Profiling of soluble and membrane-bound metalloproteinases

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A targetted proteomics approach

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Cover: lung tissue section stained with fluorescent activity-based probe ML21

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Contents

List of abbreviations

AANHS Acetic acid *N*-hydroxy succinimide
ABPP Activity-based proteomics probe
ACE Automatic cartridge exchanger
ACN Acetonitrile
ADAM A Disintegrin And Metalloproteinase
ADAMTS A Disintegrin And Metalloproteinase with Thrombospondin motifs
AP-1 Activator protein 1
APMA 4-aminophenylmercuric acetate
APP Amyloid precursor peptide
Aβ Amyloid beta
BALF Bronchoalveolar lavage fluid
BCIP 5-bromo-4-chloro-3-indoyl phosphate
BMP Bone morphogenic protein
BPC Base peak chromatogram
C_8-PEG Poly-ethylene glycol monooctylether
CD Catalytic domain
CHL Close homologue of L-1
CNS Central nervous system
COPD Chronic obstructive pulmonary disease
CUB Complement C1r/C1s, Uegf (EGF-related sea urchin protein) and BMP-1 domains
ECM Extracellular matrix
EDB Elution-digestion buffer
EDTA Ethylenediaminetetraacetic acid
EGF Epithelial growth factor
EGFR Epithelial growth factor receptor
EIC Extracted ion chromatogram
EMMPRIN Extracellular matrix metalloproteinase inducer
ESI Electrospray ionisation
ExB Extraction buffer
FEV_1 Forced expiratory volume in 1 second
GPI Glycosyl-phosphatidyl-inositol
HBE Human bronchial epithelia
HB-EGF Heparin binding epithelial growth factor
HER2 Human Epidermal growth factor Recepto
HME Human metallo-elastase
HPD High pressure dispenser
HPLC High performance liquid chromatography
IGFBP Insulin-like growth factor binding protein
IL Interleukin
L1-CAM L1 cell adhesion molecule
LPS Lipopolysaccharide
MADDAM Metalloprotease and disintegrin dendritic antigen marker
MADM Mammalian disintegrin-metalloproteinase
MDC Metalloproteinase-like, disintegrin-like, cysteine-rich proteins
MME Murine metalloelastase
MMP Matrix metalloproteinase
MRM Multiple reaction monitoring
MS Mass spectrometry
MT-MMP Membrane-type matrix metalloproteinase
NBT Nitro blue tetrazolium
NHS *N*-hydroxy succinimide
NRG Neuregulin
PACE Paired basic amino acid cleaving enzyme
PAGE Polyacrylamide gel electrophoresis
PAI Plasminogen activator inhibitor
PC Proprotein convertase
PKC Protein kinase C
PMA Phorbol 12-myristate 13-acetate
PUMP Putative metalloproteinase
RAGE Receptor for advance glycation products
RANKL Receptor activator of NF-κB ligand
SAX Strong anion exchange
SCX Strong cation exchange
SDS Sodium dodecyl sulphate
SH3 Src homology 3
SIR Selective ion recording
SNP Single nucleotide polymorphism
SPE Solid phase extraction
SPPS Solid phase peptide synthesis
SRM Single reaction monitoring
TACE TNF alpha converting enzyme
TAPI TNF alpha protease inhibitor
TBST Tris buffered saline with Tween-20
TEB Trypsin equilibration buffer
TGF Tranforming growth factor
TIC Total ion chromatogram

TIMP Tissue inhibitor of metalloproteinases
TNF Tumour necrosis factor
t-PA Tissue plasminogen activator
TRANCE TNF related activation induced cytokine
TSR Thrombospondin type I-like repeat
TTP Thrombotic thrombocytopenic purpura
TWB Trypsin wash buffer
VCAM vascular cell adhesion molecule
VEGF Vacular endothelial growth factor
vWF von Willebrand factor
WB Western blot

Chapter 1

Metzincin proteinases in health and disease

General introduction

1.1 Introduction

Metzincins are a ubiquitously expressed family of multi-domain zinc (II)-dependent endopeptidases[1] whose members include well-known metalloproteases such as the Matrix Metalloproteases (MMPs)[2], the A Disintegrin And Metalloproteases (ADAMs)[3], the ADAMs with a thrombospondin motif (ADAMTS)[4], the bacterial serralysins[5] and proteases such as the astacins (including the meprins)[6,7]. This superfamily of proteases is defined by the presence of a Zn^{2+} ion at the catalytic centre which is coordinated by three histidine residues in the zinc binding consensus sequence HExxHxxGxxH that is present in all proteolytically active metzincins, and a characteristic, strictly conserved methionine containing tight 1,4 beta turn forming a hydrophobic cleft for the catalytic zinc ion[8]. Catalysis of protein substrates is (most probably) carried out via a general base mechanism involving activation of a zinc-bound water molecule by the carboxylate group of the conserved glutamate residue in the catalytic pocket followed by attack of water on the polarized carbonyl group in the substrate's scissile bond[9].
The main physiological function of these proteases lies in the modulation and regulation of extracellular matrix (ECM) turnover by either direct proteolytic degradation of the ECM proteins (e.g. collagen, proteoglycans and fibronectin)[10] or by liberation of biologically active proteins such as cytokines, growth factors and chemokines from their membrane-anchored proforms (so-called shedding).

1.2 Matrix metalloproteinases

The largest human subfamily of the metzincins is the matrix metalloproteases or matrixins (see table 1) which consists of 23 distinct proteases in human (24 in mouse). The first MMP identified in 1962 as the protease responsible for the degradation of fibrillar collagen in tadpole tails during metamorphosis was dubbed interstitial collagenase[11]. After identification of a similar collagenase in human skin, this protease was renamed MMP-1. MMPs have since been identified as the major enzymes responsible for turnover of extracellular matrix by proteolytic degradation of virtually all proteinaceous components of the ECM[10].

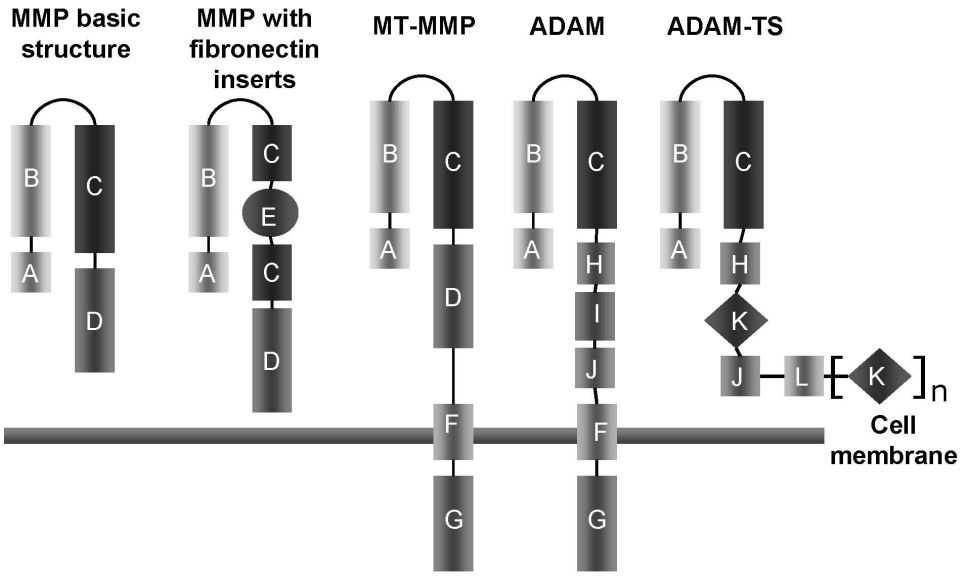

Figure **1**

Schematic representation of the domain structure of metzincin proteases

A: signal peptide; B: prodomain; C: catalytic domain; D: hemopexin-like domain; E: fibronectin type II insert; F: transmembrane domain; G: cytoplasmic tail; H: disintegrin domain; I: cysteine-rich domain; J: EGF-like domain, K: thrombospondin type I-like repeat, L: spacer region.

MMPs are largely excreted proteins with several conserved domains (see figure 1). All MMPs contain the catalytic domain, which is shielded off in the inactive form of the enzyme by the prodomain. This propeptide interacts with the catalytic region through a conserved cysteine residue and the Zn^{2+} ion in the catalytic pocket (the so-called cysteine switch)[12,13]. Except for MMP-7, MMP-23 and MMP-26 all MMPs contain a C-terminal hemopexin-like domain which functions primarily as a recognition sequence for the substrate[14]. Although MMPs retain catalytic activity towards a wide range of substrates when missing this domain, the hemopexin domain, which is structured like a four-bladed propeller structure with each blade consisting of 4 antiparallel β-sheets and 1 α-helix, is an absolute necessity for the degradation of triple helical collagens[15]. The gelatinases (MMP-2 and -9) further contain a series of three fibronectin type II inserts in the catalytic domain which facilitate binding of gelatine and collagen[16].

Table 1: *Overview of the 23 identified human matrix metalloproteinases and their common names.*

MMP	Alternative name	MMP	Alternative name
1	Collagenase-1 Interstitial collagenase	16	Membrane type-3 MMP
2	Gelatinase A 72 kDa type IV collagenase	17	Membrane type-4 MMP
3	Stromelysin-1 Transin-1	19	Human orthologue of *Xenopus* MMP-18
7	Matrilysin Pump-1	20	Enamelysin
8	Collagenase-2 Neutrophil collagenase	21	Human orthologue of *Xenopus* xMMP
9	Gelatinase B 92 kDa type IV collagenase	23	Cysteine array MMP Femalysin MMP-22
10	Stromelysin-2	24	Membrane type-5 MMP
11	Stromelysin-3	25	Membrane type-6 MMP Leukolysin
12	Macrophage metallo-elastase	26	Matrilysin-2 / endometase
13	Collagenase-3	27	None
14	Membrane type-1 MMP	28	Epilysin
15	Membrane type-2 MMP		

MMP function is regulated at several levels. Firstly induction of gene expression is controlled by a number growth factors and cytokines, and may be suppressed by transforming growth factor β and glucocorticoids[2,17]. Besides soluble factors, MMP expression may also be regulated by cell-cell contact or interaction of cells with ECM components such as EMMPRIN (extracellular matrix metalloproteinase inducer or CD147)[18]. The expressed MMPs are largely excreted as inactive zymogens with the propeptide effectively limiting entrance into and catalysis of a substrate in the catalytic pocket by blocking the catalytic zinc (II) ion via the cysteine-switch mechanism. Activation of proMMPs can occur through several mechanisms (reviewed in [19]) that all lead to disruption of the cysteine switch. Perhaps the most important mechanism is proteolytic

removal of the prodomain by other endopeptidases such as furin[20]. Removal of the prodomain of MMPs, which contains a furin-like proprotein convertase recognition site (RRKR or RxKR), has been described for 9 MMPs including all membrane-type MMPs. Alternatively the prodomain can be proteolytically removed by plasmin and other serine proteases, or even other MMPs. This mechanism is well described for MMP-2 where the proMMP2 binds the endogenous MMP inhibitor TIMP-2 (tissue inhibitor of metalloproteinases 2). This complex in turn functions as a ligand for the membrane-bound MMP-14 (or membrane-type 1 MMP) leading to activation of MMP-2[21].
The cysteine switch may also be broken by chemical reactions, either physiologically by oxidation of the cysteine by reactive oxygen species, or artificially by mercury-containing compounds such as 4-aminophenylmercuric acetate (APMA) or denaturing surfactants such as sodium dodecyl sulphate (SDS). This disruption of the thiol-zinc interaction leads to allosteric relocation of the prodomain leading to active forms of the enzyme with the propeptide still attached or to autoproteolytic removal of the relocated prodomain.
MMPs are inhibited by the general protease inhibitor α_2-macroglobulin and a small family of natural inhibitors specifically geared towards inhibiting metalloprotease activity. These TIMPs are a group of 4 proteins (21-30 kDa in size) that as a group effectively inhibit all MMPs in vivo[22].
Excreted MMPs are generally classified according to their substrate specificity, leading to four classes: the collagenases (MMP-1, -8 and -13), the gelatinases (MMP-2 and -9), the stromelysins (MMP-3, -10 and -11) and a heterogeneous rest group containing matrilysin (MMP-7), metallo-elastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26) and epilysin (MMP-28). In this nomenclature the membrane anchored MMPs (MMP-14, -15, -16, -17, -24 and -25) are considered a separate class. An alternate classification arranges the MMPs according to their domain structure[23].

1.2.1 Collagenases

MMP-1
MMP-1 or collagenase-1 was the first described matrix metalloprotease and has sparked a large volume of research on the physiological and pathological role of MMPs. MMP-1 is a secreted enzyme and contains the hemopexin-like domain necessary for degradation of triple helical collagens. ProMMP-1 is probably activated via a two-step proteolytic process involving either mast cell tryptase[24] or urokinase[25] and MMP-3. Mature collagenase-1 can be found as two distinct forms: a major 57 kDa species and a minor, glycosylated 61 kDa form[26]. The active enzyme is inhibited by TIMP-1.
MMP-1 has a wide substrate specificity and is capable of degradation of aggrecan, versican, perlecan, casein, nidogen, serpins and tenascin-C[27]. Since MMP-1 does contain the hemopexin domain, it is one of the MMPs capable of proteolytic cleavage of fibrillar collagen leading to unwinding of the triple-helical structure leaving the collagen (or gelatine) highly susceptible to degradation by other proteases. Proteolysis by MMP-1 has been implicated in release of membrane-anchored proforms of insulin growth-factor binding proteins (IGFBP-3 and -5), IL-1β and L-selectin, amongst others[27].

Since mice lack a clear orthologue of human MMP-1 definition of the physiological role by creating knock-out mice has not been possible, but transgenic mice which express human MMP-1 are known to develop lung emphysema, indicating a possible role for MMP-1 in development of this disease[28]. Other experiments with transgenic mouse strains have revealed development of skin disorders such as hyperkeratosis, and bone growth retardation[29,30]. Since MMP-1 appears to play a key role in turnover of ECM, deregulation of MMP-1 activity has been hypothesized to be involved in various diseases where excessive or insufficient ECM turnover is involved, such as arthritis[31,32], cancer[33,34], wound healing disorders[35], and fibrotic diseases[36]. Although many studies have found a positive correlation between MMP-1 expression and disease it has proven difficult to clearly identify MMP-1 as a systemic marker of the investigated disease. Experiments with local sampling have proven more accurate, for instance in analysis of MMP-1 in synovial fluid of rheumatoid arthritis patients where MMP-1 level correlated with inflammatory activity[37,38].

MMP-8

MMP-8 (neutrophil collagenase or collagenase-2) is very similar to MMP-1 in structure and physiological function, although subtle differences in substrate selectivity exist. MMP-8 has a stronger affinity towards type I collagen than MMP-1, while MMP-1 preferentially cleaves type III collagen[39]. Neutrophil collagenase was first described in 1990 when it was cloned from neutrophils obtained from a patient with granulocytic leukaemia[40]. Contrary to MMP-1, MMP-8 is not released immediately after synthesis, but rather stored in specific granules that release the active enzyme upon stimulation[41]. As with MMP-1, MMP-8 can be activated by proteolytic removal of the propeptide by stromelysin-1 (MMP-3), but also by matrilysin (MMP-7)[42]. The mature enzyme is 64 kDa in size, with glycosylation increasing the size to 75 kDa. Autoproteolytic degradation has been described, yielding a 40 kDa fragment, which retains catalytic activity, but does not cleave fibrillar collagen. MMP-8 activity can be inhibited by both endogenous inhibitors TIMP-1 and TIMP-2[43].

MMP-8 seems to have an important function during embryogenesis and postpartum remodelling of uterine tissue, as demonstrated by expression studies in mice[39]. Collagenase-2 has also been implicated as one of the major contributors to connective tissue turnover in inflammation due to oxidative auto-activation of the enzyme[44]. Experimental evidence has shown that purified MMP-8 is capable of degrading α_1-proteinase inhibitor which could imply increased MMP-8 activity in the pathology associated with pulmonary emphysema[45].

The role of MMP-8 in cancer is interesting, since recent findings have suggested this MMP to have a protective effect by suppressing tumour metastasis[46,47], and by aiding therapy by increasing the susceptibility to oncolytic viruses[48]. On the other hand studies have identified MMP-8 as a promoter of ovarian tumour proliferation[49] and high MMP-8 expression seems to be an indicator for poor prognosis[50]. This contradicting evidence of the role of MMP-8 in cancer is an indication of the complex, and so far still poorly understood biological roles of MMPs. MMP-8 is further involved in various inflammatory processes, such as atherosclerosis, where both the expression as well as the protease activity is higher in vulnerable plaques than in stable plaques[51,52], indicating that MMP-8 may be useful as a marker for plaque instability in atherosclerosis.

MMP-13

MMP-13 (collagenase-3) is the latest human collagenase described in the literature. This enzyme exhibits preference towards cleavage of type II collagen, effectively completing the substrate spectrum of the collagenases. Collagenase-3 was first cloned from breast cancer tissue in 1994[53]. MMP-13 expression can be influenced by a wide range of hormones and cytokines, such as parathyroid hormone (indicative of the important role of MMP-13 in bone development), insulin-like growth factors I and II, platelet derived growth factor, basic fibroblast growth factor, transforming growth factor β1 (interestingly both up- and downregulates MMP-13 expression depending on the tissue), interleukin-1 and -6, tumour necrosis factor α and many more[54]. ProMMP-13 can be activated by auto-proteolysis or propeptide removal by various other MMPs like stromelysin-1 (MMP-3), yielding a mature enzyme of 48 kDa which in turn can be inhibited by TIMP-1, -2 and -3[55]. Active MMP-13 is a key factor in the activation pathway of several MMPs. Besides the TIMP route of inactivation, MMP-13 can bind to a specific receptor on the surface of osteoblasts and fibroblasts resulting in internalisation and degradation of the protease[56].

MMP-13 plays an important role in bone development and remodelling, as may be anticipated from its capability to cleave type II collagen (a major component of cartilage). This rather specific function is reflected in a limited expression profile of MMP-13 during development and adulthood, which is restricted to developing skeletal tissue.

Contrary to the other collagenases, MMP-13 has a relatively high specific activity towards gelatine, indicating that the proteolytic role of MMP-13 expands past the first-step of cleavage of triple-helical collagens. Further identified substrates of MMP-13 include aggrecan and perlecan, TGFβ, biglycan, the large isoform of tenascin-C, fibrillin-1 and -2, fibrinogen and two serpins (α2-antichymotrypsin and plasminogen activator inhibitor-2)[54].

MMP-13 expression has been regularly described in literature as indicative of various cancerous processes including chondrosarcoma, breast cancer, head and neck tumours and melanoma[57]. In all cases high expression of MMP-13 seems to be related to aggressiveness of the tumour.

Regarding the important role of MMP-13 in bone turnover it is not surprising that this enzyme has been linked to various bone-related diseases. Since MMP-13 degrades both type II collagen and aggrecan, it has been linked to cartilage destruction in rheumatoid and osteoarthritis[58]. The specialized role of MMP-13 in bone development and disease have made it an interesting target for selective MMP-13 inhibitors as therapeutic compounds[59,60].

1.2.2 Gelatinases

MMP-2

MMP-2 (gelatinase A, 72 kDa type IV collagenase) is one of the two described human gelatinases in the MMP family, named for their ability to proteolytically degrade gelatine (denatured collagen). MMP-2 is ubiquitously expressed as a 72 kDa zymogen and subject to extensive glycosylation. Expression of MMP-2 is constitutive and most pro-inflammatory stimuli fail to increase the expression level since the gene, in contrast to that

of MMP-9, lacks binding sites for pro-inflammatory transcription factors such as activator protein-1[61].
The MMP-2 zymogen is activated by the MMP-activation cascade depicted in figure 2. Under favourable stoichiometric conditions TIMP-2 complexes with proMMP-2 and this complex forms a ligand for the membrane-bound membrane type1-MMP which subsequently removes the prodomain of proMMP2 by proteolytic cleavage (aided by free active MMP-2) , yielding the truncated 64 kDa active enzyme[62]. If the concentration of TIMP-2 is too high, both MT1-MMP and active MMP-2 will be inhibited, and no further activation will ensue. Besides this activation pathway, proMMP2 can also be activated by thrombin and activated protein C[63].
MMP-2 differs from other MMPs in the fact that the catalytic domain contains cysteine-rich inserts that resemble the collagen binding regions of the type II repeats in fibronectin. These inserts are required for binding and cleavage of collagen and elastin[64].
The main function of MMP-2, as with other MMPs, lies in the degradation of extracellular matrix proteins. MMP-2 is capable of cleaving gelatine, type I, IV and V collagens, elastin and vitronectin[65]. Through their ability to degrade collagen in the vascular basal membranes the gelatinases are involved in neovascularization[66] both under physiological conditions and in pathologies such as tumour metastasis. MMP-2 can also facilitate migration of cells by direct degradation of the basement membrane thus allowing infiltration of for instance neutrophils and lymphocytes, or liberation of chemo-attractants[67]. This latter process, named ‘ectodomain shedding’ is one of the important physiological functions of the membrane-bound ADAM proteases, but has also been described for many other members of the metzincin superfamily. MMP-2 has been known to be involved in both promoting and inhibiting inflammation by liberation of pro-inflammatory mediators (for instance the active form of interleukin-1β[68]), proteolytic degradation of chemoattractants (for instance transforming monocyte chemoattractant protein-3 into the truncated form with antagonistic properties on the CC chemokine receptor[69]) and a profound role in the chemotactic gradient that is necessary in clearance of recruited inflammatory cells from tissue[70]. Recent evidence from proteomics studies show that the role of MMP-2 in processing of signalling proteins may be much greater than originally anticipated which, when confirmed for other MMPs could lead to a paradigm shift in the physiological role of these proteases[71,72].
Interestingly, MMP-2 knockout mice exhibit a normal phenotype under physiological conditions, although the animals do show different response patterns in allergen challenge which may be attributed to disturbance of the important role of clearing immune cells[70]. These findings indicate that MMP-2 function may be interchangeable with other metalloproteases, a hypothesis that is supported by the observation that expression of the second gelatinase, MMP-9 is greatly increased in MMP-2 null mice[73].

MMP-9
MMP-9 (gelatinase B, 92 kDa type IV collagenase) was first discovered in neutrophils in 1974[74]. MMP-9 is expressed as a 92 kDa zymogen which can be activated to the 83 kDa mature enzyme. The larger size of MMP-9 relative to MMP-2 can be contributed to a heavily O-glycosylated collagen V-like insert that links the metalloprotease domain to the

hemopexin-like domain[75]. MMP-9 activation may be mediated by removal of the prodomain by serine proteases or other MMPs[76], or may be a direct response to oxidative stress which disrupts the cysteine switch[77]. As expected, a considerable overlap exists in the substrates degraded by MMP-2 and -9, but MMP-9 is incapable of direct proteolysis of collagen I[65].

MMP-9 has been described to release the biologically active form of vascular endothelial growth factor (VEGF) which plays an important role in angiogenesis. This process is complemented by the direct proteolytic degradation of the vascular basement membrane proteins, indicating that MMP-9 (even more than MMP-2) may play a crucial role in the formation of new blood vessels[78]. MMP-9 further has an important role in migration of immune cells as demonstrated by the reduced presence of neutrophils, lymphocytes and dendritic cells in brochoalveolary lavage fluid (BALF) of MMP-9 knockout mice after antigen challenge[79]. MMP-9 null mice show decreased fertility since MMP-9 is crucial in several stages of the female reproductive cycle (implantation of the embryo and the remodelling of endometrial tissue that occurs during the menstrual cycle)[80]. Absence of MMP-9 also leads to disorders in bone development, specifically delayed bone ossification due to insufficient angiogenesis in growth plates[81] and reduced osteoclast recruitment[82]. The biological role of MMP-9 is extensively reviewed in [83].

