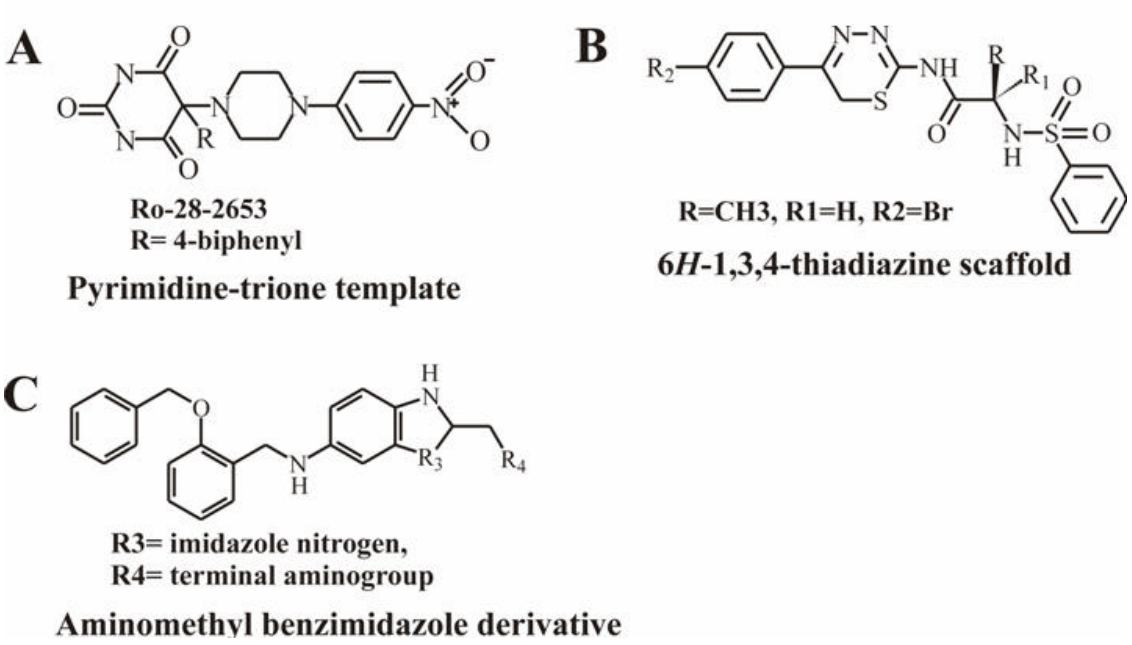



**Figure 12. Novel MMP inhibitors.** (A) Ro-28-2653 (R is 4-biphenyl) is an MMP inhibitor with a high selectivity towards MMP-2, -9 and MT1-MMP. (B) A 6H-1,3,4-thiadiazine derived compound has a high affinity for MMP-9. (C) Nonpeptide gelatinase B inhibitors with modest inhibitory activity against MMP-9.

*2.1.2.4 Synthetic peptides and pseudopeptides*

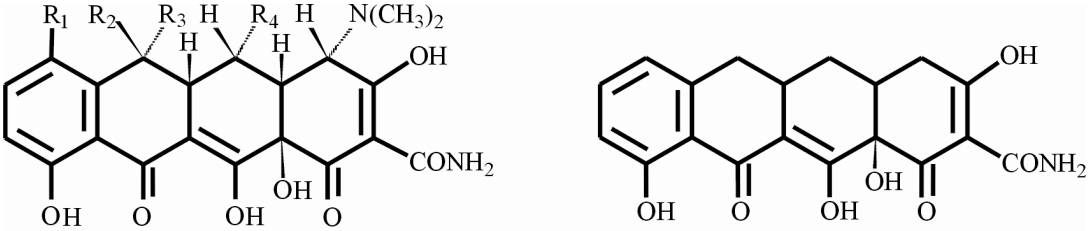
Synthesized peptides with natural and unnatural amino acids can also inhibit MMPs. The peptides or pseudopeptides were discovered by screening of combinatorial chemical synthetic libraries (58) or phage display libraries (59). The histidine-(epsilon-amino caproic acid)-(beta-alanine)-histidine (His-eAhx-ßAla-His) sequence was found to yield inhibition of both MMP-2 and MMP-9. The length of the spacer between the two terminal histidines was found to be crucial for the inhibitory potential. As no chemical optimization was undertaken, the inhibitory potential remains moderate (58). The cyclic peptide (CTTHWGFTLC) is a potent and selective inhibitor of MMP-2 and MMP-9 but not of several other MMP members (e.g. MT1-MMP, MMP-8 and -13) or of several serine protease members (e.g. trypsin-2, neutrophil elastase, or cathepsin G). Although the inhibitory mechanism is not clear yet, it is speculated that the tryptophan residue in the HWGF motif may bind to the hydrophobic pocket of the substrate cleft in the enzyme