As MMP-2, MMP-9 is capable of processing cytokines and chemokines. MMP-9 cleaves interleukin-8 to its more potent truncated form, activates IL-1β and transforming growth factor β[65]. Where MMP-2 is naturally inhibited by TIMP-2, MMP-9 is mostly inhibited by TIMP-1[84]. Contrary to MMP-2 which is expressed ubiquitously under physiological conditions, MMP-9 is only present constitutively in neutrophils[85] where it is stored in granules to be rapidly released after stimulation. Expression in many other cell types is inducible by (inflammatory) stimuli[86], is increased in malignant cell lines and correlates with the metastatic potential[87]. Neutrophil-derived MMP-9 is distinguishable from other sources since it forms a covalent complex with neutrophil gelatinase B-associated lipocalin (NGAL)[88].

The role of gelatinases in pathology has been studied extensively, especially in lung diseases (reviewed in [65]) and cancer (reviewed in e.g. [89] and [90]). The amount of both gelatinases in BALF and sputum of patients suffering from chronic asthma is higher than in healthy individuals and this increase is hypothesized to be mainly due to gelatinases originating from eosinophils and epithelial cells[91,92]. This increase may be responsible for the characteristic tissue remodelling events observed in chronic asthma such as thickening of the basement membrane, smooth muscle tissue hypertrophy and reduced epithelial thickness. MMP-2 does not seem to play an important role in the pathophysiology of acute asthma, but MMP-9 and MMP-9/TIMP-1 ratio are increased in exacerbations of acute asthma[79]. This phenomenon may be explained by the presence of a pool of MMP-9 inside the neutrophils that is released during the asthmatic attack. Since neutrophils do not produce TIMP-1, degranulation leads to a strong increase in the local concentration of proteolytically active MMP-9 which may cause several of the symptoms observed in acute asthma such as airway obstruction due to desquamation of epithelial cells and increased mucus production by goblet cells[65].

The relevance of gelatinases in the pathophysiology of chronic obstructive pulmonary disease (COPD) is not clear, but excess protease activity certainly plays an important role in development and progression of the disease. Sputum and BALF of patients contain high concentration of both MMP-2 and -9, and especially MMP-9 may be an important factor since MMP-9 activity not only causes ECM destruction itself, but has also been described to degrade α1-antitrypsin leading to increased activity of neutrophil elastase and cathepsin G[65,93]. MMP-9 is further capable of promoting infiltration of neutrophils (loaded with MMP-9 containing granules) by production of the biologically more active truncated form of IL-8, causing a vicious circle of MMP-9 activity in the diseased lung. Gelatinase presence and activity has been described as elevated in many other pulmonary diseases, such as cystic fibrosis, bronchiectasis, acute respiratory distress syndrome (ARDS) and infectious diseases (reviewed in [65] and [83]).

The obvious relation of gelatinases to tumour metastasis and angiogenesis has lead to a plethora of research papers on the role of MMP-2 and -9 in diverse malignant processes. This hypothesis was first affirmed by the observation that MMP-2 knockout mice show decreased tumour angiogenesis and progression[94] and since then MMPs have been identified as important players in angiogenesis, growth and metastasis of tumours.

The development of a new vascular system is necessary for development a tumour, since without new blood vessels the size of a tumour will be restricted. Gelatinases are primarily involved in this process by enabling proteolytic degradation of the vascular basal membrane, opening the way for endothelial migration to the formation of a new vessel[95]. MMP-2 is further capable of cleavage of laminin-5, which after degradation yields a cryptic site that increases endothelial cell migration[95], and the release of VEGF by MMP-9 stimulates angiogenesis not only under physiological conditions but also in cancer. Tumour growth can be stimulated by gelatinase activity since MMP-2 and -9 have been known to release growth factors[96]. Tumour metastasis is a process that involves release of single tumour cells, migration of these single cells to a vessel and penetration into the blood stream or lymph system and finally adhesion to vessel endothelium and extravasation into the tissue at the metastatic location. The ECM degrading properties of gelatinases are crucial in both exit of the metastatic cells from the bulk tumour as well as entrance into the new seeding site.

Increased gelatinase expression and activity has been described in hundreds of publications describing malignant diseases ranging from breast cancer[97], urogenital cancers[98-100], brain tumours[101], lung cancer[102], skin cancer[103] and many more. Interestingly many authors have found a positive correlation between gelatinase expression or activity and invasive potential of the tumour involved, again stressing the crucial role MMP-2 and -9 play in metastasis. A comprehensive review of the literature on gelatinase involvement in individual cancers is beyond the scope of this thesis, but excellent reviews are available(e.g. [83]).

Besides pulmonology and oncology gelatinase activity is under investigation in several other research fields. MMP-2 has been identified as a possible target in cardiovascular disease since it was identified as the protease responsible for degradation of the vasodilator peptide andrenomedullin, with one of the resulting fragment peptides having vasoconstrictive properties, possibly leading to hypertension[104].

1.2.3 Stromelysins

MMP-3

MMP-3 or stromelysin-1 was the first described in 1985 as a 51 kDa protein secreted by rabbit fibroblasts[105] that was able to degrade casein, and could be distinguished from collagenase by the inability to degrade type I collagen. More or less simultaneously a protease named transin was described in transformed rat cells, which later was identified to correspond to stromelysin[106,107]. Stromelysins have a basic MMP structure, with a hemopexin-like domain. The 51 kDa latent zymogen can be activated by proteolytic removal of the prodomain by for instance the serine proteases trypsin-2[108] and matriptase[109], yielding a 43 kDa active enzyme in humans. MMP-3 is upregulated by exposure to interleukin 1β, and downregulated by retinoic acid and dexamethasone[110].

The substrate specificity is broad and MMP-3 has been described to degrade many ECM proteins such as fibronectin, denatured collagens (gelatin), laminin and proteoglycans. MMP-3 is incapable of degrading triple helical collagens, but can cleave the globular portion of type IV collagen[111]. Besides degradation of ECM components MMP-3 is also involved in the activation cascade of the gelatinases and MMP-13 (see figure 2). The physiological function of MMP-3 is, surprisingly, not well described in literature, but is assumed to be mainly in turnover of extracellular matrix. MMP-3 is highly upregulated in mammary tissue during involution after the lactation period and seems to have a pro-apoptotic effect[112].

In vitro experiments with cultured cells have identified some membrane-bound signalling proteins that may be released by MMP-3 (e.g. E-cadherin[113] and Fas ligand[114]), but the physiological relevance of these finding is unclear. One well-described substrate of MMP-3 is plasminogen activator inhibitor-1 (PAI-1)[115], and proMMP-3 can form a complex with tissue-type plasminogen activator which increases the activity of t-PA[116]. These findings may be indicative of an important regulatory function of MMP-3 in the fibrinolytic pathway.

MMP-3 has been described as a factor of importance in development of arthritis[117], asthma[118], aneurism[119], impaired wound healing[120], Alzheimer's disease[121] and various cancers[122,123]. Recent insights and studies demonstrate that association of MMP-3 with disease states (especially cancer) is difficult, as many newer studies do not find a positive correlation between MMP-3 and the disease. This observation may be (partially) explained by the function of MMP-3 as an activator of other MMPs which makes identification of the protease 'culprit' difficult.

MMP-10

The cloning of rat transin/MMP-3 in 1985 quickly lead to the identification of a second stromelysin. This protease, named transin-2[124], and later identified in humans as stromelysin-2[125] or MMP-10 has 82% sequence homology with MMP-3[126]. MMP-10 is secreted as a 53 kDa zymogen, and is activated to a 47 kDa mature protease. Originally, MMP-10 production was thought to be to be relatively unaffected by stimuli such as

cytokines and hormones[127], but recent studies have shown results that indicate the contrary[128].
The physiological function of MMP-10 is poorly understood, with only a handful of publications dealing with characterization of this protease. The in-vitro substrate specificity seems similar to that of MMP-3, but catalytic activity towards type III, IV and V collagens is weaker[129]. Contrary to MMP-3, MMP-10 is not produced by fibroblasts, but is expressed in keratinocytes which in turn do not produce MMP-3[130]. MMP-10 seems to have an important role in skin wound healing and cellular migration, since it is primarily found at the front of the migrating epithelial 'tongue'[131] and has been observed in migrating enterocytes in inflammatory bowel disease[132,133]. MMP-10 is, in vitro, capable of processing laminin-5 which may be an additional mechanism in which the enzyme enables cellular migration[134].
Stromelysin-2 presence and activity at sites of resorption in developing bone has been demonstrated by histochemistry and casein in-situ zymography, and may play a role in the remodelling events taking place during ossification[135].

MMP-11
The third human stromelysin is MMP-11, which was first described in 1990 in a breast carcinoma cDNA library[136]. Although MMP-11 is often categorized as a stromelysin, it is very different from the other two proteases in this group. Production of MMP-11 is highest in fibroblasts, and is particularly observed in remodelling tissues at later stages of the process. MMP-11 has been associated with many physiological processes where ECM remodelling occurs, such as during embryonic development, female reproductive cycle and wound healing. The 56 kDa MMP-11 zymogen is activated intracellularly by furin[20] or paired basic amino acid cleaving enzyme-4 (PACE-4)[137] and is secreted as a 47 kDa active protease. The physiological role of MMP-11 is unclear, but differs significantly from other MMPs. No major ECM proteins such as collagens, gelatin and fibronectin can be degraded by MMP-11, and contrary to many other MMPs, exhibits an anti-apoptotic effect. The biological mechanism underlying this effect is not known, but probably involves proteolytic cleavage of yet uncharacterized protein substrates that promote cell survival[138]. Several possible substrates of MMP-11 have been identified, mainly being protease inhibitors such as α1 proteinase inhibitor and α2 macroglobulin. MMP-11 further has a weak caseinolytic activity and has been shown to cleave insulin-like growth factor binding protein-1 (IGF-BP-1) in a carcinoma cell line[139]. Recent research has demonstrated that although MMP-11 does not cleave many ECM proteins, degradation of type VI collagen is one of the physiological functions and is related to inhibition of adipogenesis by stromelysin-3[140].
The relatively narrow and aberrant substrate specificity of MMP-11 probably stems from a mutation that occurred in the highly conserved methionine-turn. Whereas all other MMPs contain an MxP sequence in the met-turn, in MMP-11 the proline is replaced by an alanine. This substitution has a profound effect on the structure of the S'1 selectivity pocket leading to greatly changed substrate specificity[141].
Although the physiological role of MMP-11 is still poorly understood, the involvement of this enzyme in especially the early stages of the process of tumour formation and metastasis

has been thoroughly investigated (reviewed in e.g. [142]). MMP-11 is rarely present in sarcoma tumours, but almost always expressed in carcinomas. An interesting observation is that the MMP-11 is produced not by the malignant cells themselves, but by the surrounding mesenchymal cells. High levels of MMP-11 have predictive value for tumour aggressiveness and low survival rate. MMP-11 plays a role in early invasion of the surrounding tissue by the tumour cells, a process which is dependent on the catalytic activity of the protease[143]. This is surprising since MMP-11 is not capable of degradation of the major ECM constituents. Cancer cell are able to stimulate nearby fibroblasts to produce MMP-11, a process which is associated with modification of the invaded ECM to a stroma phenotype by desmoplasia[144]. The anti-apoptotic effect of MMP-11 may play a role in establishment of the tumour, and early survival. MMP-11 deficient tumour exhibit higher levels of apoptosis, and implantation of experimental tumours is lower in MMP-11 null mice[145]. Although development of primary tumours is favourably affected by high MMP-11 levels, the metastatic potential of MMP-11 expressing tumours seems to be lower. In experiments with MMP-11 null mice, the number and size of secondary tumours was greater than in wild type mice implanted with similar sized tumours, indicating a protective effect of MMP-11[142].

1.2.4 Membrane-type MMPs

In addition to the soluble matrix metalloproteinases, a small group of membrane anchored MMPs has been described. The first member of this subfamily, MMP-14 or membrane type-1 MMP (MT1-MMP) was discovered only in 1994[146] and cloning experiments have since revealed the existence of five additional MT-MMPs. The domain structure of MT1-MMP is very similar to that of soluble MMPs (see figure 1) with the characteristic zinc binding catalytic domain, the prodomain in the inactive zymogen form of the protease, and a linked hemopexin-like domain, but most MT-MMPs are membrane-anchored by a single-pass transmembrane domain, and contain an intracellular cytoplasmic tail that contain three putative phosphorylation sites and is presumed to have significance in localization of the enzyme on the cell surface[147]. All MT-MMPs described contain the furin-like recognition site in their prodomain, allowing activation of the zymogen by proteolytic removal of this domain by furin and other proprotein convertases[148].

MMP-14

MT1-MMP is present at the cell surface as a 55-60 kDa active protease but may be processed by autocatalysis into a smaller species of 45 kDa by proteolytic removal of the catalytic domain. This truncated form, which still contains the hemopexin-like domain is assumed to play a role in autoregulation of MT1-MMP catalytic activity[149]. The substrate specificity of MT1-MMP is well described in literature (reviewed in [150]). The enzyme is capable of proteolytic degradation of type I, II and II collagens following the characteristic cleavage pathway used by collagenases[151], a finding that was corroborated by knockout experiments that confirmed the role of MT1-MMP as an important interstitial collagenase. MT1-MMP null mice die within 3 weeks, showing severe developmental abnormalities

related to deficiencies in ECM processing, such as dwarfism, skeletal dysplasia and defective vasularization[152,153]. MT1-MMP may be actually regulated by availability of type I collagen as a substrate, a hypothesis stating that migrating cells are triggered by clustering of cell surface integrins upon encountering a 3-dimensional collagen matrix leading to transcriptional activation of the MT1-MMP promotor[154]. Besides type I collagen, MT1-MMP is capable of degradation of many other ECM components, such as fibronectin, vitronectin, tenascin, nidogen, aggrecan, fibrin, fibrinogen and laminin-5 (leading to a possible stimulation of cellular migration as described earlier)[150]. Although the major proteolytic function of MT1-MMP lies in cleavage of extracellular substrates, some studies indicate a role in intracellular proteolysis after incorporation and accumulation of active MT1-MMP in the centrosomal compartment where it could contribute to development of mitotic spindle changes by degradation of pericentrin[155,156]. This mechanism could give rise to a role for MT1-MMP in malignant transformation of cells. MT1-MMP has further been identified as a cell-surface sheddase, and is capable of cleavage of many membrane-anchored proteins such as E- and N cadherin, integrins, hyaluronan receptor CD44, receptor activator of NF-κB ligand (RANKL) and several cell-surface proteoglycans and their receptors[150].

MT1-MMP was originally identified as the extracellular protease responsible for activation of proMMP-2, and this process remains the best described proteolytic function of the enzyme. In this process one of the subunits of an MT1-MMP dimer forms a trimeric complex with proMMP-2 and TIMP-2 at the cell surface leading to proteolytic removal of the propeptide of the MMP-2 zymogen by the 'free' MT1-MMP unit. Besides proMMP-2, also proMMP-13[157] and proMMP-8[158] have been identified as possible targets for activation by this mechanism, which could be complementary to the intrinsic collagenolytic activity of MT1-MMP.

The role of MT1-MMP in cancer is manifold, but the involvement in angiogenesis is well described and demonstrated by the insufficient vascularization in knockout mice. Firstly MT1-MMP is capable of degradation of the deposited fibrin matrix after vascular injury, effectively disrupting the repair mechanism and allowing endothelial invasion[159]. MT1-MMP may also be involved in migration of the endothelial cells into the ECM, by proteolytic degradation of the extracellular matrix proteins, and by processing of various adhesion molecules[160]. The formation and stabilization of the newly formed capillary tubes may also be dependent on MT1-MMp activity, as demonstrated by impaired capillary formation in knockout models and RNA interference experiments[161]. Finally, MT1-MMP is able to release vascular endothelial growth factor A (VEGF-A) by shedding, again promoting neovascularization[162].

MMP-15

MT2-MMP (MMP-15) was first described in 1995 as the second member of the membrane-anchored MMP subfamily[163]. MT2-MMP is an ubiquitously expressed enzyme with largely overlapping substrate specificity with MT1-MMP[151]. Although the physiological function of this protease is not as well described as for MT1-MMP, some studies have indicated a role in follicle rupture during ovulation[164], generation of tubular structures during

angiogenesis[165] and has anti-apoptotic properties[166]. MT2-MMP is also capable of activating the MMP-2 zymogen, but contrary to MT1-MMP the activation mechanism is not dependent on the presence of TIMP-2, but rather on interaction with the hemopexin-like domain of MMP-2[167].
Involvement of MT2-MMP in pathology is still unclear, but considering the similarity with MT1-MMP a role in cancer is expected. Indeed, MT2-MMP is present in many investigated tumours, such as glioblastoma[168], non-small cell lung carcinoma[169], ovarian[170] and breast carcinoma[171], and seems to correlate to tumour invasiveness[168].

MMP-16 and MMP-24
The two latest additions to the membrane-spanning MMP family are MT3-MMP (MMP-16), first described in 1997[172] and MT5-MMP (MMP-24), first described in 1999[173]. These enzymes are still poorly described in literature. The crystal structure of MT3-MMP has been elucidated, and shows extensive homology to MT1-MMP[174]. MT3-MMP activity is regulated by an autoproteolytic shedding process where a soluble form of the enzyme is released from the cell surface, and the active enzyme shows a high affinity to TIMP-3, as opposed to TIMP-1. Although originally anticipated to be a brain-specific enzyme[175], MT5-MMP is possibly involved in remodelling events in endometrial lesions, and endometriosis[176].
Like the other MT-MMPs, both MT3- and MT5-MMP are capable of activating proMMP-2[177,178].

MMP-17 and MMP-25
The final two MT-MMPs are structurally different from the other four with respect to their interaction with the cell membrane. MT4-MMP (MMP-17)[179] and MT6-MMP (MMP-25)[180] are linked to the cell membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor, as opposed to containing a membrane-spanning domain. This anchor moiety is linked to the hemopexin-like domain by a 35-45 amino acid long hydrophilic linker, or stem. After production of the enzyme this stem region is linked to a short hydrophobic tail, which is exchanged for a GPI anchor in the endoplasmatic reticulum[181]. The stem region further contains 2 or 3 cysteine residues, which probably have a function in formation of complexes, as demonstrated by the presence of ~120 kDa and ~180 kDa isoforms of MT6-MMP that are dissociated in the mature 57 kDa form of the enzyme under reducing conditions[182]. The GPI anchor gives the possibility of interaction of these proteases with lipid raft microstructures, and the possibility of internalization and recycling of the enzymes[181]. The TIMP-inhibition profile of the GPI anchored MT-MMPs is different than for the membrane-anchored MT-MMPs. While the latter are relatively resistant to inhibition by TIMP-1 due to incompatibility of the Thr_{98} residue with the S'1 selectivity pocket, MT4- and MT6-MMP are effectively inhibited by TIMP-1, as well as by TIMP-2 and TIMP-3[183,184]. GPI anchored MT-MMPs can be shed from various cells in exosomes, possibly leading to paracrine transfer to other cells[185].
Both MT4- and MT6-MMP are capable of degrading ECM protein, albeit MT4-MMP in a limited fashion with cleavage demonstrated for gelatin, fibrin and fibrinogen, while MT6-

MMP can cleave a wider range of ECM constituents including fibronectin, type IV collagen and proteoglycans[186]. In vitro experiments have revealed a multitude of possible substrates (including TNF alpha, indicating sheddase activity), but the physiological relevance is unclear (reviewed in [181]). Interestingly, MT4-MMP is not able to activate proMMP-2 even in vitro, making this the only MT-MMP that lacks this trait[187]. MT6-MMP does activate proMMP-2, but generates a different form of the active enzyme than the other MT-MMPs, indicating that the interaction between MMP-2 and MT6-MMP is unique[188]. This activation mechanism is possibly dependent on the tight junction protein claudin-5, since cells that do not produce this protein are incapable of proMMP-2 activation by MT6-MMP[189]. MT4-MMP has been described as an activator of the aggrecanase ADAMTS-4[190].
Both GPI-anchored MT-MMPs are highly expressed in a wide variety of cancer cells ranging from breast carcinoma to glioma and colon cancers (reviewed in [181]), but the clinical relevance of the presence of these proteins in malignant cells is not yet clear.

1.2.5 Matrilysins

MMP-7
MMP-7 (matrilysin) was originally described as PUMP-1 (putatative uterine metalloproteinase-1) in 1988, and was long considered a third member of the stromelysin family (MMP-11 was not known yet), although it appeared only distantly related to the other stromelysins[125]. The pump-1 gene identified from rat tumour cDNA cloning experiments was confirmed to code for a secreted metalloprotease in rat uterus[191], and later dubbed MMP-7. The mmp-7 gene contains a AP-1 promotor region, leaving it sensitive to upregulation by cytokines and growth factors[192]. MMP-7 is the smallest human MMP (28 kDa zymogen, 19 kDa mature active form) since it lacks the C-terminal hemopexin-like domain. This 'minimal domain structure' means the activated enzyme is comprised of only the zinc-binding catalytic domain and results in an inability of MMP-7 to degrade intact collagens, again demonstating the importance of the hemopexin-like domain in substrate recognition[193]. MMP-7 is however capable of degradation a wide array of other ECM components such as gelatin, fibronectin, laminin and elastin. MMP-7 is capable of cleaving the prodomain of the gelatinases MMP-2 and -9[194], but the relevance under physiological conditions seems debatable considering the alternative, well-described activation pathway of proMMP-2. Finally, MMP-7 is a possible sheddase, with potential to liberate TNFα, Fas ligand, heparin binding epidermal growth factor (HB-EGF), E-cadherin and β4-integrin[195].
The original biological function of MMP-7 was the involvement in involution of the endometrium after pregnancy, but as later discovered, an MMP-unique function of MMP-7 seems to be the role in innate immunity. MMP-7 knockout mice exhibit decreased resistance to bacterial gastrointestinal infection, and show decreased clearance of *E. coli* from the small intestine. MMP-7 is constitutively produced in the mucosal epithelium, and may exert its function in mice by cleavage of pro-α-defensins yielding peptides with antibacterial properties Co-localization of MMP-7 and pro-α-defensins in specialized epithelial Paneth cells seems to confirm this function[196]. The role in mucosal host defense is further confirmed by the finding that exposure of cultured mucosal epithelial tissue or cells

to pathogenic bacteria such as *E. coli* or *Pseudomonas aeruginosa* causes an increased production of MMP-7, and that germ-free bred mice show no expression of MMP-7 in the unchallenged gastro-intestinal tract[197]. The strong induction of MMP-7 by bacterial challenge is a epithelium-specialized function, since it does not occur in other cell types expressing MMP-7, and is exclusive for MMP-7 since no other MMPs are upregulated[198].
MMP-7 seems to be important in wound repair, since MMP-7 null mice show severe defects in epithelial wound healing which is probably due to a disrupted re-epithelialization[199].
MMP-7 further enables migration of neutrophils through the epithelium during inflammation, since in MMP-7 null mice show accumulation of neutrophils in the insterstitium without crossing of these cells over the epithelium, resulting in reduced mortality[200]. The mechanism underlying this effect is probably the creation of a chemotactic gradient by cleavage of the proteoglycan syndecan-1 at the epithelial cell surface, causing liberation of the syndecan-bound chemokine KC that is produced by the epithelial cells after injury.
The role of MMP-7 in cancer is well described in literature. MMP-7 seems to occupy a unique place in the MMP-cancer association, since it is one of the few MMPs that is actually produced by cancerous cells themselves, as opposed to the stroma under stimulation of the malignant cells[195]. As with other MMPs, MMP-7 has been identified in a wide range of tumours, and correlates with the agressiveness of the tumour. In a malignant state, the activation of MMP-2 and -9 by MMP-7 may have an important function, and lead to increaded invasiveness of the tumour[201,202]. MMP-7, like MMP-3 may further promote tumour invasion by shedding of E-cadherin, leading to decreases cellular adhesion. Tumour growth is likely associated with MMP-7 sheddase activity, since the release of soluble HB-EGF by MMP-7 promotes cellular proliferation[203]. MMP-7 is further capable of cleaving all six members of the insuline-like growth factor binding protein (IGF-BP) family, leading to increased availability of free IGF which again promotes cancer cell growth and survival[204]. The activation of ADAM-28 is attributed to MMP-7[205], leading to increased degradation of IGF-BP3.

MMP-26

Recently, a novel matrilysin-like enzyme was identified in an endometrial tumour and named MMP-26 (matrilysin-2, endometase)[206,207]. Like MMP-7, this is a minimal domain MMP missing the hemopexin-like C-terminus. This protease seems to have a more limited substrate specificity compared to MMP-7, not being able to degrade collagens, laminin and elastin[208]. MMP-26 is unique from other MMPs since it is the only MMP described so far that does not have a functional cysteine switch mechanism keeping the pro-enzyme in its latent conformation. This functional loss of the cysteine switch is attributed to the presence of a histidine residue n-terminal of the cysteine, a feature only observed in MMP-26[209] [marchenko 2001]. Basal expression of MMP-26 is low except in endometrium, but is increased in many carcinoma cell lines[210]. The physiological function of MMP-26 remains to be elucidated to date.

1.2.6 Macrophage metalloelastase

MMP-12 was first described in 1981[211] as murine metalloelastase and later identified as a member of the MMP family[212]. In 1993 an orthologue in human was found[213]. MMP-12 was identified as an elastolytic metalloproteinase produced by alveolary macrophages, which lead to the trivial name (murine) macrophase metalloelastase (MME) or human macrophage elastase (HME). MMP-12 is expressed as a 54 kDa inactive zymogen, and is activated to a 45 kDa active enzyme by removal of the propeptide sequence. This mature enzyme can be further truncated to a 22 kDa active form involving processing of the C-terminal sequence mediated by serine protease or autocatalytic cleavage[213]. This autolytic removal of the c-terminal domain is possible in many other MMPs, but occurs very slow in contrast to MMP-12 which is readily processed to the smaller form. Expression of MMP-12 is limited macrophages, and is not observed in blood monocytes. As the name reveals, a major substrate for MMP-12 is elastin, but MMP-12 is capable of degrading other ECM constituents (but not gelatin)[214] and many non-matrix proteins in vitro[215].
MMP-12 null mice show normal development in absence of inflammatory stress, but litter size is smaller, presumably due to placenta abnormalities during gestation. Macrophages obtained from knockout mice retain only a small fraction of their elastolytic activity, indicating that MMP-12 is indeed the most important elastin degrading enzyme (in mice). MMP-12 is a vital factor in penetration of macrophages through the basement membrane, as demonstrated by a complete inhibition of this migration in macrophages from MMP-12 null mice both in vitro as in vivo[216].
MMP-12 seems to play an interesting role in cancer which is different from other MMPs. MMP-12 is the primary protease responsible for proteolytic liberation of angiostatin from plasminogen. Angiostatin is a 38 kDa protein with anti-angiogenic properties by the selective inhibition of endothelial proliferation. MMP-2 and -9 are also capable of degradation of plasminogen in vitro, but were found to have a minor to non-existing contribution in the production of angiostatin in an animal model[217,218]. This is another example of a protective effect of MMP activity in tumourogenesis, and is an indication that knowledge of the actual biochemical mechanism of the involvement of the protease is indispensible and cannot be replaced by mere association of expression levels in tumours.
Since human MMP-12 was first cloned from alveolar macrophages it is not surprising that the role of MMP-12 in lung diseases has been extensively studied. The hypothetical involvement of MMP-12 in fibrotic processes in the lung is obvious, but studies concerning the role of MMP-12 in emphysema are diffuse. Some authors find no upregulation of MMP-12 in macrophages of patients compared to control groups, but rather of other macrophage-derived MMPs[219], while others state that macrophage elastase is an absolute requirement for the development of emphysema after cigarete smoke exposure in a knockout animal model[220-222]. The interspecies difference may have caused bias with respect to the importance of MMP-12 since, although the failure of MMP-12 null mice to develop emphysema makes a more compelling point than determination of expression levels, MMP-12 seems to be the major MMP present in murine macrophages, while in human several other MMPs are produced[198]. A polymorphism in the MMP-12 gene has

been described to have predictive value in lung function decline in COPD[223]. Elevated MMP-12 has been described in induced sputum of COPD patients[224], but a recent studie has found a slight increase of MMP-12 only in stage 0 of the disease[225], which could imply involvement of MMP-12 in the early development of COPD. Proteolytic fragments of elastin have been implied as chemotactic factors in macrophage recruitment[226], giving a possible explanation of an early role of MMP-12, with the severe tissue destruction at later stages being mainly caused by other macrophage-derived proteases.

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1.3 ADAM proteinases

The "a disintegrin and metalloproteinases" (ADAMs) or MDCs (metalloproteinase-like, disintegrin-like, cysteine-rich proteins) are membrane-anchored metzincins of the adamalysin subfamily which also contains the interesting biological close relative, the class III snake venom metalloproteases[1]. These proteases have a similar domain structure to the membrane-type MMPs (see figure 1), but with two distinct differences. The ADAM proteases do not contain the characteristic hemopexin-like domain, but instead have three additional domains; the cysteine-rich domain, the EGF-like repeat domain and the disintegrin domain from which the family derives its name. While the biological function of the EGF-like domain has not yet been fully elucidated, the cysteine-rich and disintegrin domains enable the cell surface bound ADAMs to interact with ECM proteins and ligands on neighbouring cells. This important function in intercellular interaction was already clear from the identification of the first members of the ADAM as key players in the fertilisation process, specifically in the binding and fusion between egg and sperm cells. The first adamalysins described in mammalia were found on guinea pig sperm and dubbed fertilin α and β (or PH-30 α and β)[2-4], and were renamed ADAM-1 and -2 after identification of several other homologous genes[5].

Disintegrin-integrin binding

The presence of a disintegrin domain in ADAMs is unique among cell-surface proteins, and can mediate cell-cell and cell-matrix interaction by binding integrins. The major integrin involved in interaction with ADAMs has been identified as integrin α9β1 which can interact with a conserved RX_6DEVF sequence in the disintegrin domain[1], although structural modelling of a snake venom disintegrin-metalloprotease has demonstrated that this motif is shielded by the cysteine-rich domain and may be inaccessible for integrin binding[6].

Many other integrins are capable of binding the disintegrin domain[7]. The consensus recognition site of disintegrins, first identified as integrin antagonists in snake venom, is dependent on the presence of Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequences, but surprisingly only ADAM-15 contains the RGD sequence in its disintegrin domain, and this recognition site enables interaction with a broader range of integrins such as αvβ3, αvβ5 and α5β1[8,9]. Other ADAMs (e.g. ADAM-9 and -23) are capable of binding these integrins through interaction with a Glu-Cys-Asp (ECD) sequence in the disintegrin domain, an observation that corroborates the pivotal importance of especially the aspartic acid residue in disintegrin-integrin interaction site[10].

Although the binding potential of the ADAM proteins to integrins is relatively well characterized, the biological relevance and scope is not yet clear[10]. The only physiological process where the functionality is well documented remains the fertilization process, with the two fertilin-ADAMs forming a hetero-dimer capable of binding integrin α6β1 on the surface of the egg[11], but this model is under debate[12]. The fact that in human the fertilin α/ADAM-1 gene encodes a non-functional protein is a strong indication that this model for ADAM involvement in egg-sperm fusion in mammalia may need to be reconsidered [13].

Gene deletion experiments have demonstrated an important role for ADAM-2 in fertilization since fertilin β null mice are infertile[14], but ADAM-2 is not indispensable in the sperm-egg fusion process since sperm from these knockout mice can still fuse with oocytes, albeit at a ~50% decreased fusion rate. The observed sterility of fertilin β -/- mice is postulated to be due to disruption of transit of the sperm through the female reproductive tract[15], which may be an indication that ADAM function during fertilization has a hitherto unknown mechanism[12].

Interaction via the cysteine-rich domain

Besides the disintegrin-integrin interaction, ADAMs are capable of binding several ECM constituents. The cysteine-rich domain may have an important function in binding heparan sulfate proteoglycan such as syndecans, a phenomenon described for ADAM-12[16]. Studies involving the *Xenopus*-specific ADAM-13 have further revealed an important role for the cysteine-rich domain in interaction with fibronectin[17]. An early observation on the structure of metalloprotease-disintegrins revealed the presence of a hydrophobic stretch in the cysteine-rich domain of several ADAMs which shows high sequence similarity to viral fusion peptides[18], which may implicate a role for the cysteine-rich domain in membrane fusion. This hypothesis has however not been confirmed by experimental data[1].

Function of the cytoplasmic domain

The cytoplasmic domain of the individual ADAM proteases is highly variable in length (40-250 amino acids) and sequence, but the majority of ADAMs contain cytoplasmic tails with PxxP motifs, which indicates binding sites for SH3 (Src-homology 3) domain containing proteins[19]. This structural feature enables interaction of the cytoplasmic domain with a large range of intracellular proteins and may be important in regulation of ADAM localization by interaction with cytoskeletal proteins such as α-actinin-2[20]. ADAM function may also be regulated by interaction of the cytoplasmic tail with intracellular signalling proteins, as demonstrated by the activation of ADAM-9 by phorbol esters via interaction with PKC δ[21].

The cytoplasmic domain of some ADAMs further contains putative phosphorylation sites for serine-threonine or tyrosine kinases, which may be involved in regulation of ADAM function, or supply binding sites for SH2 domain containing proteins[1].

ADAM metalloprotease function

Perhaps the best studied function of the ADAM proteins is the catalytic activity exhibited by the metalloprotease domain. The metalloprotease domain is highly homologous with the MMP catalytic domain, but interestingly not all ADAMs contain the consensus HExxHxxGxxH zinc-binding metalloprotease active site (see table 2), which leads to the conclusion that from the 22 human ADAMs only 12 (possibly) may exert endopeptidase activity. Interestingly, in mice an additional subfamily of possibly catalytically active ADAMs (ADAM-24-26, -34 and 36-40) has been described and named testases, referencing the testis-specific expression pattern[22]. In human no orthologues of the testases have been identified to date.

Table 2*: Overview of the 22 identified human ADAM proteases. (t)MDC: (transmembrane) metalloprotease-like, disintegrin-like, cysteine-rich protein.*
‡: although full length ADAM-1 contains a metalloprotease domain with active site sequence, this domain is removed in the mature processed form[4].

ADAM	Alternative names	MP active site?	Remarks
1	Fertilin α, PH-30 α	yes ‡	Non-functional pseudogene Testis specific
2	Fertilin β, PH-30 β Cancer/testis antigen 15	no	Testis specific
3	Cyritestin, tMDC I	no	Non-functional pseudogene Testis specific
6	tMDC IV	no	Testis specific
7	EAP-1, Sperm maturation-related glycoprotein GP-83	no	
8	Cell surface antigen MS2, CD156a	yes	
9	MDC9, Meltrin γ Myeloma cell metalloproteinase	yes	
10	Kuzbanian protein homolog, CD156c	yes	
11	MDC	no	Brain specific
12	Meltrin α	yes	
15	Metargidin, MDC-15	yes	
17	TACE, CD156b Snake venom-like protease	yes	
18	tMDC III, ADAM-27	no	Testis specific
19	Meltrin β, MADDAM Metalloprotease and disintegrin dendritic antigen marker	yes	
20	None	yes	Testis specific

21	ADAM-31	no	Testis specific
22	MDC 2	no	
28	MDC-L, ADAM-23	yes	
29	Cancer/testis antigen 73	no	Testis specific
30	None	yes	Testis specific
32	None	no	Testis specific
33	none	yes	

The ADAM metalloprotease domain is shielded off by a prodomain in the inactive zymogen in a similar structural conformation as in MMPs involving a cysteine switch mechanism. The primary activation pathway of the ADAM zymogens involves removal of the prodomain by proprotein convertases (PCs) such as furin in the trans-Golgi network as demonstrated by strongly diminished activation of ADAM-9 and -15 in the presence of early-secretory pathway inhibitors brefeldin A and monensin, increased activation of ADAM-10 after overexpression of PC-7 and blocked processing of proADAM-10, -12 and -19 after mutational modification of the furin Rx(R/K)R recognition site (discussed in [1]). Some ADAMs may require autoproteolytic processing for activation, as demonstrated for ADAM-8[23] and -28[24]. Interestingly, the prodomain may act as a chaperone and is required for proper folding of the ADAM protein, and especially the metalloprotease domain. Removal of the prodomain prior to folding yields an inactive form of ADAM-17[25]. For ADAM-10 a similar effect has been observed in cells expressing the protease without the prodomain, an effect that could be reversed by cotransfection of the prodomain[26].
Although, similar to the MMPs, the catalytically active ADAMs are capable of degradation of ECM proteins such as collagens and fibronectin *in vitro*, the physiological relevance of this observation is not clear. The endopeptidase activity for which the ADAMs are best known is the so-called shedding; liberation of biologically active proteins from their membrane-anchored proforms. This function of the ADAMs was first described simultaneously by two groups in 1997, identifying a disintegrin-metalloprotease as an important contributor to the production of soluble TNFα from the membrane-bound precursor[27,28]. The responsible protease was named TACE (TNF-alpha converting enzyme) and gene-silencing experiments demonstrated a dominant, but not exclusive role for TACE since TNFα production was significantly diminished but not completely shut down in the knock-out mice.
Considering the scope of this thesis only the ADAMs containing the metalloprotease active site will be discussed in further detail.

ADAM-8

ADAM-8 (CD156) was first described in 1990 as antigen MS2 in mice[29], and was later confirmed as a member of the human ADAM family in 1997 [30]. ADAM-8 is a transmembrane glycoprotein primarily expressed as a 120 kDa zymogen by immune cells, but is also found in neurons and oligodendrocytes in the central nervous system. The promoter region of the *adam-8* gene contains response elements for lipopolysaccharide (LPS) and cytokines such as interferon γ, interleukin-6 and TNF α[31].

ProADAM-8 does not contain the furin cleavage recognition site and depends on autoproteolytic removal of the prodomain for activation, yielding a 90 kDa mature enzyme. The mature cell-surface ADAM-8 may be further processed by autoproteolytic shedding yielding a 60 kDa soluble remnant form of the protein missing the metalloprotease domain[23]. ADAM-8 may also exist as a soluble, catalytically active form which is presumably released from the cell by autoproteolytic shedding[32]. Contrary to the MMP and MT-MMP families, ADAM-8 catalytic activity is not inhibited by any of the four known TIMPs *in vitro*[33].

The biological function of ADAM-8 is far from clear, but the protein has been associated with neuron-glia interactions in the central nervous system[34], cell-cell fusion in osteoclast differentiation (an effect presumably mediated by the remnant form)[32] and recent work has shown a possible role in the ovulation process involving hormonal regulation of ADAM-8[35]. ADAM-8 sheddase activity *in vivo* is still rather poorly characterized, but *in vitro* experiments involving cleavage assays of 10-mers containing known recognition sites for ectodomain shedding have revealed multiple possible substrates such as β-amyloid precursor protein (APP), the low affinity IgG (CD16) and IgE (CD23) receptors, L-selectin, P-selectin glycoprotein ligand, transforming growth factor α (TGF α) and TNF α[36]. ADAM-8 has also been implicated in shedding of the neural adhesion molecule CHL-1 (close homologue of L-1), yielding a biologically active fragment that enhances neurite outgrowth and suppresses neuronal cell death, indicating an important role in the development of the central nervous system[37].

Knock-out experiments in mice have identified ADAM-8 as a non-essential protein during development since ADAM-8 null mice show no pathological defects[38]. This finding may be explained by a rather large redundancy with other ADAM proteases or by a specific role for ADAM-8 not in development but rather in processes such as inflammation, a hypothesis that seems to be substantiated by the responsiveness of ADAM-8 expression to cytokine stimulation.

ADAM-8 has been associated with development of asthma following the observation that ADAM-8 is highly upregulated in experimental ovalbumine-induced asthma models in mice, showing strong induction in peribronchial and perivascular inflammatory cells and bronchiolar epithelial cells[39]. In an asthma model with transgenic mice producing a soluble form of ADAM-8 a protective effect of sADAM-8 was found, presumably related to suppressed trafficking of leukocyte migration[40], which may be mediated by shedding of vascular cell adhesion molecule 1 (VCAM-1) by ADAM-8[41]. The amount of clinical evidence linking ADAM-8 to development of pulmonary diseases is limited, but soluble

ADAM-8 and VCAM-1 are increased in BAL fluid of patients suffering from eosinophilic pneumonia[42] and ADAM-8 mRNA is increased in asthma patients with a positive correlation with disease severity[43].
ADAM-8 is expressed in human neutrophils where it is stored in granules, and is transported to the cell surface upon stimulation. The cell surface exposed ADAM-8 is then rapidly released into the extracellular space by a metalloprotease activity dependent process. These in vitro findings have been confirmed in neutrophils retrieved from synovial fluid from patients suffering from rheumatoid arthritis, again indicating a possible role for ADAM-8 activity in inflammatory processes[44].
ADAM-8 expression is increased in neutrophils that are in contact with endothelial cells, and since L-selectin is a possible substrate ADAM-8, and ADAM-8 colocalizes with L-selectin on the neutrophil surface ADAM-8 mediated L-selectin shedding may play an important role in regulation of neutrophil rolling and trans-endothelial extravasation[44].
Since ADAM-8 is involved in osteoclast differentiation it is not surprising that several studies have focussed on the role of this protease in the development of pathological bone destruction. ADAM-8 expression is positively associated with tissue destruction in rheumatoid arthritis, and ADAM-8 protein is mainly localized at the edge of the eroded cartilage and bone (the so-called pannus)[45]. A similar induction of ADAM-8 at the edge of healthy and eroded tissue is described in loosening hip replacements[46].
ADAM-8 has further, as most metzincins, been associated with various malignant processes. In pancreatic duct adenocarcinoma, ADAM-8 expression and protein level is correlated with the invasiveness of the tumour and with reduced survival rate[47], a phenomenon that may involve tumour hypoxia[48]. The same correlation has been described for primary brain tumours and ADAM-8 activity, as measured by conversion of a peptide substrate, is higher in cells obtained from gliomas with higher invasive potential[49].
Overexpression of ADAM-8 has also been associated with poor prognosis in lung cancer, with high expression significantly more common in stage IIIb/IV adenocarcinomas compared to lower stages. The soluble isoform of the protein has been suggested as a useful diagnostic serum marker for lung cancer[50]. ADAM-8 overexpression has finally been described in prostate cancer[51] and renal cell carcinoma[52,53].

ADAM-9
ADAM-9 or meltrin γ was first identified in 1996 as MDC-9 from breast carcinoma[54] and as myeloma cell metalloproteinase in myeloma[55]. ADAM-9 is ubiquitously produced as a 110 kDa glycosylated zymogen which can be activated in the medial Golgi apparatus by prodomain removal by proprotein convertase activity to the 84 kDa mature form that is found on the cell surface. The cell membrane anchored form can be further truncated by proteolytic processing to a 47 kDa soluble form which contains the metalloprotease domain and retains its catalytic activity as demonstrated by the ability to cleave insulin B-chain *in vitro*. Purified soluble ADAM-9 is further capable of cleaving peptides containing the membrane-proximal cleavage region of β-APP, proTNFα, the p75 TNF receptor and c-kit ligand-1 (KL-1), but not IL-6 receptor, the p55 TNF receptor, transforming growth factor α and L-selectin[56]. Recombinant ADAM-9 is capable of degradation of fibronectin *in vitro*,

but the physiological relevance is not proven[57]. Like ADAM-8, ADAM-9 activity is not inhibited by any of the four TIMPs[33].
As with ADAM-8, knockout models have demonstrated that ADAM-9 is not vital for development and survival since ADAM-9 null mice show no major abnormalities[58]. ADAM-9 is capable of processing β-APP, and is widely considered to be one of the three alpha-secretases from the ADAM family (next to ADAM-10 and -17)[59]. The ADAM-9 knockout mice do however show no aberrant APP processing, indicating functional compensation by or redundancy with other ADAMs. Similarly, shedding of HB-EGF which is known to be decreased in cells overexpressing a mutant form of ADAM-9[21] is unaffected by ADAM-9 knock-out.
In rat kidney, ADAM-9 is mainly found in the basolateral surface of the tubular cells. The expression in glomerular epithelia is restricted to areas in contact with the underlying basement membrane indicating an important role in cell-cell and cell-matrix interaction[60] which may be mediated by binding of the disintegrin domain to beta-1 integrins which co-localize with the renal ADAM-9 distribution[61].
The soluble isoform of ADAM-9 has been reported as an important player in tumour metastasis in the liver. After shedding from the cell surface of activated hepatic stellate cells, sADAM-9 is hypothesized to bind to α6β4 and α2β1 integrins on the surface of carcinoma cells and promote stroma-tumour interaction. Addition of the soluble ADAM-9 to Matrigel invasion assays showed an increased invasive potential in several cancer cell lines. This effect is blocked by the broad-range metalloprotease inhibitor 1,10-phenantroline, indicating a possible role for an active metalloprotease, although it remains unclear whether this is activity of ADAM-9 itself. Histological examination of tumour sections obtained from liver metastases revealed that ADAM-9 expression was highest at the invasive front, and in regions of tumour-stroma contact while the tumour cells and hepatocytes themselves were negative for ADAM-9[62].
ADAM-9 expression is higher in malignant prostate tumours, and expression in carcinoma cell culture is increased in response to oxidative stress. This effect is likely mediated by an androgen receptor (AR) involving mechanism, since AR-negative prostate cancer cells do not exhibit increased expression after exposure to radical oxygen species (ROS), and preincubation of the cells with the anti-androgen bicalutamide abrogates the higher expression of ADAM-9[63]. By blocking ADAM-9 production apoptotic cell death could be induced in the cultured prostate cancer cells, indicating a role for ADAM-9 in tumour survival[64]. Concurrently, high tumour ADAM-9 expression has been identified as a significant prognostic marker for relapse in prostate cancer[65].
Since ADAM-9 is a putative α-secretase of β-APP this protease has gained some interest from the Alzheimer's disease research field (although less than the two other α-secretases in the ADAM family). Alpha secretases supposedly have a protective effect in the development of Alzheimer's disease, which is amongst others characterized by accumulation of neurotoxic amyloid-beta peptides (Aβ) and plaque formation in the brain. These peptide fragments are the result of sequential β- and γ-secretase processing of APP, while α-secretases have a cleavage site within Aβ leading to degradation of the neurotoxic peptides. Decreased sheddase activity of the ADAM α-secretases may therefore play an

important role in the development of the disease[66]. The physiological relevance of ADAM-9 as an α-secretase has however been debated in recent years, especially after findings that APP cleavage is unaltered in ADAM-9 knockout models, and the consensus seems to point to a minor role of ADAM-9 in APP processing *in vivo*[67,68].