and that the histidine residue may act as a ligand for the catalytic zinc ion. Opening of the cyclic structure by replacing the cysteines with serines results in a 10-fold lower inhibitory activity (59). Serendipitously, we found that polyhistidine in the micromolar range can inhibit both gelatinase A and gelatinase B (31), presumably by chelating the active site zinc and by simulating His-eAhx-ßAla-His.

*2.1.2.5 Tetracyclines and chemically modified tetracyclines (CMTs)*

Apart from their role as antibiotics, tetracyclines (TCs) have been shown to inhibit connective tissue breakdown. Initial studies were done with minocycline and doxycycline. Doxycycline was also extensively studied as an antitumor drug. Chemically modified tetracyclines (CMT) have several potential advantages over conventional TCs. Long-term systemic administration does not result in gastrointestinal side effect and toxicity, and higher plasma concentrations can be reached for prolonged time periods (60). In addition, on the basis of their chemical properties, TCs and CMTs may cross anatomical barriers such as the blood brain barrier and blood retina barrier. TCs/CMTs (Figure 13) have been applied in animal models of periodontitis (61), cancer cell metastasis (62), multiple sclerosis (63) and adjuvant arthritis (64).

The proposed mechanisms of action of CMTs results from their ability to bind metal ions (e.g. $Ca^{2+}$ and $Zn^{2+}$) which are required to maintain proper enzyme conformation and hydrolytic activity. The binding of TCs/CMTs with pro- or active MMPs results in the disruption of the normal conformation of the protein structure and leaves the enzymes inactive while being vulnerable to degradation into small molecular fragments (e.g. within the tissue context of an inflammation) (65). CMTs have also been shown to downregulate expression of MMPs (63), in particular gelatinases, and thus to reduce the production of the pro-enzyme. Other mechanisms include inhibition of oxidative activation and increase in degradation of pro-MMPs (63), inhibition of production of TNF-a and IL-8 (61) and reduction of the expression of the serine proteinase trypsinogen-2 (66).




CMT

| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Tetracycline | H | OH | $CH_3$ | H |
| Doxycycline | H | H | $CH_3$ | OH |
| Minocycline | $N(CH_3)_2$ | H | H | H |

**Figure 13. Tetracyclines (TCs) and chemically modified tetracyclines (CMTs).**

**2.2 Macromolecular inhibitors**

2.2.1 Tissue inhibitor of metalloproteinases (TIMP)

Once an MMP, e.g. gelatinase B, is secreted and activated, its activity can still be regulated by degradation or inhibition. TIMPs are shown to be important in regulating the activity of MMPs, including gelatinase B (67).

TIMPs are stable glycoproteins with a relative molecular weight of 20 to 30 kDa. They contain six conserved disulfide bridges (68). These disulfide bridges define six protein loops, of which the first three form an aminoterminal domain and the others take part in a carboxyterminal domain. TIMP-1 is a glycosylated protein. The glycosylation seems to play a role in various functions including correct folding of the nascent protein, transport of the molecule to the cell surface and enhanced stability of the protein (69).

Four different TIMP genes and proteins have been described in man, of which TIMP-1 binds with high affinity to gelatinase B, and TIMP-2 and -3 with low affinity. TIMP-2, -3 and -4 bind with high affinity to gelatinase A. TIMP-1 is an inducible protein, in contrast to TIMP-2, which is constitutively expressed (70). The inhibition by TIMPs follows slow tight-binding kinetics and is highly complex as different binding sites for TIMPs exist on gelatinase B. Not only the activated gelatinase B can bind to different TIMPs, but the proenzyme is also able to bind TIMP-1 and -3 (71).