ADAM-10

ADAM-10 was first isolated in 1995 as a membrane-bound metalloproteinase capable of cleaving myelin basic protein[69], later found to be expressed by various cell types and named MADM (mammalian disintegrin-metalloproteinase), and have significant sequence homology with several other earlier discovered mammalian disintegrin-metalloproteinases such as meltrin-α (ADAM-12) and MS2 (ADAM-8) in 1996[70]. MADM/ADAM-10 was discovered to be a mammalian counterpart of the *Drosophila* protein Kuzbanian which plays an important role in neurological development by proteolytic activation of the Notch-receptor and shedding of the Notch receptor ligand delta[71].

ADAM-10 is produced as an inactive zymogen which is activated by proprotein convertases in the trans-Golgi network to its 56-58 kDa glycosylated mature form[72]. Contrary to most other metzincins, the cysteine switch mechanism which normally links the prodomain to the catalytic centre keeping the enzyme inactive seems to be non-functional in ADAM-10[73]. ADAM-10 catalytic activity is inhibited by TIMP-1 and -3[74].

ADAM-10 is one of the better characterized ADAMs to date, with many studies focussing on the proteolytic function of this enzyme. ADAM-10 is capable of degradation of type IV collagen in vitro[75] and has been identified as a possible sheddase of cell-surface bound proteins such as epidermal growth factor receptor ligands EGF and betacellulin[76], ephrin-A2[77], cellular prion precursor protein[78], chemokines CX3CL1 and CXCL16[79,80] adhesion molecule L1[81] and many others, although some caution has to be taken in interpreting these results since most target substrates have been identified *in vitro*, an many ADAM-10 mediated shedding events in cell lines are validated by incubation with supposedly ADAM-10/ADAM-17 selective hydroxamate-based inhibitors (like TAPI, TNF alpha protease inhibitor) that were later shown to actually be broad-range metzincin inhibitors. ADAM-10 is believed to be primarily involved in the constitutive shedding of cytokines, chemokines and their receptors, while ADAM-17 mediated shedding is more responsive to stimuli[82]. The release of TNFα from cells was thought to be (partially) mediated by ADAM-10 but recent insights have demonstrated that ADAM-10 probably plays a minor role[83].

The best studied shedding activity mediated by ADAM-10 lies within the Notch signalling pathway. Notch signalling is a highly conserved, ancient pathway that is essential in intercellular contact and cell fate determination in all stages of neural development. The membrane-bound Notch receptor is activated by either membrane-bound ligands delta or serrate/jagged on adjacent cells leading to an intracellular signalling cascade involving a series of proteolytic cleavages (S1-3), internalization of the processed Notch receptor, transport to the nucleus and finally activation of gene transcription. The precise outcome of Notch receptor activation is highly dependent on other extra- and intracellular signals and can lead to inhibition of neuronal cell and oligodendrocyte differentiation, or promotion of differentiation of glial progenitor cells (reviewed in [84]).

Although the role of the invertebrate ADAM-10 homologues Kuzbanian (*Drosophila*) and Sup-17 (*C. elegans*) in Notch signalling is clear[85,86], involving proteolytic activation of the Notch receptor-ligand complex (stage S2), attempts to identify ADAM-10 as a key player in mammalian Notch signalling have yielded variable results. Some studies point to ADAM-17 as the essential protease for Notch processing, at least by proteolytic cleavage assays using ADAM-17 in vitro[87] and cotransfection of DTACE (the Drosophila ADAM-17 homologue) in delta-expressing Drosophila S2 cells, but ADAM-17 knockout models show little similarity to Notch knockouts while ADAM-10 deficient mice die early in embryogenesis showing major defects in development of the central nervous system and the vascular system with similar histological abnormalities observed in complex Notch deficiency induced in mice[88,89].
This apparent discrepancy between *in vitro* substrate specificity and biological relevance is also apparent from the unclear role of ADAM-10 as an APP α-secretase. ADAM-10 is widely expressed in neuron in the CNS[90], is capable of cleaving APP-derived peptides *in vitro* and overexpression of ADAM-10 increases the release of cleaved APP from HEK 293 cells, while transfection with a non-functional ADAM-10 mutant inhibited APP processing[91]. On the other hand most preserved embryonic fibroblast cell lines obtained from ADAM-10 knockout mice show no decreased or altered APP processing compared to control, although two out of 17 lines do have severe APP processing defiencies[88].
Several shedding events possibly mediated by ADAM-10 have been associated with cancer pathology. Proteolytic removal of the extracellular domain neuronal cell adhesion receptor L1-CAM by ADAM-10 promotes metastasis in colon cancer[92], ADAM10 may be involved in metastasis by shedding of CD44 promoting migration of cancer cells through the ECM by interaction with hyaluronic acid[93] and ADAM-10 mediated shedding of EGF receptor ligands may promote tumour growth. ADAM-10 activity may be associated with poor prognosis in breast cancer by shedding of membrane-anchored HER2 receptor leaving a fragment with constitutive kinase activity leading to ligand independent cell growth and resistance to apoptotic signals[94].
ADAM-10 proteolytic activity has been associated with a protective effect in inflammatory processes by shedding of the receptor for advance glycation products (RAGE), yielding a soluble form which acts as a decoy receptor for RAGE ligands[95]. ADAM-10 may on the other hand stimulate inflammatory processes in allergic response by shedding of the low affinity IgE receptor CD23 yielding the soluble form that is described to stimulate differentiation of germinal B-cells into plasma cells, cytokine release by monocytes and IgE production by B-cells[96]. ADAM-10 is involved in shedding of FAS ligand resulting in a soluble form which can have both pro- and anti-apoptotic effects, depending on the microenvironment. sFAS-L concentration in serum correlates with progression of inflammatory diseases such as arthritis and colitis indicating a possible role for ADAM-10 activity in these diseases [97,98].

ADAM-12
ADAM-12 was first described in mouse in 1995 as meltrin α as a disintegrin-metalloproteinase involved in myoblast fusion[99]. The human homologue was cloned 3 years

later and experiments revealed a peculiarity of this ADAM compared to most other members of the family with respect to the existence of an alternative splicing variant yielding a soluble form of the enzyme (ADAM12-S)[100]. For most other ADAMs soluble forms have been described that are the result of autoproteolytic ectodomain shedding, but for most of these proteins the biological relevance is unclear since many experiments demonstrating these soluble forms have been performed with cell lines expressing recombinant ADAMs. Soluble ADAM-12 has a very similar ectodomain structure compared to the membrane-anchored form but lacks the transmembrane and cytoplasmic domains, but instead possesses a unique 33 amino acid C-terminus. Both the membrane-anchored and the soluble ADAM-12 are expressed a inactive zymogens that are activated by proprotein convertase cleavage of the prodomain[101]. Interestingly, the prodomain remains attached to the activated enzyme after PC activation, giving sADAM-12 a four-leaf clover like structure[102]. ADAM-12 expression is relatively resistant to transcriptional upregulation, only TGFβ has been reported as an inducer in hepatic stellate cells[103,104].
Interaction of the disintegrin domain of ADAM-12 and integrins α9β1 and α7β1 may be involved in cell differentiation during muscle development[105]. ADAM-12 has been suggested to be involved in modulation of the cytoskeleton by altering β1 integrins on the ECM, which causes reorganisation of the actin-integrin binding between cytoskeleton and ECM[106]. A second mechanism by which ADAM-12 can reorganize the cytoskeleton is mediated by interaction with actinin-1 and -2[20]. ADAM-12 knockout mice show only limited abnormal muscular development, which could be an indication that ADAM-12 function overlaps with and can be compensated by other (ADAM) proteins[107]. Overexpression of ADAM-12 improves muscle regeneration after injury[108], and induces adipogenesis due to enhanced adipocyte proliferation. ADAM-12 efficient mice are resistant against high-fat diet-induced obesity[109].
ADAM-12 is capable of degrading several ECM proteins such as fibronectin, type IV collagen and gelatin *in vitro*[110], but the physiological relevance is probably minor[111]. The membrane-bound isoform of ADAM-12 is reported in ectodomain shedding of several substrates. ADAM-12 is capable of cleaving insulin-like growth factor binding proteins (IGFBP) 3 and 5 in vitro[112], HB-EGF[113], the oxytocinase placental leucine aminopeptidase[114], EGF and betacellulin[115] and notch ligand delta-like 1[116].
ADAM-12 is highly expressed in placenta, and sADAM-12 levels in serum are markedly increased during pregnancy. Interestingly, this rise in serum sADAM-12 is not observed in women carrying trisomy-18 and -21 foetuses, making ADAM-12 a possible prenatal marker for Down syndrome[117].
ADAM-12 has been associated with various pathological states. As expected from the postulated biological function, ADAM-12 is implicated in development of musculoskeletal disorders. Polymorphisms in ADAM-12 have been associated with osteoarthritis susceptibility[118], and although ADAM-12 seems to play a role in muscle regeneration, prolonged high expression of ADAM-12 is associated with muscular dystrophy[119]. In heart muscle tissue ADAM-12 is increased in patients with hypertrophic cardiomyopathy, and ADAM-12 may contribute to tissue remodelling observed in heart failure by shedding of HB-EGF[113,120].

The role of ADAM-12 in cancer has been extensively reviewed[121] and ADAM-12 overexpression is observed in many tumours, ranging from breast and lung cancer to glioblastoma and osteoclastoma. An interesting difference with the majority of MMPs and ADAMs is that in most tumours, the cancerous cells themselves and not the stroma are the main source of ADAM-12 in the tumour[122]. ADAM-12 is often hypothesized to stimulate tumour growth, as demonstrated by a strongly reduced tumour progression in ADAM-12 deficient mice with experimental prostate cancer[123], but anti-ADAM-12 antibodies have a stimulating effect on breast and gastric carcinoma proliferation *in vitro*[124,125].The latter effect may be effectuated by ADAM-12 induced apoptosis-resistance in tumour cells[126]. *Adam-12* is a candidate breast cancer gene, with two possibly functional polymorphisms in the metalloprotease and disintegrin domains showing strong association with development of breast cancer[127].
The existence of a soluble variant makes ADAM-12 a possibly interesting biomarker compared to the membrane-bound ADAMs since sADAM-12 can be found in a functional form in biofluids such as serum and urine. A positive correlation has already been demonstrated between breast cancer progression and urinary level of sADAM-12[110], and urine levels of patients with bladder cancer are higher than in healthy individuals[128].
ADAM-12 has recently been comprehensively reviewed[129].

ADAM-15
ADAM-15 was first described in 1996 as metargidin, referring to its RGD-motif in the disintegrin domain unique within the ADAM proteases[130]. ADAM-15 is produced by many cell types as an inactive glycosylated zymogen with the prodomain linked to the catalytic domain via a cysteine switch. The prodomain contains a proprotein convertase recognition site, so the activation process is presumed to involve PC mediated removal of the prodomain, yielding the 85 kDa mature protease.
ADAM-15 deficient mice are viable and fertile, but show reduced neovascularization after hypoxia-induced retinopathy, and growth of implanted melanoma is reduced in null mice compared to wild type, which could indicate inhibited tumour angiogenesis, but histological examination of the tumours revealed no difference in vascularization[131].
The consensus integrin binding site RGD in disintegrins was first found in snake venom derived disintegrin, but until the discovery of ADAM-15 no similar site was found in the mammalian ADAMs. Since ADAM-15 does contain this sequence it is expected to have a much broader binding selectivity towards integrins, and therefore more possibilities for cellular adhesion to neighbouring cells and the ECM than the other ADAMs, where the interaction is preferentially mediated through RGD independent interaction with integrin $\alpha 9\beta 1$[132]. The ADAM-15 disintegrin domain containing the RGD sequence is found to have a strong affinity towards integrin $\alpha v\beta 3$ after expression as a recombinant fusion protein with glutathione S-transferase[8]. Recombinant ADAM-15 is capable of binding $\alpha v\beta 3$ on a monocytic cell line, and $\alpha 5\beta 1$ on a T-cell line[133]. The putative role in intercellular adhesion is confirmed by the observation that overexpression of ADAM-15 leads to increased adhesion in cultured NIH3T3 cells[134]. In concurrence with this result is the observation that overexpression of ADAM-15 inhibits wound healing in monolayers of intestinal epithelial

cells by increasing cellular adhesion and inhibition of migration[135]. RGD-mediated integrin αvβ3 binding has been postulated to inhibit cellular adhesion and motility by blocking integrin αvβ3 so contact with ECM proteins such as vitronectin, as demonstrated by reduced migration of ovarian cancer cells when overexpressing ADAM-15[136]. This anti-invasive function of ADAM-15 was also found in melanoma cells, and is possibly linked to p38 kinase activation[137].

The pro-adhesive properties of ADAM-15 may provide the protein with a protective or homeostatic function in degenerative diseases such as osteoarthritis. ADAM-15 deficiency accelerates cartilage destruction, while overexpression has a protective effect[138]. The disintegrin domain of ADAM-15 has further been demonstrated to inhibit migration of airway smooth muscle cells through binding to β1-integrins[139].

Integrin binding mediated by ADAM-15 is a possible player in inflammatory processes such as inflammatory bowel disorder. ADAM-15 expression is higher in diseased area, and histological examination shows binding of ADAM-15 expressing endothelia and crypt epithelia to leukocytes. Interestingly, immunochemistry shows mainly the active, mature form to be present in these cells. In regenerating tissue, ADAM-15 positive epithelia cells are in close contact with integrin-positive myofibroblasts[140].

ADAM-15 may play a role in cardiovascular disease. ADAM-15 is expressed in endothelium, and is capable of binding of platelets by interaction with platelet integrin α(IIb)β3. Platelets are further activated by adhesion to ADAM-15 and thrombus formation, leading to a possible role in thrombotic diseases[141]. ADAM-15 expression in arterial cells is markedly higher in atherosclerotic tissue than in healthy control. ADAM-15-integrin binding may play a modulating role in formation of neo-initima[142].

Although most interest in biological function of ADAM-15 has been directed towards the integrin binding capacity, the metalloprotease domain does contain the consensus zinc-binding sequence, and is thus expected to be proteolytically active. Purified ADAM-15 is capable of proteolytic degradation of type IV collagen and gelatin[143]. Experiments with recombinant ADAM-15 and a peptide library have demonstrated broad substrate specificity in vitro, with a pattern similar to that of ADAM-8. Transfection experiments in HEK 293 cells exposed to recombinant ADAM-15 did reveal cleavage of the low affinity IgE receptor CD23 [144]. Only recently the first report of a possible biologically relevant substrate was published. ADAM-15 was found to be the sheddase responsible for E-cadherin, yielding a soluble form that interacts with, and stabilizes HER2 and HER3 receptors. This finding may be a first clue of a role for ADAM-15 in the development of breast cancer[145].

ADAM-17

ADAM-17 or TNF alpha converting enzyme (TACE) is undoubtedly the ADAM that has attracted the most interest in research right from its discovery as the protease responsible for the release of soluble TNFα from cells in 1997[27,28]. The shedding of TNFα had already been described earlier, and was reported to be inhibited by metalloprotease inhibitors[146,147], but with the identification of ADAM-17 as the principle TNF sheddase the first physiological substrate for ADAM-mediated shedding was discovered.

TACE is constitutatively produced by many cell types as an inactive zymogen with a cysteine switch motif, and is activated by prodomain removal by proprotein convertase proteolysis. The prodomain of ADAM-17 appears to be essential for correct folding of the metalloprotease domain in proADAM-17 and may have a chaperone function in transport through the subcellular structures[25]. After activation in the late Golgi apparatus, active ADAM-17 may be sequestered in lipid rafts, an observation supported by the increased processing of TNF after cholesterol depletion and after incubation of ADAM-17 expressing cells with high-density lipoproteins[148,149].
One interesting observation is that recombinant soluble ADAM-17 is highly sensitive to salt (NaCl), with complete inhibition observed at concentration where no structural or conformational changes in the protein occur. This effect has been attributed to disturbance of the electrostatic interaction between protease and substrate, but the physiological relevance is puzzling. Perhaps this effect is a safety precaution regulating the proteolytic activity of TACE after its shedding from the cell surface, but experimental proof is not available[25]. ADAM-17 activity is inhibited by the endogenous metalloprotease inhibitor TIMP-3, but not by TIMP-1, -2 and -4[150].
ADAM-17 proteolytic activity is essential during embryonic development. Mice with a targeted deletion lacking the zinc-binding region in the catalytic domain generally die perinatally, an effect that seems independent on decreased TNF processing. The mice exhibit anatomical abnormalities like open eyelids, missing conjuctival sac and attenuated corneas. Surviving mice display lower body weight, epithelial dysgenesis in may tissues, perturbed hair coats caused by disorganization of the hair follicles, and irregular pigmentation[151].
ADAM-17 (together with the closely related ADAM-10) is not a typical member of the ADAM family when examining the catalytic domain: the catalytic domain is much longer than in most adamalysins, leading to two unique protuberances in the structure of the catalytic pocket. The S'3 pocket is very deep and merges with the S'1 pocket, and the characteristic Ca^{2+} binding motif is not found in TACE[152].
ADAM-17 sheddase activity is a well-studied phenomenon, with the liberation of soluble TNFα being the first substrate. ADAM-17 (as ADAM-10) cleaves proTNF at Ala_{76}-Val_{77} and is the dominant TNF sheddase, since genetic deletion of the zinc-binding sequence in mice results in monocytes that are incapable of releasing soluble TNFα[27]. Other ADAMs may compensate for decreased ADAM-17 activity as ADAM-9, -10 and -19 are capable of processing of proTNF *in vitro*, but the physiological relevance of this redundancy seems limited, especially under stimulating conditions[83]. ADAM-10 has been postulated to be responsible for the constitutive release of TNFα, while after stimulation ADAM-17 takes over the shedding from the cell surface[82].
ProTNFα de novo production in monocytes can be induced with LPS but for TNF release (a measure for enhanced ADAM-17 activity) stimulation by phorbol esters is necessary, indicating a PKCδ mediated mechanism involving phosphorylation of the cytoplasmic tail of TACE leading to activation or translocation of the enzyme[25]. Other studies have found that the cytoplasmic tail is not necessary for phorbol ester induced shedding[153], again contradicting a possible role for intracellular phosphorylation. The effect of phorbol ester

stimulation on ADAM-17 mediated shedding is intriguing: shedding is induced within minutes after exposure, but prolonged stimulation results in downregulation of ADAM-17 involving internalization of the cell surface bound TACE, a process which appears to be dependent of metalloprotease activity[154].

Besides TNFα, ADAM-17 is also capable of shedding of soluble TNF receptors p55 and p75 TNF-R[151]. Shedding of p55 TNF-R is inducible by hydrogen peroxide[155]. ADAM-17 is implicated in shedding of L-selectin[151], and ADAM-17 relocalizes to L-selectin on the neutrophil cell surface after interaction with endothelial E-selectin[156].

ADAM-17 may function as an α secretase[157], but as with ADAM-9 and -10 the physiological relevance and role in development of Alzheimer's disease is under debate. There is some additional evidence identifying ADAM-17 as a relevant α secretase, since ADAM-17 activation downstream of muscarinic receptor 1 stimulation has been described to decrease the accumulation of neurotoxic Aβ peptides, a hallmark of α secretase activity[158]. The activation pathway of ADAM-17 via muscarinic receptor activation is also implicated in processing of cellular prion protein[159].

The *adam17* $^{\Delta Zn/\Delta Zn}$ mouse model bears striking similarity with transforming growth factor α (TGFα) deficient phenotype. The role of ADAM-17 in TGFα shedding was confirmed by a strong decrease in soluble TGFα production in the ADAM-17 mutant mice[151]. The anatomical abnormalities in heart valves observed in ADAM-17 deficient mice may also arise from decreased shedding of another EGFR ligand, HB-EGF which is also a possible substrate for ADAM-17[160,161].

Since TNFα is an important proinflammatory mediator, ADAM-17 activity has been implicated in many diseases involving inflammation such as rheumatoid arthritis, Crohn's disease and inflammatory bowel disorder. An interesting finding is that inhibition of ADAM-17 by conditional knockout has a strong protective effect in endotoxin shock in mice, and significantly reduces mortality. This observation may find use in the clinic where septic shock is still a condition with high mortality[162].

ADAM-17 mediated TNF processing may be important in atherosclerosis, since atherosclerotic plaques express both ADAM-17 and substrates TNFα and p55 TNF-R. Shedding is stimulated by microparticles, small vesicles that are present in atherosclerotic lesions as a result of cellular apoptosis[163].

ADAM-17 expression is upregulated in many tumours[164]. ADAM-17 mediated shedding of EGFR ligand TGFα and amphiregulin may be involved in invasion of breast cancer cells, and high expression of both ADAM-17 and TGF α correlates with poor prognosis[165]. Experiments with renal carcinoma cells have demonstrated ADAM-17 may be an essential factor in tumour formation since cells are unable to form solid tumours and loose their invasive potential after ADAM-17 silencing[166]. ADAM-17 (and -10) may be involved in early steps in the malignant transformation and tumour growth. ADAM-17 is shown to be a major sheddase of the natural killer cell receptor ligand MICA, thereby inhibiting the immunological clearance of transformed cells[167].

ADAM-19

ADAM-19 or meltrin β was first cloned in human in 2000 as a disintegrin-metalloprotease involved in 1α,25-dihydroxy vitamin D_3 induced differentiation in primary monocytes and named MADDAM (metalloprotease and disintegrin dendritic antigen marker). Expression was found to be maintained in monocytes differentiating to dendritic cells, while macrophages do not produce MADDAM[168]. The authors also identified the novel protein as a human homologue of the earlier discovered murine meltrin β (ADAM-19)[99,169]. ADAM-19 is closely related to ADAM-12 (meltrin α) and both proteins appear to be involved during development in mesenchymal cells, with ADAM-19 expression observed mainly in areas where peripheral neural cell lineages are formed. ADAM-19 expression is also found in the developing lung, intestine, heart and skeletal muscle, while ADAM-12 gene activity is found mainly in mesenchymal cells giving rise to bone, visceral organs and skeletal muscle[170]. Knockout experiments revealed ADAM-19 to be essential for heart development with ADAM-19 null mice dying perinatally with severe cardial abnormalities, mainly in the ventricular septum and the heart valves[171]. ADAM-19 may exist as an alternative splice variant missing the disintegrin and metalloprotease domain, dubbed meltrin β mini. This isoform is capable of initiating neural outgrowth in mouse neuronal cells, implicating a possible role for this alternative form in the development of the nervous system[172].

ADAM-19 is widely expressed in adult mammals, with high expression in bone, lung and heart. ADAM-19 is expressed as an inactive zymogen which is activated by cleavage of either one of two furin-recognition sites locate between the pro- and metalloprotease domain[173]. Indications exist that autoproteolytic truncation of ADAM-19 by cleavage within the cysteine-rich domain is necessary for proteolytic activity[174].

ADAM-19 is catalytically active and is capable of cleaving α2 macroglobulin in vitro, but was not able to cleave proteins such as type I and IV collagen, gelatin, casein and laminin, nor was cleavage of tested synthetic fluorogenic MMP and TACE substrates observed[175]. Later studies using a soluble recombinant ADAM-19 revealed a wide substrate specificity, with potential targets for proteolysis including myelin basic protein, insulin B-chain TNFα, TRANCE (TNF related activation induced cytokine) and kit ligand-1, but not TNF receptor-55 and -75 and IL6-receptor. The proteolytic activity of recombinant ADAM-19 is not inhibited by TIMP-1, -2 and -3. Cell-based shedding assays show increased release of TRANCE (TNF related activation induced cytokine) in COS-7 cells overexpressing ADAM-19, and kit ligand-1 shedding seems to be negatively regulated by ADAM-19[176]. The proteolytic activity towards TRANCE, a member of the TNF family is interesting since TRANCE signalling is associated with dendritic cell survival by inhibiting apoptosis[177].

ADAM-19 has been implicated in shedding of neuregulin (NRG), with a preference for the beta-isoform, with observed shedding of NRG β1 and β4, but not α2. Overexpression of ADAM-19 increases shedding, while shedding is markedly decreased in ADAM-19 deficient mutants. The shedding of NRGs seems in line with the essential role of ADAM-19 in the development of heart and central nervous system[178]. The sheddase activity of ADAM-19 towards NRG has been attributed to presence of the protease in lipid rafts within the neurons, an localization which is not common in the ADAMs which are mostly membrane-anchored[179]. Recently the NRG-processing activity of ADAM-19 has been

demonstrated to occur within the Golgi apparatus, and not the cell surface, again hinting at a unique localization of ADAM-19 mediated proteolysis[180]. The role of ADAM-19 in the developing heart and the shedding of NRG β is not exclusive, and may be compensated by other members of the ADAM family, as demonstrated in a study investigating the role of individual ADAM proteases. Apparently there is significant redundancy between the individual family members, which may complicate interpretation of results of individual knockout models[181]. ADAM-19 has recently been described having α-secretase activity towards APP, since overexpression increased release of the cleaved APP fragment increased while RNA interference decreased shedding. This may implicate a (partial) role for ADAM-19 in the processing of APP[182].
High ADAM-19 expression has been associated with invasive potential of primary brain tumours, with detectable proteolytic activity present on tumours with the highest expression[49]. ADAM-19 expression is high in renal cell carcinoma[52] and ADAM-19 expression is significantly higher in patients with chronic allograft nephropathy compared to healthy graft recipients, but is also increased in acute rejection and in non-allograft related diseases. Interestingly, ADAM-19 colocalizes with CD4+ T-cells (T-helper cells) indicating a role in the allograft rejection process[183]. ADAM-19 may be involved in development of fibrotic lesions in kidney disease. Expression is markedly higher in many renal structures in patients compared to healthy adults, and may be related to infiltration of macrophages[184].

ADAM-20 and -30
These two proteins are interesting with respect to being the only human testis-specific ADAMs with the metalloprotease active site sequence. Unfortunately, almost no research has been aimed at elucidating the biological roles of these proteases. ADAM-20 was first described in 1998, and has high sequence similarity to the fertilins (ADAM-1 and -2) and surprisingly, ADAM-9[185]. Since mature ADAM-1 loses its metalloprotease domain during processing and is considered non-functional in human, ADAM-20 was hypothesized to be the functional equivalent of ADAM-1. ADAM-30 was cloned in 1999[186], but the publication describing the cDNA sequence remains the only study published to date. Considering the expression pattern these two ADAMs may be functional homologues of the murine testases, although no evidence for this is published.

ADAM-28
ADAM-28 was first described in 1999 as eMDC II[187] in human and macaque epididymis, and as MDC-L in human lymphocytes[188]. There is some confusion about the identity of ADAM-23 and ADAM-28, there is some consensus that they correspond to the same protein, but why the annotation ADAM-28 is preferred is unclear. ADAM-23 was described as a disintegrin-metalloprotease (MDC-3) without the zinc-binding catalytic site sequence, and thus probably does not have proteolytic activity[189], while ADAM-28 does contain the zinc-binding site.
ADAM-28 is expressed as a 115 kDa zymogen that is activated to an 88 kDa mature form that can be detected on the cell surface. A smaller, soluble variant of ADAM-28 has been

observed. Interestingly, ADAM-28 lacks the furin recognition site, and may depend on autolytic activation, or activity of other metalloproteases (MMP-7) for prodomain removal[24,190].
Purified ADAM-28 has proteolytic activity in vitro, and is able to cleave myelin basic protein, but not ECM constituents like collagen I-IV, fibronectin, and laminin. The substrate specificity *in vitro* seems to overlap largely with ADAM-8 and ADAM-15, as demonstrated by screening against a library of synthetic peptides, and ADAM-28 is capable of cleavage of the low affinity IgE receptor CD23[144]. The soluble form of ADAM-28 is capable of *in vitro* cleavage of insulin-like growth factor binding protein-3[190]. ADAM-28 catalytic activity is not inhibited by TIMP-1, and only weakly inhibited by TIMP-2[191]. ADAM-28 can bind integrin α4β1 by interaction with its disintegrin domain[192,193].
The expression of ADAM-28 in the epididymis suggests a role in reproduction. The soluble splice variant was found in mouse epididymus, but no functional role could be attributed to this protein in sperm[194]. ADAM-28 may have a function in development of dental tissue, since expression in the developing tooth, and appears to be associated with tooth root hypoplasia in patients[195]. The mechanism behind this biological effect is postulated to be a survival promotion mediated through ADAM-28 induced proliferation of dental papilla mesenchymal cells, while silencing of ADAM-28 results in apoptosis of the dentals mesenchymal cells[196]. Some studies have included ADAM-28 in screening for overexpressed protein in cancer. Expression of ADAM-28 was found to be high in non-small cell lung carcinoma, and correlated with proliferation and metastasis[197]. ADAM-28 expression in breast cancer is also high, and proteolytic activity of ADAM-28 in shedding of IGFBP-3 may contribute to cell proliferation in breast cancer[198].

ADAM-33
ADAM-33 was first described in 2002 as a novel disintegrin-metalloprotease in mouse and human[199]. ADAM-33 in quite unique within the metzincin family in the fact that within 6 months after its discovery, even before ADAM-33 was well characterized at the protein level, it was already associated with a disease state[200]. ADAM-33 is now widely regarded as an asthma susceptibility gene and many studies have tried to find associations of ADAM33 polymorphisms with asthma and bronchial hyperresponsiveness.
ADAM-33 is widely expressed, except in liver. An interesting structural peculiarity is that ADAM-33 lacks SH3 binding sites in its cytoplasmic domain, which may indicate that the role in intracellular signalling is different than for other ADAMs[199]. ADAM-33 is produced as a 123 kDa glycosylated zymogen, and may be activated by cleavage of one of three putative furin recognition site between the pro- and metalloprotease domain resulting in a 100 kDa mature enzyme that can be detected at the cell surface. ADAM-33 contains the zinc-binding metalloprotease active site, and recombinant ADAM-33 has been demonstrated to possess proteolytic activity and soluble ADAM-33 is capable of cleaving α2-macroglobuline[201]. The disintegrin domain of recombinant ADAM-33 has affinity towards integrin α9β1, but not α4β1 and α4β7 (implicated in ADAM-28 mediated interaction with lymphocytes)[202], but later studies have revealed a possible mechanism for

inhibition of cell migration by ADAM-33 mediated interaction with integrins α4β1 and α5β1[203].

An in vitro cleavage specificity screening of recombinant ADAM-33 has revealed limited substrate specificity compared to other ADAMs. Only four peptides, based on the putative cleavage sites of kit ligand-1, APP, TRANCE and insulin B-chain were cleaved while broadly recognized sheddase cleavage sites such as in EGF, HB-EGF, proTNFα, p55 and p75 TNF receptors and TGFα were not hydrolyzed by ADAM-33[204]. The cleavage sites of the four recognized peptides are similar to those observed in ADAM-19, an observation already expected from the phylogenic tree identifying this ADAM and *Xenopus* ADAM-13 as having the highest sequence homology[199]. The catalytic activity is quite different from ADAM-17, and can be explained by marked differences in the catalytic pocket as demonstrated by crystallographic resolution of the structure of both proteins[205]. Cellular shedding of KL-1 was evaluated by cotransfection of full length ADAM-33 into COS-7 cells. After transfection KL-1 shedding was slightly higher, and transfection of a non-functional ADAM-33 mutant slightly decreased KL-1 release. A similar experiment with APP shedding resulted in no increase in soluble αAPP release from the cells, leading to the conclusion that ADAM-33 is not a relevant α secretase *in vivo*. ADAM-33 activity is inhibited in vitro by TIMP-3 albeit at much higher concentration than ADAM-17[204].

As expected from the genetic association with asthma, most research efforts involving ADAM-33 have been focussed on the biological and pathophysiological function in the lung. Pulmonary ADAM-33 expression has been found in cells of mesenchymal origin like smooth muscle cells and fibroblasts, but expression in epithelia has been subject to debate. Early reports show no expression in airway epithelia, while the consensus now seems to be that epithelia does indeed produce ADAM-33[43,206], but expression in epithelia is sensitive to gene silencing by hypermethylation of the promoter region[206]. ADAM-33 is found in smooth muscle tissue and airway epithelia while its presence is limited in the immune system, which may be an indication that the role of ADAM-33 lies more in tissue remodelling than in the allergic component of asthma[207]. ADAM-33 may be expressed as a number of isoforms as the result of alternative splicing. Originally ADAM-33 was already described as having a β-isoform as result of a 26 amino acid deletion in the region linking the cysteine-rich region to the EGF-like domain[199], but a study investigating the production of ADAM-33 isoforms by fibroblasts from a healthy volunteer has revealed a large number of isoforms may be expressed in fibroblasts, with 6 forms missing the metalloprotease domain. The alternative mRNA sequences are partially translated into protein as demonstrated by Western blot with at least 5 specific bands corresponding to protein of smaller molecular size are visualized. Several of these bands in the 50-60 kDa region are hypothesized to correspond to the isoform lacking the metalloprotease domain, although no validation for this hypothesis is supplied[208]. Later investigation of the different splice variant expressed in biopsies of asthmatic lung revealed a similar result showing transcripts corresponding to many isoforms being present, with a minority actually containing the metalloprotease domain region[209]. These findings, combined with the observation that the alternatively spliced ADAM-33β appears to be insensitive to proteolytic activation of the zymogen may indicate a limited functionality for the catalytic domain *in vivo*[210].

The 55 kDa isoform of ADAM-33 has been detected in BAL fluid of patients, and levels were higher in patients with moderate and severe asthma. The level of ADAM-33 correlated inversely with the measured FEV_1 % predicted, which associates ADAM-33 production in the lung with airway obstruction[211]. Expression of ADAM-33 in airway smooth muscle cells obtained from asthma patients was found to be higher than in healthy control, and the overexpression could be reversed by incubation of the cells with interferon γ[212]. Expression of ADAM-33, like ADAM-8 was found to increase with disease severity in asthma patients[43].

Knockout experiments in mice have demonstrated that ADAM-33 is not essential for development and survival, ADAM-33 null mice show no morphological abnormalities and are fertile. Interestingly, the knockout mice response in allergen sensitization/challenge experiments is normal compared to wild type and pharmacological bronchoconstriction gives a normal response. These results have demonstrated that, at least in mouse, the role of ADAM-33 in allergic airway disease may be limited, although the model may not correspond perfectly to asthma in human[213].

The genetic association of ADAM-33 with asthma and bronchial hyperresponsiveness has been extensively studied, with group worldwide attempting to reproduce the association found in 2002 in other populations. Many groups do find polymorphisms that are associated[214-216], while others fail[217,218], and there seems to be a rather high heterogeneity in SNPs that appear to be responsible for the association, a trait that is observed more often in population-wide genetic screening. In recent years, several reviews have been published summarizing the state of events[219,220].

Since the physiological function of ADAM-33 in not clear to date, interpretation of the effect of individual SNPs on protein function are complicated. In theory both dysfunction of the metalloprotease domain (shedding of EGFR ligand and inflammatory cytokines) and the disintegrin domain (cellular adhesion and migration) are possible causes of smooth muscle remodelling observed in asthma. Much evidence points to a minor role of the metalloprotease domain, but most studies have found expression of metalloprotease domain containing ADAM-33 low, while on the protein level no distinction is made. More research on the biological role of ADAM-33 and elucidation of its endogenous substrate (if any) are necessary to explain the genetic association found in many studies.

Although almost all research on ADAM-33 has been focussed on asthma, there appears to be an association of an ADAM-33 polymorphism in the cytoplasmic and development of chromic obstructive pulmonary disease (COPD), with increased airway hyperresponsiveness and higher infiltration of inflammatory cells[221].

Two recent studies have found an association of ADAM-33 with psoriasis susceptibility, with the second study reproducing two out of three SNPs identified in the first, making a strong case for involvement of ADAM-33 in psoriasis[222,223].

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1.4 ADAMs with a thrombospondin type I-like motif (ADAMTS)

The latest discovered subfamily of the metzincins are the ADAMTS proteases whose first members were cloned in mouse in 1997, and were immediately associated with inflammation[1]. The human ADAMTS family comprises 19 secreted disintegrin-metalloproteases (ADAMTS-1 to -20, with -11 being identified as a literature alias of ADAMTS-5) with a similar domain structure to the membrane-anchored ADAMs but with some marked structural differences in the C-terminal region. ADAMTS proteins lack the EGF-like domain (and naturally the transmembrane and cytoplasmic regions), but contain a central thrombospondin type I-like repeat (TSR) between the disintegrin domain and the cysteine-rich domain. The cysteine-rich domain is followed by a spacer region of unknown function, and (with the exception of ADAMTS-4) a variable number of additional TSR's (see figure 1)[2]. The TSR motifs have a putative function in protease-substrate interaction and association with the extracellular matrix, as demonstrated for the interaction of ADAMTS-1 with sulphated glycosaminoglycans like heparan[3].
ADAMTS's are expressed as inactive zymogens that can be activated intracellularly, a process that is likely dependent on proprotein convertase activity in the late-Golgi network since all ADAMTS's contain the furin recognition site within their prodomain[4]. Autocatalytic processing has been described in the C-terminal domain, effectively releasing several of the TSR's from the protein[5].
The ADAMTS's can be roughly divided into four functional groups: the hyalectanases or aggrecanases (ADAMTS-1, -4, -5, -8, -9 and -20), the pro-collagen N-endopeptidases (ADAMTS-2, -3 and -14), ADAMTS-13 as a unique member and a heterogeneous group of proteases with unknown function. The biochemical and biological characterization is limited for the majority of ADAMTS's especially when looking at the most recently decribed members. Although several studies were published on the biological and pathological role of the pro-collagen peptidases, which are implied in processing of pro-collagens prior to fibril formation and therefore have an important role in formation of connective tissue[6,7], most research effort so far has been directed to only a few submembers: the aggrecanases ADAMTS-1, -4 and -5 and the putative von Willebrand-factor cleaving protein ADAMTS-13.

1.4.1 Aggrecanases

ADAMTS-1 or METH-1 was the first identified human ADAMTS in 1998[8]. ADAMTS-1 is a proteolytically active enzyme as demonstrated by the α2-macroglobuline trapping assay[9], and is capable of degradation of aggrecan by cleavage in the chondroitin sulphate attachment domain[10]. ADAMTS-1 knock-out mice are viable, but are considerably smaller than wildtype, with growth retardation continuing after birth. The smaller size of the null mice is contributed mainly to disfunction in fat metabolism, since the knockouts are extremely lean, although no histological abnormaliteis are visible in adipocytes (besides the smaller size). ADAMTS-1 knockout mice show abnormalities in the structure of the adrenal medulla, but no functional deficiency is observed. The mice further exhibit abnormal development of the kidney, with enlarged calices and fibrotic interstitial lesions, indicating

a role for ADAMTS-1 in ECM turnover during development[11,12]. This role in ECM homeostasis is confirmed by the observation that ADAMTS-1 is markedly downregulated in the liver of chirrotic rats[13]. ADAMTS-1 has been implicated in ECM turnover during bone formation[14,15], and may play a role during follicular development and ovulation[16,17], a function that is corroberated by the observed sub-fertility in ADAMTS-1 null mice. ADAMTS-1 inhibits fibroblast-growth factor-2 VEGF mediated vascularization, an effect presumably mediated by autocatalytically processed fragments, while full-length ADAMTS-1 has sheddase activity towards HB-EGF and amphiregullin and may promote angiogenesis in tumours[18]. ADAMTS-1 expression is lower in patients suffering from asthma [19] and non-small-cell lung carcinoma[20].

Although ADAMTS-1 is capable of aggrecan processing, this process is attributed more to ADAMTS-4 (aggrecanase-1) and -5 (aggrecanase-2) activity, with ADAMTS-1 only fulfilling a minor role *in vivo*[21]. ADAMTS-4 was first described in 1999[22], as was ADAMTS-5/ADAMTS-11[23,24]. Since the specific Glu^{373}-Ala^{374} cleavage of aggrecan mediated by the ADAMTS's is observed in cultured explants undergoing cartilage destruction[25,26] and the resulting aggrecan fragments are detectable in synovial fluid of patients with arthritis[27] the role of ADAMTS-4 and -5 in arthritic diseases is well investigated. ADAMTS-4 expression is sensitive to pro-inflammatory factors such as IL-1, TGFβ and TNFα, but ADAMTS-5 is not, or to a much lesser extent ([28] and references therein), which is indicative that ADAMTS-5 functions as a constitutative aggrecanase while ADAMTS-4 functions more under inflammation. IL-1 stimulation may induce production of a 37 kDa variant form of ADAMTS-4 in chondrocytes which is capable of aggrecan degradation[29], while a truncated form resulting from C-terminal processing yields an inactive form, presumably due to disrupted substrate binding[30]. Another interesting alternative processing pathway has been described in *in situ*, extracellular activation by the proprotein convertase PACE4 in cultured cartilage explants[31].

Findings in knockout experiments investigating the major aggrecanase in murine osteoarthritis however contradicted the role of ADAMTS-4 under inflammatory stimuli as observed during arthritis, with knockout yielding no protective effect[32], while the ADAMTS-5 null mice exhibited a decreased cartrilage destruction[33,34]. The relevance of these observations in human arthritic diseases is not entirely clear, since indications exist that the cytokine-sensitivity of ADAMTS-4 is not present in murine cells, making the translation of the knockout results difficult[35], but recent characterization of the proteolytic profile of both aggrecanases have provided indication that ADAMTS-5 is indeed the major aggrecanase in arthritis, since the aggrecanolytic activity is around a 1000-fold higher than of ADAMTS-4 under physiological conditions[36], but this may not be true under inflammatory conditions observed in arthritis. The consensus seems to be that both aggrecanases have a significant effect in the pathological cartilage destruction observed in human osteo- and rheumatoid arthritis[28,37], but recent evidence from double-knockout mice has shown that aggrecanase activity from ADAMTS-4 and -5 may have redundance with another, yet unidentified protease which has a different substrate specificity under inflammatory conditions. Neither ADAMTS-4 nor -5 was found to be essential in both

development and cartilage turnover, indicating that the role of these aggrecanases may be more limited than found in earlier studies[38].

1.4.2 ADAMTS-13

The most studied ADAMTS to date is perhaps ADAMTS-13 or the van Willebrand factor cleaving protease. This protein was first identified in 2001, and immediately associated with processing of ultralarge multimeric form of von Willebrand factor (vWF)[39,40].
In the phylogenic tree ADAMTS-13 stand apart from the other family members, mainly due to the presence of C-terminal CUB (Complement C1r/C1s, Uegf (EGF-related sea urchin protein) and BMP-1 (bone morphogenic protein-1)) domains which are presumed to play a role in protein-protein interaction with other CUB-containing proteins[2]. The prodomain of ADAMTS-13 is unusually short and, contrary to other ADAMs is not necessary for accurate folding of the metalloprotease domain. Removal of the prodomain by proprotein convertases is furthermore not required for proteolytic activity, since proADAMTS-13 is well capable of processing of vWF[41].
Defects in vWF processing are the underlying pathology in thrombotic thrombocytopenic purpura (TTP), a rare syndrome involving microangiopathic haemolytic anaemia and thrombocytopenia, and may lead to neurological dysfunction, tissue infarction and renal failure. The mortality is high (90%) when untreated, but the reduced processing of the ultra-large vWF multimers can be compensated by plasma exchange essentially supplementing the patient with active vWF cleaving protease (ADAMTS-13)[42,43]. The inactivity of ADAMTS-13 in TTP may be congenital, or the result of an acquired auto-immune response targeting ADAMTS-13. The pathophysiology of TTP and the role of ADAMTS-13 has recently been extensively reviewed[44].

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1.5 Scope of the thesis

This thesis describes the development of novel methods for activity-based proteomics based on the selective interaction of active metalloproteinases such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) with newly synthesized inhibitor molecules.
The general introduction (**chapter 1**) gives an overview of the current literature on the biological and pathophysiological role of the MMPs, the ADAMs and the recently discovered subfamily of ADAMs with a thrombospondin repeat (ADAMTSs).
Excessive metalloproteinase activity is a possible underlying cause for the development of many diseases ranging from inflammatory conditions to pathological tissue remodelling and cancer the profiling of metalloproteinase activity could provide a valuable diagnostic tool and shed light on the complicated role of the MMPs and ADAMs in health and disease.
As metalloproteinase activity is highly regulated in vivo, determination or profiling of the actual active isoforms that are present could provide a better picture of the (patho)-physiological situation. Several analytical techniques for distinguishing active from non-active, either in pro-protease, or zymogen form or inactivated by endogenous inhibitors, have been described in literature. **Chapter 2** gives an overview of the traditional methods for activity determination based on monitoring of substrate conversion and expands to the recently developed field of activity-based proteomics (ABP).
ABP methodology uses small molecular weight inhibitors as ligands and roughly can be divided into two approaches; using inhibitor probes labelled with either biotin or a fluorophore for tagging and visualisation of active proteinases or by using immobilized reversible inhibitors for activity-based solid phase extraction.
In **chapter 3** we describe the first approach where the base structure of a successful reversible hydroxamate-based inhibitor was modified to include a photoreactive moiety for crosslinking of the probe to the active metalloproteinase and a biotin group for visualisation and pulldown using biotin-streptavidin interaction. A similar probe with an incorporated fluorescent moiety was tested alongside the biotinylated probe and the probe is optimized by transfer of the photocrosslinking group from the P'2 to the P'1 position in the molecule.
In **chapter 4** we test a library of reversible peptide-based hydroxamates as inhibitors against three model metalloproteinases, MMP-9, MMP-12 and ADAM-17. The best performing inhibitors are immobilized on Sepharose beads and used for activity-based enrichment of metalloproteinases. This approach is used to enrich active endogenous ADAM-17 from a complex proteome (a total cell lysate of A549 lung carcinoma cells).
Solid phase extraction has the advantage of being susceptible to automation, and this approach is described in **chapter 5**. By packing the immobilized inhibitor beads into a cartridge suitable for the PROSPEKT automated solid phase extraction module, and coupling the automated extraction to inline tryptic digestion and LC-MS analysis we show a system for fully automated activity-based analysis of metalloproteinases. The enrichment platform is coupled to a chip-based LC interface for improved robustness and the system is tested with the model proteinase MMP-12.

In **chapter 6** we summarize the results and give future perspectives. A preliminary approach for the use of the inhibitor probes for in-vivo imaging using a 99m-technetium label is discussed and the challenging task of proper pre-analytical sample handling to preserve the activity status of the proteinases in the samples is discussed.

Chapter 2

Functional proteomics on zinc-dependent metalloproteinases using inhibitor probes

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Abstract

Metzincins are a family of Zn^{2+} dependent metalloproteinases, with well known members such as the MMPs and the ADAMs. Metzincin function is mainly geared towards modulation and regulation of the extracellular matrix by proteolytic degradation of ECM proteins, and by liberation or production of biologically active proteins from their pro-forms. Since metzincin activity is strictly regulated in vivo novel analysis methods are necessary to elucidate the role of the active enzymes in health and disease. This concept gives an overview of available methods, and describes an approach to use synthetic metzincin inhibitors as affinity-probes for selective determination of active metzincins in biological and clinical samples.