The interactions between progelatinase B and TIMP-1 seem to occur mainly through the C-terminal domains of both molecules (72, 73). Complexes of progelatinase B and

TIMP-1 are able to inhibit other MMPs by the formation of gelatinase B/TIMP-1/MMP complex. This indicates that the inhibitory N-terminal domain of TIMP-1 is still available for interaction in the progelatinase B/TIMP-1 complex (74). Inhibition of active gelatinase B occurs through interaction between the N-terminal domains of TIMP-1 and the active site of the enzyme (72, 75).

Interaction of gelatinase B with TIMP-2 is mediated by the N-terminal domains of the enzyme but not by the hemopexin domain. Moreover, the inhibition of gelatinase B by TIMP-2 is less effective than by TIMP-1 (75). No interactions between TIMP-2 and progelatinase B were observed yet.

TIMP-3 is an insoluble ECM-bound MMP-inhibitor with, like TIMP-2, a higher affinity for gelatinase A than for gelatinase B. It can bind to both progelatinases and activated gelatinases, and the carboxyterminal domains of both enzymes are important for interaction with TIMP-3 (76).

### 2.2.2 Monoclonal antibodies and derivatives specific for MMP-9

Human neutrophil gelatinase B was used, in our laboratories, as an antigen to prepare mouse monoclonal antibodies (mAb). By selection, five mAbs were found to display binding capacity to gelatinase B. None of the mAb displayed cross-reactivity to gelatinase A in a direct ELISA. One of these, the mAb REGA-3G12, was unique in that it inhibited catalysis by gelatinase B *in vitro* (77). For the application of gelatinase B-inhibitory monoclonals in patients, derivatization may be necessary, e.g. to minimize the human anti-mouse antibody (HAMA) response and to prevent classical complement activation by antigen antibody complexes. Single-chain variable fragment, for mAb REGA-3G12 (REGA-3G12 sc Fv) were expressed in *Escherichia coli* (78). The inhibitory activity of the purified sc Fv was found to inhibit the gelatin degradation by gelatinase B with a similar specific activity as that of the intact monoclonal antibody and of the pepsin-clipped $F(ab')_2$ derivative. *Pichia pastoris* yeast was used to produce REGA-3G12 sc Fv derivatives [with an oligohistidine $(His)_6$ tag at 3´ side or without a tag]. By the use of a recently developed assay with fluorescent gelatin as substrate, the dimer form of REGA-3G12 sc Fv with a $(His)_6$ tag inhibited gelatinase B (66% inhibition at 5 µM) and gelatinase A (75% inhibition at 5 µM). The monomer REGA-3G12 sc Fv

with a tag specifically inhibited gelatinase B (44% inhibition at 5 μM) in the same assay (31). According to recent unpublished results, the selective inhibition of gelatinase B/matrix metalloproteinase-9 by the monoclonal antibody REGA-3G12 is determined by interactions with part of the active site domain, different from $Zn^{2+}$-binding and fibronectin domains.

Similar work on monoclonal antibodies was also performed in other laboratories than ours. In one example, four monoclonal antibodies for human gelatinase B, named 1G5, 4C6, 4G6 and 4H4 were generated of which 4G6 and 1G5 were found to cross-react with human gelatinase A. None of the four antibodies inhibited gelatinase activity. Selective detection of gelatinases with these monoclonal antibodies was successfully demonstrated by immunostaining with the combined use of 4H4 and 4G6 clones (79). In another example, human MMP-9 was used to immunize mice for the isolation of monoclonal antibodies. Three IgG1 mAbs were identified by immunoreactivity with purified MMP-9 and designated 6-6B, 7-11C and 8-3H. These mAbs reacted specifically with MMP-9 in ELISA and Western-blot analysis. The 6-6B mAb inhibited the activation of MMP-9, but had no effect on MMP-2 activation. It was suggested to be useful for examining the autolytic and catalytic activity of MMP-9 in normal and abnormal biological processes (80).

## 3. Testing MMP inhibitors in animal models

Because of their potential in therapeutic approaches, MMP inhibitors have been tested in many animal models of acute or chronic inflammation or of invasive cancer. The literature is extensive as is exemplified in a recent overview of models used to study the pathophysiology of MMP-9 (6). For this reason, we have organized this part of the Introduction section by describing a few prototypic examples of animal models.