Introduction

Metzincins are multidomain proteins with endopeptidase activity that are dependent on a Zn^{2+} ion in their catalytic pocket for proteolysis. The two best-described members of this superfamily are the Matrix Metalloproteinases (MMPs) and A Disintegrin And Metalloproteinases (ADAMs)

The main physiological role of MMPs lies in the regulation of extracellular matrix (ECM) by promoting turnover through direct proteolytic degradation of ECM proteins such as collagens, or by the production of bioactive signalling proteins through proteolytic activation (MMP biological function has been extensively reviewed in the literature, for instance in [1]). ADAM proteinases have a bimodal function: They mediate cell-cell and cell-matrix interaction through integrin binding to their disintegrin domain, and more than half the known human ADAMs contain a catalytic domain that enables modulation of the cellular environment by activation of signalling pathways through proteolytic release of bioactive proteins from their membrane anchored proform. This ectodomain shedding is well described for many ADAM proteinases and their natural substrates such as cytokines (soluble TNF release by ADAM-17 or TNF alpha converting enzyme, TACE) and growth factors such as TGFα (ADAM-9). For a review on ADAM proteinases see [2].

Metzincins are expressed as inactive zymogens, where the catalytic pocket is shielded by a prodomain that interacts with the zinc ion in the catalytic centre via a highly conserved cysteine residue (the so-called cysteine switch). Through proteolytic removal of the propeptide by furin proprotein convertases or other MMPs the catalytic site becomes accessible to substrates. MMP activity is controlled by a small family of endogenous specific inhibitors, the Tissue Inhibitor of Metalloproteinases (TIMP 1-4) or the generic proteinase inhibitor α_2-macroglobulin.

Endopeptidase activity is mediated by a Zn^{2+} ion coordinated between three histidine residues in the consensus zinc-binding HExGHxxGxxHD motif that is present in the catalytic centre of all known proteolytically active MMPs and ADAMs. The exact mechanism of proteolysis is under debate, but probably involves polarization of an active-site-bound water molecule to function as a nucleophile that attacks the polarized carbonyl-group of the scissile peptide bond. Proton transfer to the peptidic nitrogen atom, which may be facilitated by the adjacent glutamate residue in the catalytic pocket acting as a base, leads to cleavage of the peptide bond.

Disregulation of MMPs can lead to a wide range of disease states, mainly correlated to proteolytic destruction, aberrant development or repair of tissue. MMPs are implicated in cardiovascular disease such as atherosclerosis and aneurism, rheumatoid and osteo-arthritis and cancer progression by promoting metastasis and angiogenesis. The (patho)physiological roles of MMPs have been reviewed by Malemud [3]

ADAM proteinases have been implicated in many of the same pathologies as MMPs due to their similar proteolytic function, but are also linked to development of Alzheimer's disease (ADAM-10 functions as an α-secretase for amyloid precursor peptide), tissue remodelling in heart failure and gastro-intestinal diseases linked to TNF (such as inflammatory bowel

syndrome). ADAM-33 has been implicated in airway diseases e.g. asthma and emphysema, although it is not yet known if this is due to an altered proteolytic activity.

Activity-based metalloproteinase analysis

The majority of hypotheses associating metzincins to disease states have been focussed on disregulation of the catalytic activity *in vivo*. This poses a challenge to the analysis of these enzymes, since the strict regulation of proteolytic activity means that traditional protein analysis techniques may not provide the required information.
Since any given sample can contain three different forms of the metalloproteinase, the inactive zymogen, the inactivated TIMP-proteinase complex and the activated mature enzyme, a mere determination of gene expression by quantitative PCR or protein amount by immunochemical techniques or conventional proteomics may be of limited diagnostic value.
To overcome this limitation several analytical techniques, that determine actual metalloproteinase activity, have been under development since the late 1980's. Table 1 gives an overview of available 'traditional' methods for measuring metalloproteinase activity. The earliest methods (preceding the identification and nomenclature of the MMPs) were already described in the 1950's and 1960's and are based quite simply on conversion of a known (protein) substrate of the metalloproteinase. Substrate degradation may be monitored by techniques such as liquid chromatography, radioactivity measurement in the supernatant after incubation of the proteinase with a radiolabeled substrate, gel electrophoresis or even weight loss of a solid piece of collagen over time. Variations on this theme include zymography, where the substrate is incorporated in an SDS-PAGE gel, which after visualisation yields not only a measure for the activity, but also the apparent molecular size of the proteinase, which in turn gives an indication of its identity.
For improved quantification several reporter molecules have been incorporated into substrates, being either whole protein (e.g. biotinylated gelatine, which yields singly biotinylated fragments after proteolytic degradation that can be distinguished from the multiply biotinylated original substrate in a rather elegant two-step capture-labelling procedure)[4] or small synthetic peptide sequences with internally quenched fluorophores. These substrates are readily available for most MMPs and give the opportunity for relatively precise measurement of total activity of metalloproteinase subfamilies with similar substrate specificity, but unfortunately give no information about the identity and relative abundance of individual MMPs.
Finally activity-based analysis of metalloproteinases may be based on the hypothesis that MMP-related diseases are caused by an imbalance between proteolytic activity and the TIMP-based inhibition system. An excess of activated proteinase may over time deplete the pool of available TIMP and cause unwanted degradation of tissue since the proteinase is no longer controlled. Although elegant, this technique may yield results of limited value since it depends on accurate quantification of the amount of active, mature proteinase that is present in a complex biological sample. A second difficulty is that the enzyme activities play a role within a tissue, while is not sure that the quantities of MMPs and TIMPs

measured in the soluble biological sample will reflect the situation in the tissue. Therefore, often parallel studies are performed using tissue samples and histological staining techniques. This problem is particularly important in case of ADAMs, since many of the ADAMs are strong cell surface bound molecules that are not easily released into biological fluids. Another problem is the lack of specificity of TIMPs for individual MMPs, which may lead to incorrect conclusions when only one pair (such as MMP-9 / TIMP-1) is determined.

MMP inhibitor design and evaluation

Since MMPs and ADAMs are potentially highly interesting targets for therapeutic intervention, attempts at producing synthetic MMP inhibitors with therapeutic value have been made, especially in the field of oncology. Several chemical entities have entered clinical trials, but finally failed due to undesirable side-effects or lack of efficacy during phase II/III clinical trials. One major factor determining the failure of MMP inhibitors so far seems to be the strong homology between catalytic sites within the MMP and ADAM families which meant that the majority of the first generation inhibitors lacked selectivity. This lack of selectivity was likely responsible for the observed side-effects and the lack in clinical benefit, since it has become clear that some metalloproteinases, for instance MMP-8, exert a protective effect against development and metastasis of tumours[5].
Most MMP inhibitors synthesized to date are based on a Zn^{2+}-chelating group fitted onto a peptide (like) backbone mimicking the endogenous protein substrate of the proteinase. Following the Schechter and Berger nomenclature[6] three distinct inhibitor types can be distinguished: inhibitors with a peptide sequence N-terminal (P_1-P_3) or C-terminal (P'_1-P'_3) of the scissile peptide bond (i.e. the Zn^{2+} ion in the catalytic site) and compounds
with a zinc binding group (ZBG) located in the middle of the inhibitor with a backbone protruding on either side.
The "N-terminal" approach has proved little successful, although one well-known inhibitor (Pro-Leu-Gly-NHOH) is characterized in the literature and is capable of inhibiting MMP activity with low µM K_i values. Although inhibitors with a ZBG in the centre of the molecule are being investigated[14] the most popular approach has been the synthesis of inhibitors, which interact at the "C-terminal" side of the catalytic zinc ion.

Table 1: *Overview of analytical methods to determine MMP activity and their advantages and their limitations*

	Technique	Advantages	Limitations	Ref
Monitoring of substrate conversion	An endogenous (protein) substrate of the proteinase of interest is added to the sample, and degradation is measured by gel electrophoresis, HPLC or mass spectrometry.	Easy technique that can be performed in any lab. Continuous monitoring of enzyme activity is possible.	Depends on the availability of an appropriate substrate. The technique is limited by the overlapping substrate specificity of metzincin subfamilies. Quantification of individual proteinases is not possible.	7
Gelatine or collagen zymography	Co-polymerization of an MMP substrate (usually gelatine) into a polyacrylamide gel. After SDS-PAGE the enzymes are renatured and the incorporated substrate is degraded. Coomassie staining yields a bright blue gel with transparent bands indicating gelatinase or collagenase activity.	Discriminates zymogen and the mature, active form of enzyme by molecular size resolution through electrophoresis.	Gelatine zymography shows only gelatinases (mainly MMP-2 and -9), so profiling is limited. No confirmation of proteinase identity. The technique does not discriminate between free and TIMP-inhibited mature enzyme. Quantification is possible with densitometry, but not very accurate.	8
Conversion of biotinylated gelatine	After degradation by gelatinases singly-biotinylated degradation products can be distinguished from the original substrate containing multiple biotin moieties by capture on immobilized streptavidin and incubation with horse radish peroxidase conjugated streptavidin. The resulting signal is inversely proportional to the enzymatic activity.	Quantitative technique with good linearity. Determines the actual active enzyme in the sample. Similar in technical difficulty to ELISA assays, so accessable for most labs.	Limited by substrate specificity. Gives only the total gelatinase activity in the sample.	4
Fluorogenic substrate conversion	An internally quenched synthetic substrate yields a fluorescent product after degradation by active proteinase. The substrate is usually a small peptide containing the recognition sequence of the proteinase (family) of interest.	Quantitative technique with good linearity. Determines the actual active enzyme in the sample. Similar in technical difficulty to ELISA assays, so accessible for most labs.	Limited by substrate specificity. No information about the identity of individual metalloproteinases	9

	Technique	Advantages	Limitations	Ref
Activity-based ELISA	The activation sequence of pro-urokinase is modified to be cleaved by the proteinase of interest. A given MMP is captured by an immobilized antibody and incubated with modified pro-urokinase. Pro-urokinase is activated and the activity can be measured by monitoring conversion of a chromogenic substrate.	Quantitative technique with good linearity. Determines the actual active enzyme in the sample. The antibody-capture step ensures the activity is related to the proteinase of interest. Similar in technical difficulty to ELISA assays, so accessible for most labs.	Determination of single MMPs. Limited profiling possibilities.	10,11
MMP/TIMP ratio	Quantification of proteinase expression by PCR or proteinase amount by zymography or ELISA, and of the expression level of the corresponding natural inhibitor by PCR, or the inhibitor amount by ELISA. Ratio >1 indicates an excess of active MMP over TIMP.	Demonstrates imbalance between proteinase and natural inhibitor, which could be more physiologically relevant than mere determination of protease activity.	Although profiling is possible quantification of active MMPs is difficult. Many authors use artificial activation procedures prior to analysis. Since individual TIMPs are not selective for one MMP the ratio between a given MMP and a given TIMP may be not indicative of an actual disturbance in the enzyme-inhibitor balance.	12,13

Several functionalities can be used as effective ZBGs (for instance carboxylic acid derivatives, thiol groups, phosphinates) and can also be used to modulate selectivity[15], but the most effective ZBG for producing high-affinity inhibitors has been the hydroxamate moiety. Hydroxamates are capable of strong bidentate ligation of the Zn^{2+} ion, effectively replacing the water molecule and inhibiting the catalytic mechanism. The binding is further strengthened by a hydrogen bond that is formed between the hydroxamate NH-group and the carbonyl oxygen of the highly conserved alanine (Ala-182 in MMP-1)[16].
The strong interaction of the hydroxamate group with the catalytic zinc ion combined with the homology of the catalytic centre in MMPs has led to the development of a large number of highly efficient broad spectrum inhibitors with low-to-sub nanomolar affinity (reviewed in [17]). Although variation in other positions can also lead to differences in inhibition pattern[18], the major determinant of affinity has proven to be the substituent at the P'_1 position which enters the hydrophobic pocket at the S'_1 position in the catalytic site. The depth of this pocket (also named the specificity pocket) varies considerably across the MMP family, from being very shallow in MMP-1 to forming an open 'pore' through the entire catalytic domain in, for instance, MMP-3[19]. This variation opens the possibility of modulating selectivity when using P'_1 substituents that are larger than an iso-butyl group, which can still be comfortably accommodated by all MMPs.
In order to tailor novel metalloproteinase inhibitors to a given set of enzymes, it is pivotal to develop analytical methods to profile the activity of MMPs and ADAMs on a family-wide scale in clinical samples to assess their respective roles in disease and to follow the activity of individual members during therapy.

Activity-based probes (ABPPs) to profile metalloproteinases

Since the analytical methods described above each have their shortcomings when applied to the family-wide profiling of metalloproteinase activity, investigators have searched for alternatives. Most presently available MMP inhibitors have broad spectrum affinity for MMPs. Although this may be less suitable for therapeutic intervention, broad-spectrum inhibition allows the profiling of proteinase activity on a family-wide scale. When an inhibitor has affinity for (almost) all metzincin proteinases in the human body one can imagine using such an inhibitor as a ligand for selective analysis of active metzincin proteinases. This approach, termed activity-based or chemical proteomics was pioneered by Bogyo and Ploegh, and Cravatt, developing probes respectively for serine hydrolases [20], the proteasome [21] and cysteine proteinases [22] where active site directed molecules were fitted with a reactive group or 'warhead' to enable covalent tagging of the active site of the proteinase. Incorporating a suitable reporter molecule such as a fluorophore or biotin enabled visualisation and enrichment of labelled, active proteinases from complex proteomes (see Figure 1).
Taking into account that the catalytic mechanism of metalloproteinases is somewhat different than that of the above mentioned proteinases, since the nucleophile is not part of the protein itself but rather an active-site bound water molecule, the development of covalent inhibitor probes for these enzymes has to rely on a different principle. Described

probes for MMPs [14,23,24] and for ADAM proteinases [25] all rely on inhibitor probes that have an interaction with the catalytic zinc ion (usually a hydroxamate zinc-chelating group) combined with a reactive photocrosslinking group that forms a covalent bond with the enzyme after UV irradiation. The reporter molecule can be biotin, a fluorophore, or a radiolabel for increased sensitivity and quantification.

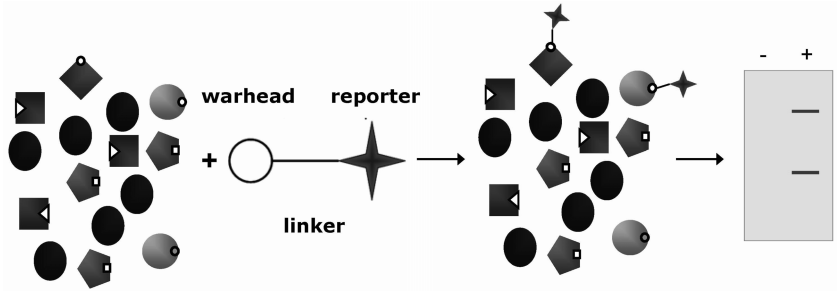

Figure 1: *Schematic representation of labelling of active proteinases in a complex proteome by activity-based probes. The probe selectively recognizes the active site of the proteinase and forms a covalent bond with the active enzyme. By incorporation of a reporter molecule such as a fluorescent dye or biotin the labelled proteinases can be visualised after gel electrophoresis, or enriched on affinity beads for further analysis*.

Sieber and Cravatt recently succeeded in coupling this approach to mass spectrometric identification of the labelled proteinase by enriching ABPP-labelled proteomes on immobilized avidin beads followed by tryptic digestion and identification after 2D-LC-MS/MS [26]. Using a cocktail of metalloproteinase-selective probes they were able to identify endogenous active members from several subfamilies in human cancer cell lines.
This approach provides investigators with a tool for profiling metalloproteinases in biological samples, but is still far from trivial. Although it gives superior enrichment possibilities, using a naturally occurring molecule like biotin as a reporter molecule gives rise to interference from endogenously biotinylated proteins. Preclearing of the sample by pull-down of biotinylated proteins with immobilized streptavidin is an option to overcome this problem, but the carrier material (usually crosslinked agarose beads) can give rise to loss of proteins due to non-specific interaction (unpublished observations). An additional problem with photocrosslinking probes is the fact that the crosslinking step is not a quantitative process, and may be influenced by many experimental factors. This limits the use of this technique to semi-quantiative or even only qualitative studies.

Activity-based solid-phase extraction (SPE)

Although the ABPP methodology provides an elegant platform for the family-wide activity-based analysis of metalloproteinases, it is advantageous to use immobilized synthetic inhibitors for the enrichment of active enzymes that are present at very low concentrations in large volumes of biofluids such as urine. When considering that the highly effective reversible inhibitors have K_i values in the low nanomolar range one can envision using them to create a platform to enrich active metalloproteinases. This approach was first described in 1986 by Moore to purify human collagenase from fibroblasts and synovial fluid using the low-affinity inhibitor PLG-NHOH as affinity ligand [27]. More recently, Freije *et al.* demonstrated the activity-based enrichment of MMP-12 using Sepharose beads with this immobilized inhibitor [28]. This lead to the analysis of active gelatinases in synovial fluid of a rheumatoid arthritis patient [29] and the finding that membrane-type 1 MMP (or MMP-14) was present in its active form only in breast carcinoma tissue but not in tissue from benign breast tumours [30]. Figure 2 shows a schematic representation of the process of activity-dependant enrichment using immobilized reversible inhibitors.
Since this technique is basically a solid-phase extraction (SPE) method, it is very suitable for development of an automated analysis platform. Freije *et al.* have demonstrated this potential by using the commercially available high affinity inhibitor TAPI-2 as an affinity ligand for the online analysis of active MMP-12 [31].
By using combinatorial synthesis for introducing variation in the peptide backbone, a wider range of reversible inhibitors has become available, which allows optimization of the ligand prior to immobilisation. The inhibitors can be screened for their inhibitory potential in a standard high-throughput enzyme inhibition assay and promising candidates selected for immobilization. Since immobilization may change the affinity of the inhibitor for the enzyme, the SPE material should be tested with a standard sample, for instance containing one or more recombinant active metalloproteinases prior to application to complex biological samples. Using this approach activity based analysis of ADAM-17 in an extract from a human lung carcinoma cell line was recently demonstrated [18].

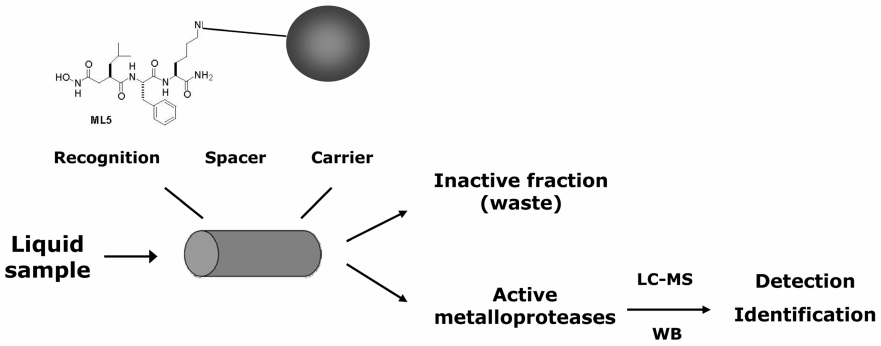

Figure 2: *Schematic representation of activity-based solid phase extraction using immobilized inhibitors as affinity ligands. A suitable inhibitor is immobilized on a solid support (e.g. Sepharose) and packed into a column. Aqueous samples containing active metalloproteinases are pumped over the column, where selective interaction between the catalytic site of active metalloproteinases and the immobilized inhibitor causes retention. Elution by either a zinc-chelating compound (e.g. EDTA) or a competitive inhibitor removes the bound metalloproteinase from the column. The activity-enriched fraction can be further analysed by immunological techniques (Western blot; WB), or processed for protein identification by liquid chromatography - mass spectrometry (LC-MS).*

Methodological prospects

The analytical use of small molecule inhibitors of metalloproteinases has the potential to greatly improve our understanding of the roles of these proteinases in health and disease. By using these functional proteomics techniques, as described in Figure 3, the analysis may move from correlating known and well-described MMPs to disease towards profiling of all active metalloproteinases. This may lead to new insights, and potentially to new targets for pharmaceutical intervention and the opportunity for a better follow-up of therapy. On the other hand, this methodology may also be used to identify unknown protein targets that interact with newly developed inhibitors thus allowing better optimization of inhibitors prior to entering clinical trials.

Affinity screening using immobilized inhibitors may also be used for determination of selectivity profiles of inhibitor libraries towards individual MMPs as recently demonstrated. [32] By using chip-based arrays of immobilized inhibitors the binding- or affinity profile of fluorescently labeled MMPs was evaluted leading to better optimalisation of inhibitor selectivity. By evaluating the binding profiles of catalytically active MMPs and inactivated enzymes the specific interaction of the inhibitor to the enzyme can be determined and distinguished from non-specific binding.

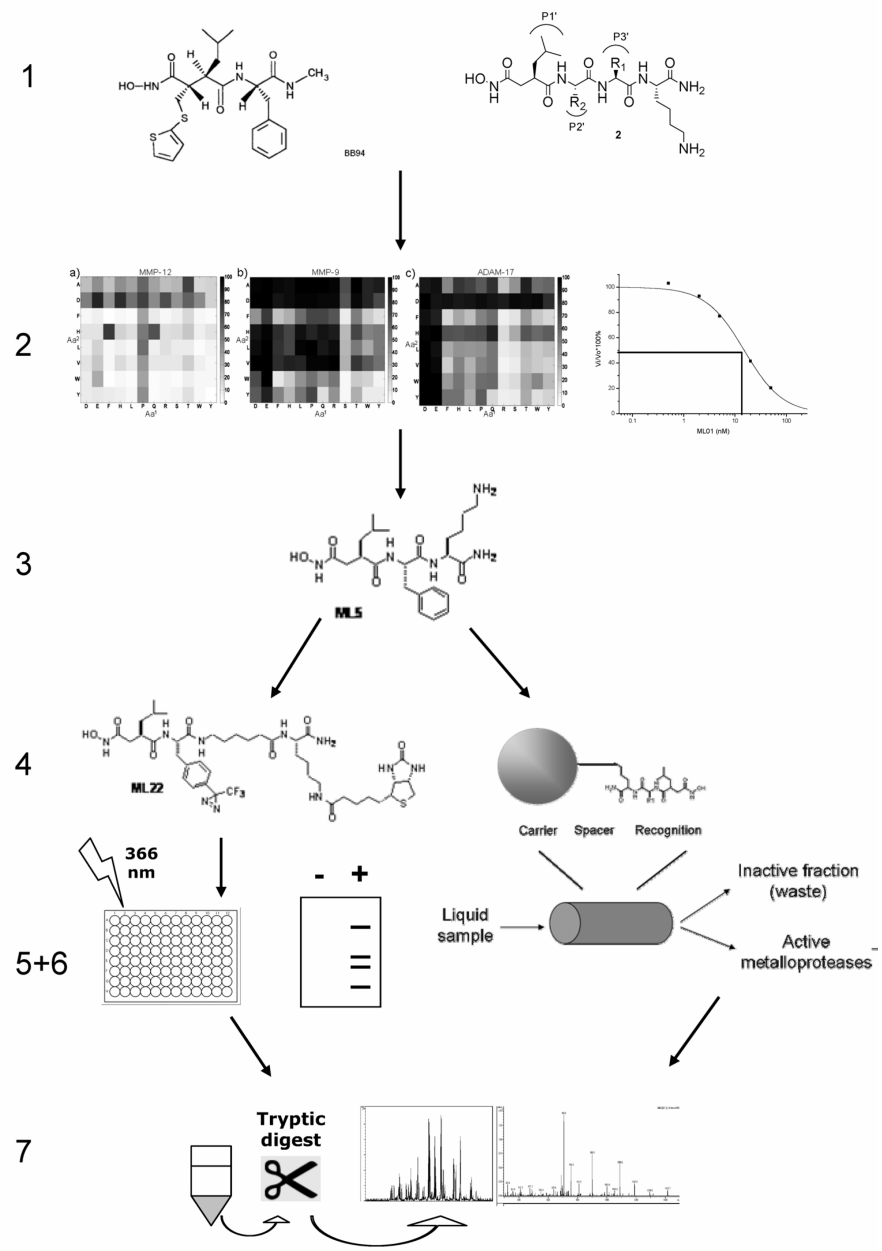
1
BB94
P1'
P3'
P2'
2
2
a)
MMP-12
b)
MMP-9
c)
ADAM-17
ML01 (nM)
3
ML5
4
ML22
Carrier
Spacer
Recognition
5+6
366 nm
- +
Liquid sample
Inactive fraction (waste)
Active metalloproteases
7
Tryptic digest

Figure 3 (previous page):
Schematic protocol for functional proteomics of metalloproteinases using inhibitor probes.

1: Selection of a suitable available inhibitor, or synthesis of novel inhibitors
2: Screening of the inhibitory profile using a panel of recombinant metalloproteinases
3: Selection of best-suited inhibitor (e.g. for profiling or selective tagging / enrichment)
4: Conversion of inhibitor into suitable structure for activity-based proteomics
- *a: Synthesis of activity-based probe based on the selected inhibitor by introducing photocrosslinker and a reporter molecule*
- *b: Immobilisation of the selected inhibitor(s) on a solid support after introducing a primary amine and a spacer arm*

5: In-vitro testing of newly synthesized probes for activity-based proteomics with recombinant proteases
- *a: Labelling of samples of interest with the activity based probe and visualisation of active metalloproteinases after gel electrophoresis*
- *b: Extraction of samples of interest with the immobilized inhibitor and visualisation of enriched active metalloproteinases by Western blot*

6: Isolation and enrichment of active proteases from biological samples
- *a: Enrichment of photolabelled proteins with immobilized streptavidin*
- *b: Extraction of active proteases from a biological sample by activity-based solid phase extraction*

7: Tryptic digestion of enriched proteins and mass spectrometric identification

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Chapter 3

Chemical proteomics on zinc-dependent metalloproteases using photoactivatable inhibitor probes

Michiel Leeuwenburgh, Paul Geurink, Theo Klein, Henk Kauffman, Gijs van der Marel, Rainer Bischoff & Hermen Overkleeft: Solid-phase synthesis of succinylhydroxamate peptides: functionalized matrix metalloproteinase inhibitors. *Org. Lett.* **8**, 1705-1708 (2006).

Klein T, Geurink PP et al, manuscript in preparation

Introduction

Matrix metalloproteases (MMPs) and A Disintegrin And Metalloproteinases (ADAMs) are catalytically active Zn^{2+}-dependant proteins that belong to the metallo-endopeptidase superfamily.
MMPs are soluble proteins that have an important physiological function in degradation, and turnover of extracellular matrix components, such as collagens and fibronectin. MMPs are subdivided according to their substrate preference in gelatinases (MMP-2 and -9), collagenases (MMP-1, -8 and -13), stromelysins (MMP-3, -10 and -11) and a number of MMPs with other natural substrates such as MMP-12 (macrophage metallo-elastase). Besides this substrate proteolysis some MMPs are capable of activation of other metalloproteases by proteolytic removal of the activity-inhibiting prodomain, allowing substrate access into the catalytic pocket. A well-known example of this is the TIMP-dependent activation of MMP-2 by membrane-bound MMP-14 [1].
Disregulation of MMPs can lead to a wide range of disease states, mainly correlated to proteolytic destruction or aberrant development and repair of tissue. MMPs are implicated in cardiovascular disease such as atherosclerosis [2] and aneurism [3], rheumatoid [4] and osteo-arthritis [5] and cancer progression by promoting metastasis and angiogenesis [6].
ADAMs are membrane-anchored metalloproteases members of the metzincin superfamily. These multidomain proteins display adhesive properties through their integrin-binding disintegrin domain and interaction of the cysteine-rich region with glycoproteins [7] and extracellular matrix proteins (e.g. fibronectin) [8]. Many members of the ADAM family contain the consensus zinc-binding catalytic sequence HEXGHXXGXXHD in their metalloprotease domain [9]. The catalytic activity enables these enzymes to process extracellular matrix components and plays an important role in proteolytic activation of membrane-anchored precursors of, for instance, growth factors and cytokines. This so-called 'ectodomain shedding' is exemplified by the release of soluble TNFα from the cell membrane through proteolysis by ADAM-17 or TACE (TNF-alpha converting enzyme) [10], the release of heparin-binding epidermal growth factor by ADAM-9 [11] and the α-secretase function of ADAM-10 in amyloid precursor protein APP processing [12] as well as the convertase activity of the same protein in the Notch/delta signalling pathway [13]. Disregulation of metalloprotease catalytic activity has been linked to inflammatory processes such as arthritis [14] and inflammatory bowel disease [8], Alzheimer's disease [15], cancer [16] and cardiac hypertrophy [17].
Conventional proteomic and genomic approaches to determine the relation of metalloproteases to disease states are limited by the fact that they only take the total amount of protein or mRNA into account, while in many cases the functionality, i.e. the catalytic activity, is more relevant. Several elegant options to determine proteolytic activity in biological samples have been developed, such as zymography and activity-linked ELISA techniques [18]. Although these techniques are able to visualize and quantify active protein, application to a family-wide proteomic approach is difficult since the techniques are inherently limited to sub-classes of enzymes due to substrate specificity (e.g. gelatinases in gelatine zymography) and antibody specificity in immunoassays.

Due to these problems the interest in family-wide, functional proteomic probes has greatly increased in recent years. Probes that selectively target active enzymes have been developed for a great number of protein classes, such as cysteine proteases [19], serine hydrolases [20], proteasome subunits [21], metalloproteases in yeast [22] and matrix metalloproteases in human samples [23-25]. In this chapter novel, photoactivatable inhibitor probes for activity-based MMP and ADAM labelling will be described.

Materials and methods

Recombinant ADAM-8, -9, -10 and -17 (ectodomain) and recombinant human TIMP-3 (Tissue Inhibitor of Metalloproteases-3) were purchased from R&D Systems (Minneapolis, MN, USA). ADAM-8 was autocatalytically activated by incubation at 37°C for 5 days according to the manufacturer's instructions.
Recombinant catalytic domains (CD) of human MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-10, MMP-11, MMP-13 were from Biomol International (Butler Pike, PA, USA). Recombinant human MMP-12 CD and recombinant human MMP-9 CD without fibronectin type II inserts (expressed in *E. Coli* as described [26,27] were a kind gift from AstraZeneca R&D (Lund & Moelndal, Sweden). TIMP-1 from human neutrophil granulocytes was from Calbiochem (La Jolla, CA, USA)
Alkaline phosphatase conjugated streptavidin was from Sigma-Aldrich (Zwijndrecht, The Netherlands). 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were from Duchefa (Haarlem, The Netherlands).
Unless otherwise mentioned all other chemicals were from Sigma-Aldrich.

Inhibitor probes[1]

The photoactivatable inhibitor-probes **ML22** (HAPhe(Tmd)AhxLys(Bio)NH_2) and **ML28** (Hydroxamic acid-Phe(Tmd)-Ahx-Lys(Bodipy-Tmr)-peptide) were synthesized as described by Leeuwenburgh [28]. A non-biotinylated control probe **ML29**, lacking the biotin or fluorescent moiety, was synthesized in the same manner (see **Fig. 1**).
Second generation probes **PPG1** and **PPG3** were synthesized according to the schemes depicted in figure 2. In these probes the trifluoroazirine photocrosslinker moiety is transferred from the amino acid residue in the P'2 to the residue in the P'1 position.

[1] Details of the synthesis will be reported in the thesis of Paul Geurink (Leiden University) and a manuscript that is presently in preparation.

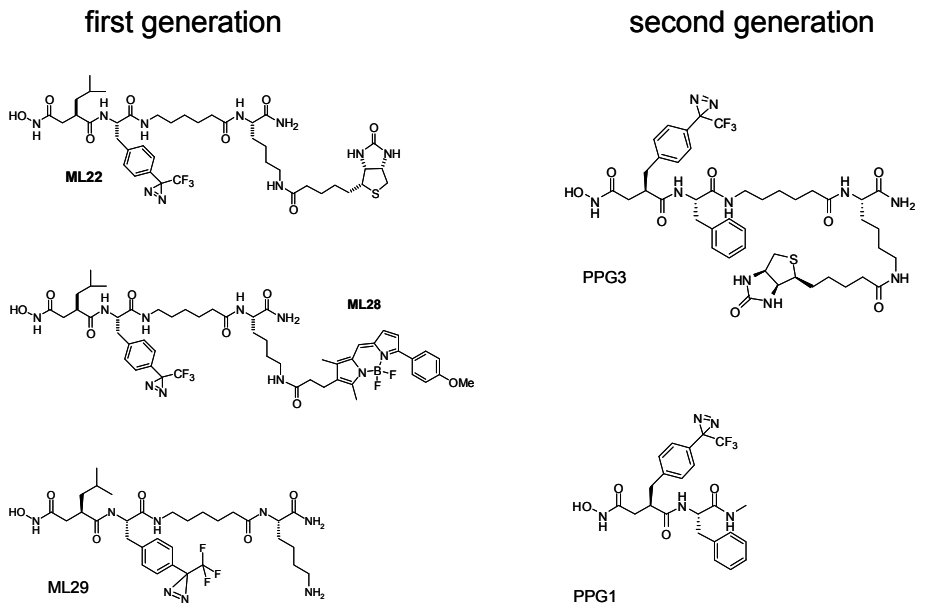
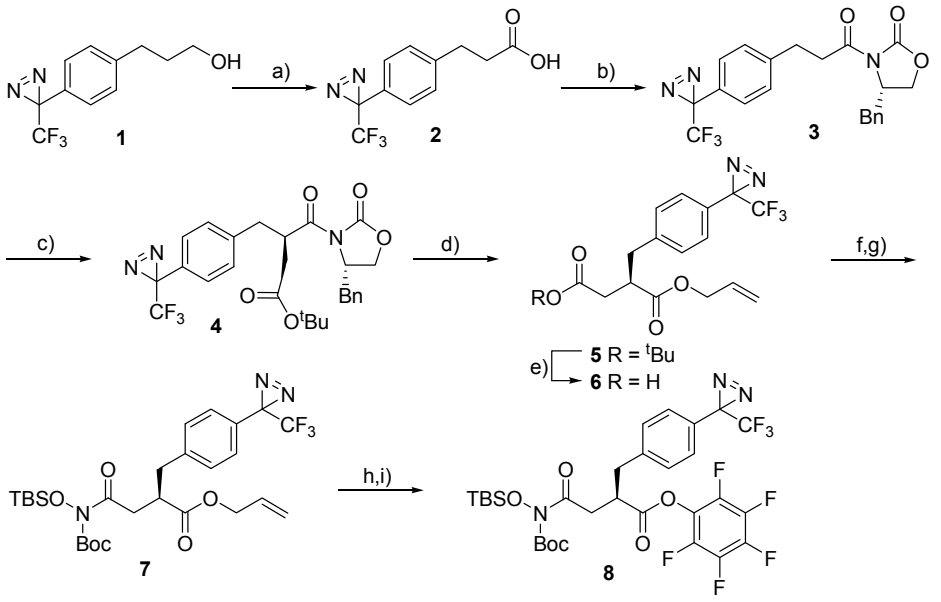
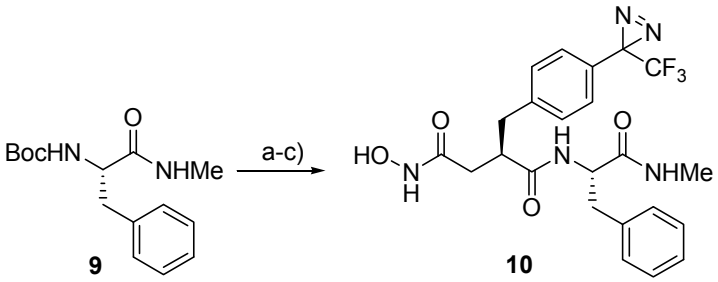


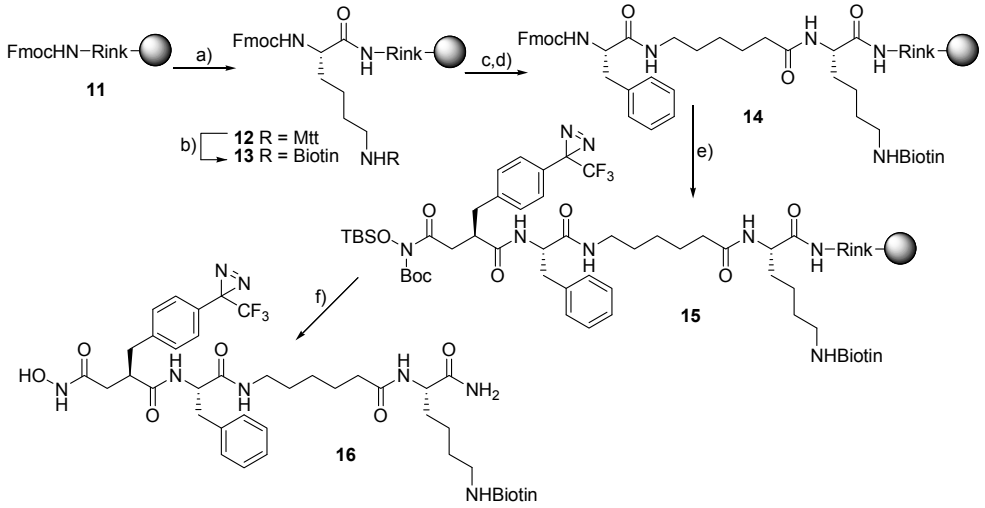
Figure 1:
Chemical structures of biotinylated, photoactivatable inhibitors (**ML22**) and (**PPG3**), BODIPY-TMR conjugated fluorescent, photoactivatable inhibitor (**ML28**) and the non-labelled photoactivatable control probes (**ML29**) and (**PPG1**).



[a] Reagents and conditions: a) TEMPO, BAIB, DCM/H_2O, 96%; b) *i*. $(ClCO)_2$, DMF, DCM, 0°C to RT; *ii*. Evans template, ^{n}BuLi, THF, 0°C, 82%; c) LiHMDS, ^{t}BuBrAc, THF, -78°C to -10°C; d) AllylOH, ^{n}BuLi, THF, 0°C to RT, 65%; e) TFA, DCM, 97%; f) $(ClCO)_2$, DMF, DCM; g) TBSO-NHBoc, ^{n}BuLi, THF, 0°C, 77%; h) $(PPh_3)_4Pd$, *N,N'*-dimethylbarbaturic acid, THF; i) PFPOH, EDC, DCM, 88%.



[a] Reagents and conditions: a) TFA, DCM; b) compound **8**, DiPEA, DCM; c) TFA:H_2O:TIS 95:2.5:2.5, HPLC purification, 6%.



[a] Reagents and conditions: a) *i*. 20% piperidine/DMF, *ii*. Fmoc-Lys(Mtt)-OH, HCTU, DiPEA, NMP; b) *i*. 1% TFA/DCM, *ii*. Biotin-OH, HCTU, DiPEA, NMP; c) *i*. 20% piperidine/DMF, *ii*. Fmoc-Ahx-OH, HCTU, DiPEA, NMP; d) *i*. 20% piperidine/DMF, *ii*. Fmoc-Phe-OH, HCTU, DiPEA, NMP; e) *i*. 20% piperidine/DMF, *ii*. compound **8**, DiPEA, NMP; f) TFA:H_2O:TIS 95:2.5:2.5, HPLC purification, 2.3%.

Figure 2:
*Top panel: synthesis route of the protected, pentafluorophenol-activated intermediate (**8**)*
*Middle panel: conversion to the photoactivatable building block for solid-phase synthesis of the PPG series (equivalent to PPG1) (**10**)*
*Lower panel: solid-phase synthesis of biotinylated inhibitor probe PPG3 (**16**)*

Determination of IC_{50} values

The affinity of the photoactivatable probes for ADAM and MMP proteases was determined in a competitive enzyme activity assay monitoring conversion of the fluorogenic substrate Mca-PLAQAV-Dpa-RSSSR-NH_2 (R&D systems) by recombinant ADAM-9, -10 and -17 in presence of increasing concentrations photoactivatable probe. For MMP-9 and MMP-12 inhibition of the conversion of fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem, Bubendorf, Switzerland) was determined. Measurements were performed in Costar White 96-well plates (Corning, Schiphol-Rijk, The Netherlands), where each well contained either 10 ng ADAM-17, 100 ng ADAM-10 or 200 ng ADAM-9 and a final concentration of 10 µM substrate in a final volume of 100 µL ADAM assay buffer (25 mM Tris pH 9.0, 2.5 µM $ZnCl_2$, 0.005% w/v Brij-35). Inhibition of MMP proteolytic activity was determined with 10 ng of MMP-9 or MMP-12 per well with a final concentration of 2

μM substrate in 100 μL MMP assay buffer (50 mM Tris pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$, 2.5 μM $ZnCl_2$, 0.05 % (v/v) Brij-35). Proteolysis rates were followed by measuring fluorescence ($\lambda_{ex,em}$ = 320, 440 nm) increase using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) at 37°C. Six-point inhibition curves (0-10 μM) were plotted in Origin 7.0 (Micronal) and IC_{50} values were determined by sigmoidal fitting.

Labelling of active recombinant metalloproteases by biotinylated, photoactivatable inhibitors ML22 and PPG3

Recombinant MMP catalytic domains and recombinant ADAM ectodomains were incubated with photoactivatable biotinylated inhibitor probe **ML22** or **PPG3** in 96-well plates (Costar White). Each well (final volume 30 μL) contained 4 pmol enzyme and a final concentration of 250 nM (unless indicated otherwise) inhibitor probe in MMP or ADAM assay buffer. The plate was irradiated by UV light under a CAMAG universal UV lamp (20W, distance to plate 4 cm) with a 366 nm filter for 30 minutes. The reaction was stopped by adding 10 μL 5× non-reducing SDS-PAGE sample buffer.

Western blotting

Samples were analyzed by SDS-PAGE on 0.75 mm thick 12.5% polyacrylamide gels. Electrophoresis was carried out at 20 mA per gel using a mini-Protean III electrophoresis system (Bio-Rad, Veenendal, The Netherlands). The proteins were transferred to an Immun-Blot PVDF membrane by wet Western blotting in a mini Trans-blot cell at 350 mA for 60 minutes in 25 mM Tris, 190 mM glycine with 20% v/v methanol. Membranes were blocked overnight at 4°C in TBST (25 mM Tris buffer pH 7.5 containing 150 mM NaCl, 0.05% v/v Tween-20) supplemented with 5% w/v non-fat dried milk (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) and incubated for 1 hour in a 1:1500 dilution of streptavidin-alkaline phosphatase (0.67 μg/ml) in TBST supplemented with 1% non-fat dried milk. Biotinylated proteins were visualized by staining with an NBT/BCIP substrate solution (0.1 M Tris buffer, pH 9.5 containing 5 mM $MgCl_2$, 0.15 mg/ml BCIP and 0.30 mg/ml NBT).

Effect of TIMPs on labelling

Aliquots of 4 pmol ADAM-10 and ADAM-17 were incubated overnight at 4°C with different molar equivalents of recombinant TIMP-3 in assay buffer, and 4 pmol aliquots of MMP-9 and MMP-12 were incubated overnight with an equimolar concentration of recombinant TIMP-1. Control aliquots were also kept at 4°C overnight. Photoactivatable inhibitor **ML22** was added to TIMP-incubated and control ADAM-10 and -17 aliquots to a final concentration of 250 nM. Photoactivatable inhibitor **PPG3** was added to TIMP-incubated and control aliquots of MMP-9 and MMP-12 to a final concentration of 200 nM. Labelling and analysis were performed as above.

Competition with non-biotinylated photoactivatable inhibitors

The control photoactivatable probes **ML29** and **PPG1** were structurally similar to inhibitor probes **ML22** and **PPG3**, respectively, the main difference being the absence of the biotin moiety (see Figure 1). Aliquots of 4 pmol ADAM-9 and -10 in assay buffer were preincubated for 15 minutes with 2.5 μM of the control inhibitor under UV irradiation. Positive controls were treated the same, but without control inhibitor added to the solution. Next, photoactivatable probe **ML22** was added to a final concentration of 250 nM. Labelling and analysis were performed as above. This procedure was repeated for MMP-9 and MMP-12, were the enzyme solutions were preincubated with 400 nM control probe **PPG1** and subsequently labelled with inhibitor probe **PPG3** at 200 nM.

Preparation and labelling of lung carcinoma cellular proteomes

Lung carcinoma cell line A549 (ATCC, Manassas, VA, USA) was cultured in 75 cm^2 culture flasks (Corning Costar, Cambridge, MA, USA) in RPMI 1640 culture medium with l-glutamine (Cambrex, Vervier, Belgium) with addition of 10% Fetal Calf Serum (FCS; Cambrex), penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL). Cells were grown (37°C, 5% CO_2) to 80-90% confluence (determined by light microscopy) and then split or used for analysis.
Cells were serum starved for 24 hours and subsequently either stimulated with phorbol 12-myristate 13-acetate (PMA) at 50 ng/mL for 2 hours or left for 2 hours without stimulation.
Cells were harvested in ice-cold lysis buffer (50 mM Tris pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$, 2.5 μM $ZnCl_2$, 2% (w/v) CHAPS) and incubated on ice for 1 hour with regular vortex mixing. Insoluble material was removed by centrifugation at 20.000 x *g* and the supernatant was used for the experiments.
Stimulated cell lysate supernatants were incubated with 1 μM photoactivatable probe **ML22** and irradiated as described above. A control lysate was treated identically but without addition of probe. Protein labelling was analyzed by Western blotting as described above.
The same lysates were also incubated with BODIPY-TMR conjugated probe **ML28**.
Lysates of basal and PMA-stimulated cells were incubated with 1 μM of probe **PPG3** and analyzed as above.

Labeling of cell lysate samples with ML28 and fluorescence scanning detection

Cell total proteome samples prepared as described above were incubated with 1 μM of photoactivatable probe ML28 and labeled by UV irradation at 366 nm for 30 minutes as described earlier for labeling with ML22. The reaction was stopped by adding non-reducing SDS-PAGE sample buffer and the samples were analyzed by gel electrophoresis as described above (10% polyacrylamide gel). The gels were scanned using a Biorad FX pro fluorescence imager with λ_{ex} at 532 nm and λ_{em} filtered through a 555 nm long pass filter.