### 3.1 Endotoxin shock – a model of acute inflammation

Bacteraemia and septic or endotoxin shock are among the most frequent causes of motality in modern hospitals. These clinical syndromes with multi-organ failure are caused by an excessive host inflammatory response to the invading microorganisms and their products (81, 82). Bacterial cell wall constituents, such as endotoxins/

lipopolysacchrides (LPS) and peptidoglycans (PGN), are active agents in the inflammatory response. The biological effects of LPS and PGN are mediated by the activation of Toll-like receptors (TLR) on multiple leukocyte types (83). In the case of endotoxinemia, excessive TLR activation leads to exaggerated stimulation of leukocytes and excessive production of inflammatory mediators, including cytokines and enzymes. Genetic defects in the TLR, cytokine, enzyme and complement factor genes thus have been found to result in the resistance against endotoxin shock. For instance, C3H/HeJ and C57Bl/10ScCr mice, homozygous for a mutation in the TLR4 gene, are resistant to endotoxin shock (83-85). This role of TLR4 in the observed hyporesponsiveness to LPS challenge was confirmed in TLR4-deficient mice (86). Similarly, mice deficient in cytokine signalling (87-89) and the enzyme gelatinase B/matrix metalloproteinase-9 (MMP-9) (14) have an increased resistance to LPS-induced toxicity, whereas mice deficient in protease inhibitors are more susceptible to LPS shock (90). Similarly, the complement cascade (91) and the plasminogen activator-plasmin system (92) have been implicated in the pathogenesis of sepsis. These data imply that TLRs, cytokines and enzymes are not unique targets in such disease and that the pathophysiology of endotoxin shock is quite complex.

Since neutrophils are the most abundant cell type in the human circulation, LPS will mainly, directly and immediately act on these cells in the event of endotoxinemia. This interaction results in the release of various categories of neutrophil effector molecules, including enzymes, reactive oxygen intermediates which contribute to the activation of MMPs (93) and release of lysozyme which further generates PGN fragments. An important aspect of endotoxinemia and septic shock is its acuteness, which may be based on the fast release of mediators by degranulation. In this respect, it was demonstrated that the release of gelatinase B/MMP-9 precedes the production of chemokines and TNF-a in baboons with sepsis (94) and that in human volunteers MMP-9 plasma levels were already maximal 1.5 to 3 hours after LPS challenge (95), a quite early time point for mRNA transcription, translation and secretion of MMP proteins, which is compatible with degranulation of preformed enzymes.

As outlined in the following chapters, a peptide inhibitor, Regasepin1 (with sequence Pro-Arg-Cys-Bip-Cys-Gly-Glu), against gelatinase B was developed in our laboratory

and shows inhibitory effect against MMP-8 and -9, which are the main MMPs in neutrophil degranulates during acute inflammation. This peptide inhibitor also inhibits TACE/ADAM-17 activity. By intravenous injection, Regasepin1 was shown to protect mice against lethal endotoxin shock (96). Regasepin2 (with sequence Pro-Pyr·Ala-Cys-Bip-Arg-Gly-Glu, Pyr·Ala represents pyridylalanine) is another peptide inhibitor with a similar inhibitory rational and sequence with Regasepin1. Regasepin2 also protects mice against lethal endotoxin shock by intravenous injection (97).

### 3.2 EAE – a model of chronic inflammation

Multiple sclerosis (MS) is a multifactorial disease influenced by genetic predisposition, environmental factors and important immunological effector mechanisms (chronic inflammation) that damage the central nerve system (CNS). Pathogenetic mechanisms, such as chemotaxis, subsequent activation of autoreactive lymphocytes, and skewing of the extracellular proteinase balance, are targets for new therapies.

Gelatinase B is an important immune effector molecule in MS pathogenesis. This enzyme not only functions in cell migration through connective tissues, and into and from blood and lymph vessels, but also lyses protein substrates that are relevant in MS and other neurological diseases. These substrates include myelin proteins, cell adhesion molecules, cytokines, and chemokines. Additional evidence supporting a detrimental role of gelatinase B in inflammatory CNS damage has been obtained with animal models. In murine Experimental Autoimmune Encephalomyelitis (EAE), which is used as animal model for multiple sclerosis, gelatinase B was upregulated during the development of the disease syndromes (98-101). Knockout mice that do not express a functional gelatinase B have been developed. Whereas young gelatinase B-deficient mice were resistant to the development of EAE, in adult mice this effect seemed to be lost, and both young and adult littermate controls developed severe EAE (12). This study showed that gelatinase B is involved in EAE development, especially early in life, and that other factors may compensate for this function at a later age.

Inhibition of gelatinase B activity improved the course of EAE. Several studies have been done with gelatinase B inhibitors with different degrees of selectivity. The hydroxamates type inhibitors have been found to be protective in animal models of EAE

(102, 103). These drugs were developed for the treatment of invasive and metastatic cancer, but were gradually abandoned because of side-effects, presumably caused by their lack of selectivity. The sulfhydryl-containing D-penicillamine, which mimics the blockage of the cysteine-switch mechanism (4), is also an unselective MMP-9 inhibitor (104), and was previously used for the treatment of rheumatoid arthritis. D-penicillamine also protects mice against the development of acute EAE, but causes side-effect rather than beneficial effects in patients with MS in control trials (105). Tetracyclines, of which minocycline and metacycline are prototypic examples, are inexpensive antibiotics that are able to penetrate the blood-brain barrier and have direct and indirect inhibitory effects on MMP-9 (65). Minocycline is one of the most potent tetracycline derivatives with gelatinase B-inhibitory activity and effective against EAE (65, 106, 107).

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## Aims and general outline of the experimental work

The general theme of this work is inhibition of gelatinase B. As is clear from the introduction section, the development of MMPIs and specific inhibitors of gelatinase B is in high demand. Many efforts into this direction have already been done in industrial and academic laboratories. Small molecular weight inhibitors of gelatinase B seem to be unselective and to cause side-effects. Previously, an inhibitory monoclonal antibody was isolated in our laboratory and shown to be selective for MMP-9 (74). The first aim was to express new recombinant derivatives of this monoclonal antibody and to study the inhibitory activity. These studies are described in Chapter 1.

Whereas macromolecular MMP-9 inhibitors, such as TIMP-1 or monoclonal antibodies, may confer some selectivity, production and use of proteins may be problematic. A vast number of small molecular inhibitors, which inhibit MMP members in the micromolar to nanomolar range, have been developed by pharmaceutical companies. The use of combinatorial chemical synthesis and the development of new high-throughput screening methodologies to deconvolute molecules with different zinc-binding groups at different positions, together with structure-function studies, are a promising approach to develop novel small-size MMP-9 peptide inhibitors. The second aim was to develop a new platform for high-throughput screening of peptide inhibitors, with the use of hardware and software of an existing DNA-sequencer. We applied the strategy of peptide inhibitor development on the basis of the consensus cleavage sequences of denatured collagen II by gelatinase B. In fact, denatured collagen II is one of the best substrates of gelatinase B (9). We synthesized thousands of substrate-based peptide compounds. With the use of our new platform novel MMP-9 inhibitors were selected. These studies are outlined in Chapter 2.

The third aim was to evaluate compounds with inhibitory effect against gelatinase B, selected from the synthetic libraries, for their *in vivo* effects. Although we did not reach the stage of development of any peptide inhibitor with selectivity against MMP-9, a number of analogs of a peptide inhibitor with *in vitro* and *in vivo* activity were synthesized and tested. The inhibitory activity of these novel compounds was corroborated and extended and, the presented novel technology was validated. We used

for our *in vivo* studies the animal model of lethal endotoxin shock as it provides a swift readout. These results are discussed in Chapter 3 and Chapter 4.

These three major aims of the doctoral work are discussed in the section of Discussion and Perspectives. An important discovery of our work on inhibitors was the finding that the best peptide gelatinase B inhibitor from the synthetic libraries simulated the evolution-selected natural propeptide sequence. It has been shown that the prodomains of metzincins, including MMPs and ADAMs (**a d**ysintegrin **a**nd **m**etalloproteinase) act as inhibitors of the catalytic domains. As a consequence we initiated the cloning and expression of the prodomain of MMP-9 for further studies. Since the chemical synthesis of the propeptide was not successful, recombinant expression in insect cells was evaluated. Fragments of gelatinase B that retain gelatin binding through the fibronectin repeats can be readily purified by gelatin-Sepharose affinity chromatography. However, the propeptide, by the lack of fibronectin repeats, can not be purified in this way. Therefore, we also discuss the development of a useful purification strategy for the propeptide and outline future work and perspectives within the context of MMP inhibition.