Pull-down of labelled, biotinylated proteins

Cell lysate labelled with probe **ML22** and control lysate (40 µL each) were incubated with 20 µL streptavidin-agarose beads (ImmunoPure Immobilized Streptavidin, PIERCE) for 30 minutes in a cooled shaking incubator (Eppendorf Thermomixer, 4ºC, 1200 rpm). Supernatants were collected and beads were washed once with 1 mL lysis buffer, 2-times 1 mL washing buffer with Tween (50 mM Tris pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$, 2.5 µM $ZnCl_2$, 0.05 % v/v Tween-20) and 5-times 1 mL washing buffer without Tween. All washing steps were performed in an Eppendorf thermomixer for 10 minutes at 1200 rpm and 15ºC. Beads were sedimented by centrifugation (3 minutes at 3000 x *g*). Wash fractions were collected. Beads were boiled for 15 minutes with 30 µL of non-reducing sample buffer for SDS-PAGE and sedimented by centrifugation. The supernatants were collected. Original samples, supernatants formed after incubation of lysates with beads, second wash fractions and the fractions obtained after boiling were analyzed by SDS-PAGE on 10% polyacrylamide gels followed by Western blotting as described above. Immunodetection was performed by incubation with a monoclonal anti-ADAM 10 antibody (clone 163003, R&D systems) at 1 µg/mL in TBST with 1% Protifar for 2 h, and alkaline phosphatase conjugated goat-anti-mouse IgG (Sigma-Aldrich) at 0.67 µg/mL in TBST with 1% Protifar. ADAM 10 bands were visualized with NBT/BCIP.

Labelling of urine from a kidney-graft recipient

An aliquot of fresh urine from a transplant patient that visited the clinic for a check-up was obtained with informed consent of the patient, kindly provided by Wynand Meelenhorst, Dept. of Pathology, University Medical Center Groningen.
The urine was centrifuged at 20,000 xg for 5 minutes to remove debris, and subsequently labelled with 1 µM probe **PPG3.** A control aliquot was treated the same with omission of **PPG3**. Both samples were analysed by SDS-PAGE and biotin Western blot as described above.

Results and discussion

Characterization of first generation probe ML22

The design of first-generation probe ML22 was a result of earlier successful experiments with the novel reversible hydroxamate-based probes, which yielded a highly effective broad-spectrum inhibitor with IC50 values comparable to, or even below those of commercially available inhibitors like TAPI-2[28,29]. Incorporation of a UV-reactive trifluoroazirine moiety on the P'2 position was hypothesized to enable photocrosslinking and thus covalent, irreversible labelling of active metalloproteases.
Both probe and control compound retain their high inhibition efficacy compared to the basic reversible inhibitor ML5, as shown in table 1 although introduction of the

trifluoroazirine group increased IC_{50} values for all three tested ADAMs. The IC_{50} values are still within the nM range, and probe ML22 seems particularly effective in inhibiting MMP catalytic activity.

Table 1: *IC_{50} values (nM) of photoactivatable inhibitor probe **ML22** and control probe **ML29** (see figure 1). For comparison the values of the non-functionalized inhibitor ML5 are included. Values were determined in a standard enzyme inhibition assays with recombinant enzymes. nd: not determined.*

	MMP-9	MMP-12	ADAM-9	ADAM-10	ADAM-17
ML5	nd	5.27	nd	nd	10.7
ML22	25.1	3.60	148	114	20.6
ML29	nd	nd	22.2	48.8	4.32

To test whether photocrosslinking is effective and to determine the conditions necessary for optimal covalent tagging some preliminary experiments were carried out. From the first tests with recombinant ADAM proteases it was apparent that labelling of ADAM10 was most efficient (see figure 3b). Figure 3a shows that the labelling process is dependent on UV irradiation, as expected. Figure 3 further shows that labelling was dependent on irradiation time, and that maximum crosslinking is reached after 30 minutes. This finding was confirmed by mass spectrometric analysis of a solution of ML22 after different incubation times, which showed that after 30 minutes only photolyzed probe remained in the sample (data not shown). A third observation was that the required concentration of probe to achieve maximum labeling is actually much lower than the 2.5 µM that was originally used, as a labeling concentration of 0.5 µM already yields the same band intensity. Additional experiments show that even lower concentrations are still tolerated, with even 100 nM still giving a good signal (data not shown). For further experiments a concentration of 250 nM was chosen as a compromise. For later labeling of biological samples the concentration of probe was increased to 1 µM since the total concentration of protein is expected to be much higher.
Figure 3a shows the effect of denaturing the enzyme prior to incubation with the photocrosslinker probe. In lane 8 (after denaturation) the intensity of the biotinylated, and thus labelled ADAM10 was strongly decreased. A more detailed study (figure 3b) cvonfirmed that all three tested ADAM proteases showed clearly decreased labelling after the enzymes had lost their tertiary structure due to denaturation. This is an indication that the labelling is indeed dependant on strong interaction with the catalytic pocket of the enzyme, since the crosslinking process itself is non-selective, and could also occur by nucleophilic attack of the carbene moiety to amino acids in the denatured enzyme.

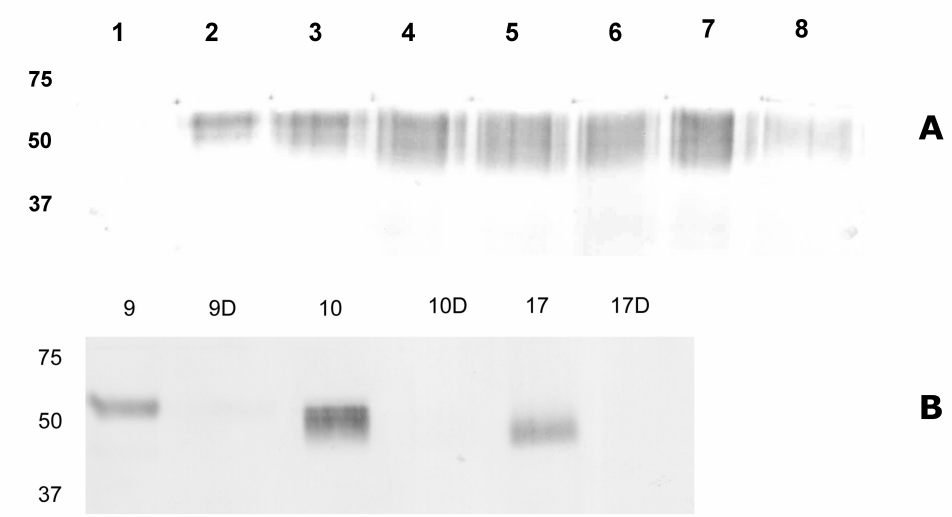


Figure 3:
a) *Biotin blot of recombinant ADAM-10 ectodomain labelled with 2.5 µM photoactivatable inhibitor probe* ***ML22****. The effect of UV irradiation time at 366 nm is shown (lanes 1-5 resp. 0; 5; 15; 30; 60 minutes) as well as the effect of competition with 100 µM reversible MMP inhibitor TAPI-2 (lane 6). Lane 7 shows labelling at 0.5 µM* ***ML22*** *for 30 minutes. Lane 8 shows decreased labelling of ADAM-10 after denaturing by preboiling the sample in a 2% SDS solution.*
b) *Effect of denaturation on labelling of ADAM proteases with inhibitor probe* ***ML22****. Aliquots of 4 pmol ADAM-9, -10 and 17 were labelled with 0.25 µM* ***ML22*** *in either native form, or after denaturation by boiling in 2% SDS for 5 minutes (D).*

Competition with a known reversible inhibitor with high affinity towards metalloproteases did however not yield the anticipated result (Fig. 3A, lane 6). Since one of the competing inhibitors in this experiment is actually covalent while the other is reversible, this may indicate favourable kinetics (K_{on}/K_{off}) for ML22 (no off rate once it is covalently bound) over the reversible inhibitor (TAPI-2) preventing effective competition.

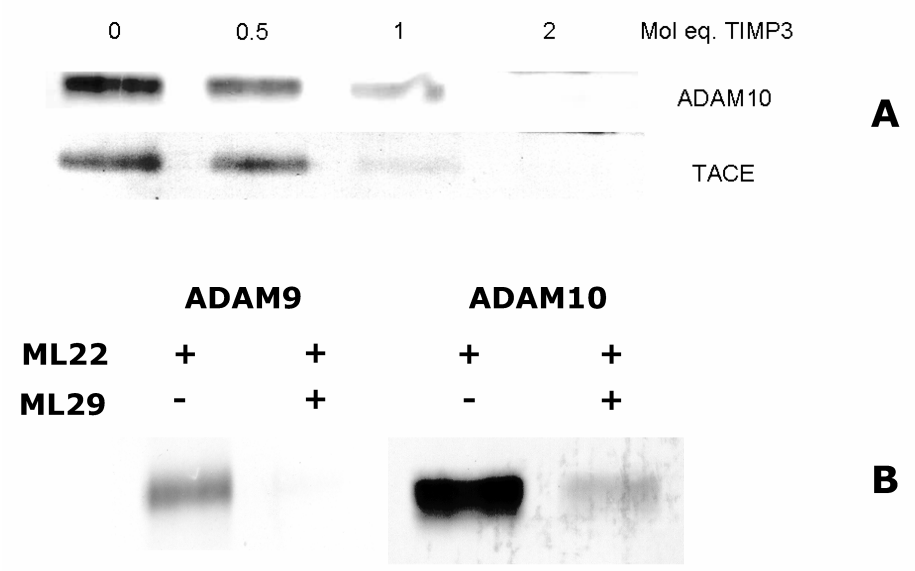


Figure 4:
A) *Biotin blot of recombinant ADAM-17 and -10 incubated with increasing molar equivalents of TIMP-3 overnight prior to photoaffinity labelling with probe* ***ML22****.*
B) *Biotin blot of recombinant ADAM-9 and -10 labelled with 250 nM photoactivatable probe* ***ML22****, with (+) and without (-) preincubation with 2.5 µM non-biotinylated, photoactivatable control probe* ***ML29****.*

To further substantiate that labelling is not only dependant on an intact three-dimensional protein structure but in particular on a functional active site, aliquots of ADAM-10 and ADAM-17 were incubated with their endogenous inhibitor TIMP-3 prior to photoaffinity labeling. Figure 4a shows a concentration-dependant decrease of labelling in the presence of TIMP-3, which is indicative of competition between TIMP-3 and the photoactivatable probe. At a two-fold molar excess neither ADAM-10 nor ADAM-17 are detectably labelled. The ability of the endogenous inhibitor to decrease the labelling of ADAMs demonstrates the specificity of the probe for the active enzyme.
TIMP-3 is a rather bulky protein compared to the small size of the inhibitor probe, which could imply that most binding sites for the probe are simply shielded off and the probe cannot access these sites. To prove that labelling is indeed active site-specific, a non-biotinylated control of the photoactivatable probe was synthesized (see figure 1). Pre-incubation with an excess of this inhibitor should occupy the available binding sites and thus preclude labelling with the biotinylated probe. Since detection by Western blotting is based on the biotin-streptavidin interaction, enzyme labelled with only control inhibitor will

not be visible. Figure 4b shows a clear decrease of labelling of both ADAM-9 and ADAM-10 after preincubation with a 10-fold molar excess of control probe, further indicating that labelling of these ADAMs with the biotinylated probe is indeed active site-specific. These results show also that the lack of competition with a high-affinity, reversible inhibitor were most likely due to the fact that the photoactivatable probe binds irreversibly to the active site. Taken together, these experimental results demonstrate that the photoactivatable probe ML22 binds to the active site of the tested metalloproteases.

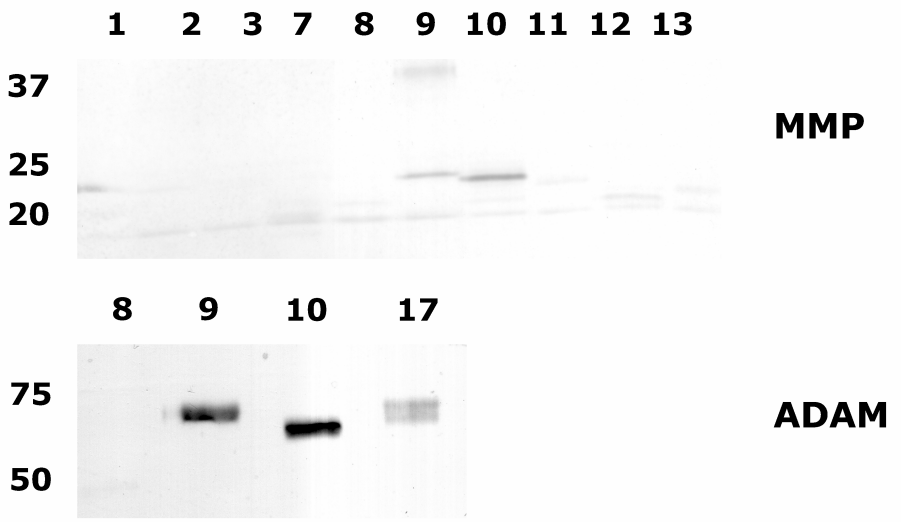


Figure 5:
*Biotin blot of 10 recombinant MMP proteases (4 pmol each; numbers above the lanes correspond to the respective MMP), and 4 recombinant ADAM proteases (ditto) labelled with 250 nM of biotinylated, photoactivatable inhibitor **ML22**.*

By testing a wider range of metalloproteases, we subsequently wanted to investigate whether ML22 is a truly family-wide, activity-dependent photoaffinity label. Activity-dependent photoaffinity labelling of metalloproteases on a family-wide scale requires a probe that interacts with the active site of any MMP or ADAM. So far such a probe has not been described in the literature. The results shown in figure 5 demonstrate that **ML22** labels several active ADAM proteases resulting in the covalent incorporation of biotin, but as shown in figure 5, this does not hold true for all MMPs nor for ADAM-8. Interestingly, efficiency of photolabelling does not correlate with the measured IC_{50} values (see Table 1) indicating that there are additional structural factors involved in arriving at an efficient photoactivatable probe that is capable of labelling the entire family of MMPs and ADAM

proteases. To investigate this further, a new synthetic approach was developed to produce the PPG series of inhibitors.

Characterization of second generation probe PPG3

To facilitate family-wide labelling a new probe (**PPG3**; see Figures 1 and 2 for details) was designed by placing the photoactivatable trifluoroazirine group in the P'1 rather than in the P'2 position. Based on the 3-dimensional structures of metalloprotease inhibitor complexes, it is hypothesized that the photocrosslinker on this position enters the hydrophobic S'1 pocket in the catalytic site of MMPs [30]. This should bring the photoactivatable group into more intimate contact with the enzymes. Since the labelling mechanism, a nucleophilic attack after activation of the photolabile group by UV irradiation to generate a carbine, is by nature not selective, close proximity to amino acids in the active site is critical for efficient labelling to avoid inactivation of the reactive carbene through reaction with water. Transfer of the photocrosslinker moiety to the P'1 position resulted indeed in improved labelling efficiency for most MMPs although there were still gradual differences (Figure 6). The labelling of ADAM-10 increased further, while labelling of ADAM-9 and -17 was comparable. ADAM-8 was also labelled, albeit at a lower yield. The efficient labelling of all tested MMPs (10 types) and ADAMs (4 types) makes **PPG3** an excellent candidate for family-wide labelling of these classes of enzymes. It is noteworthy that most labelled MMPs resulted in multiple bands upon SDS-PAGE analysis. This is likely due to degradation (auto-proteolysis) prior to or during photoaffinity labelling. Further experiments are planned to investigate this.

Table 2: *IC_{50} values (nM) of photoactivatable inhibitor probe **PPG3** and control probe **PPG1**. Values were determined in a standard enzyme inhibition assays with recombinant enzyme ND: not determined.*

	MMP-9	MMP-12	ADAM-10	ADAM-17
PPG3	24.2	12.5	54.1	490
PPG1	ND	9.38	ND	530

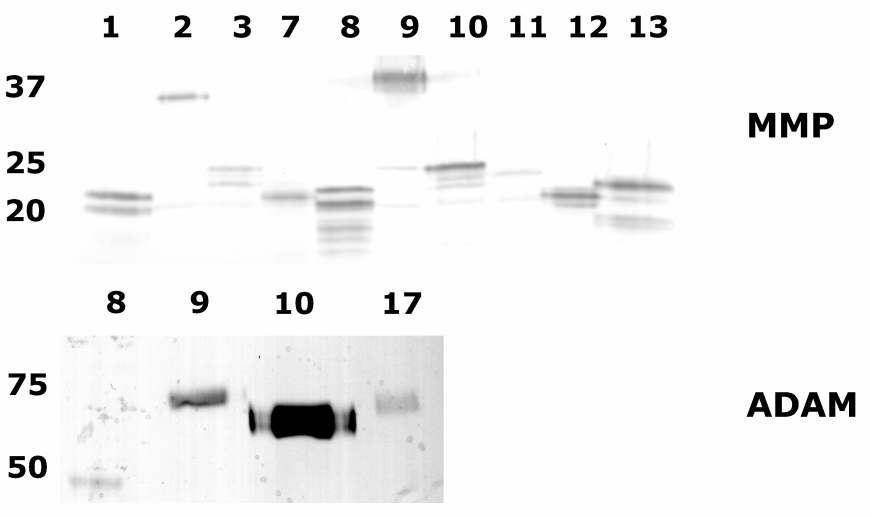


Figure 6:
Biotin blot of 10 recombinant MMP proteases (4 pmol each; numbers above the lanes correspond to the respective MMP), and 4 recombinant ADAM proteases (pmol each; numbers above the lanes correspond to the respective MMP) labelled with biotinylated, photoactivatable inhibitor PPG3.

Inhibition data show again a rather poor correlation between inhibitor affinity as expressed in IC_{50} values (**Table 2**) and photolabelling efficacy. IC_{50} values determined for MMP-9 and -12 and for ADAM-10 and -17 show that **PPG3** is more potent against MMP-12 than against ADAM-10 while ADAM-10 is more strongly labelled. Comparison of the IC_{50} values between **ML22** (Table 1) and **PPG3** shows furthermore that the lower IC_{50} value for **ML22** does not translate into more efficient photolabelling rather the contrary. While ML22 is a better inhibitor of MMP-12 and ADAM-17 activity than **PPG3**, labelling of MMP-12 with PPG3 is stronger and labelling of ADAM-17 is not decreased. The inhibition constant of **PPG3** for MMP-9 is similar to that of **ML22** but labelling is more efficient with **PPG3**. Another interesting observation when comparing labelling of MMP-9 in figures 5 and 6 is the decreased autocatalytic degradation of MMP-9, indicating that PPG3 is in fact an inhibitor with strong binding properties., or that one of the autolytic fragments is preferentially labeled by **PPG-3**.
This lack of a clear correlation of IC_{50} values and labelling yield confirms the greater importance of placing the photoactivatable trifluoroazirine in close proximity to amino acids in the enzymes' active sites rather than trying to increasing inhibitor affinity. More work is ongoing to probe this structure-labelling efficiency relationship further to arrive of more effective, family-wide photoactivatable probes.

Activity-dependence of photoaffinity labelling with PPG3

To prove that labelling with of MMPs with **PPG3** is activity-dependent, we followed a similar strategy as for ML22. MMP-9 and -12 were incubated with a twofold molar excess of TIMP-1 prior to labelling with the probe. Figure 7a shows that pre-incubation with TIMP-1 completely inhibited the labelling of both MMP-9 and MMP-12, indicating that labelling by **PPG3** was selective for the catalytically active isoform of the enzyme and that it is possible to distinguish free active enzyme from TIMP-complexed, inactive MMP.
The effect of competition with a non-biotinylated control probe (**PPG1**) was also investigated. As shown in figure 7b, preincubation with a 2-fold molar excess of competing probe is capable of preventing labelling with **PPG3** almost completely, as compared to the 10-fold excess that was necessary in the case of **ML22** and its control **ML29**. This is a further indication that the **PPG** probes have higher crosslinking efficiency and that less of the probe is lost due to non-productive photolysis of the inhibitor. The fact that a 2-fold excess completely inhibits binding of **PPG3** to the recombinant enzymes makes also a strong point for the site-specificity of the probes, since many places for non-selective reaction with the enzyme would still be available after preincubation with **PPG1**.

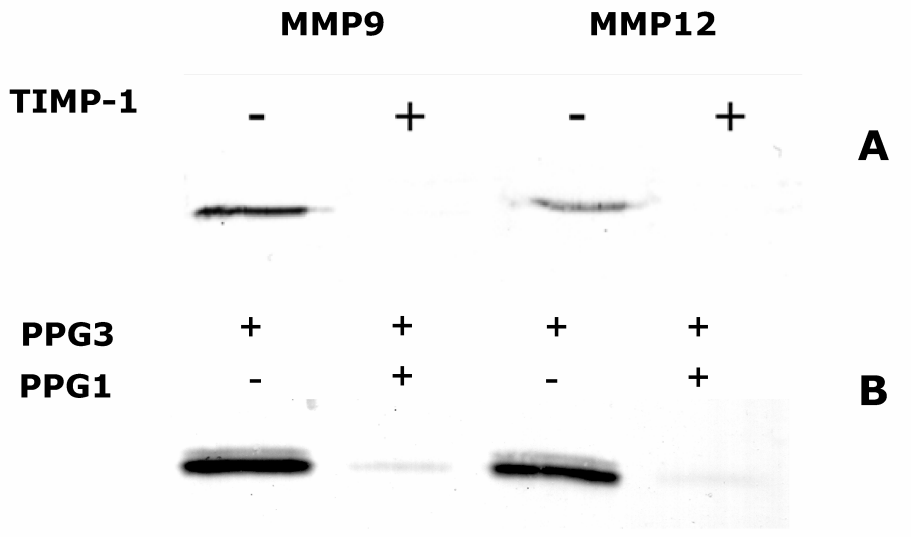


Figure 7:
A) *Biotin blot of recombinant MMP-9 and -12 incubated with a two-fold molar excess of TIMP-1 overnight prior to photoaffinity labelling with 200 nM probe* ***PPG3****.*
B) *Biotin blot of recombinant MMP-9 and -12 labelled with 200 nM photoactivatable probe* ***PPG3****, with (+) and without (-) preincubation with 400 nM non-biotinylated, photoactivatable control probe* ***PPG1****.*

Labelling of proteins in complex proteomes with probe ML22

To test the applicability of the probes, functional proteomics labelling experiments were performed in detergent-based total cell lysates of a human lung carcinoma cell line (A549 cells) stimulated with phorbol 12-myristate 13-acetate (PMA). This matrix was chosen as a "model sample" since previous experiment had already demonstrated the presence of several (catalytically active) metzincins, such as ADAM-10 and ADAM-17.
Early experiments using the non-denaturing surfactant Triton X-100 for solubilisation of membrane-anchored proteins (such as ADAMs) yielded disappointing results, which was explained by an unknown interference of Triton with the photocrosslinking process (as demonstrated by labelling of recombinant ADAM-10 in increasing concentrations of Triton X-100, data not shown). After switiching to CHAPS for solubilisation of membrane proteins several bands were visualized on a biotin blot as shown in figure 8a. One interesting observation is the presence of three intense bands in both the labelled and the unlabeled sample, which correspond to endogenously biotinylated proteins. Since the staining method is based on streptavidin-biotin binding it is unable to discriminate between naturally biotinylated proteins and proteins that are biotin conjugated due to the inhibitor probe.
Mass spectrometric analysis of a pull-down experiment with immobilized streptavidin revealed that the endogenously biotinylated proteins were mainly CoA carboxylases involved in the cellular energy metabolism. The presence of these highly abundant biotinylated proteins interfered with the mass spectrometric identification of photolabelled proteins, since the chromatograms were 'flooded' with tryptic peptides resulting from the naturally biotinylated proteins.
To demonstrate that labelling of endogenous ADAM proteases is in fact possible in a complex proteome with these probes (as was already shown by the group of Cravatt using different probes [24]), a similar pull-down experiment was performed on labelled and unlabeled A549 lysate. The bound and unbound fractions were subsequently analysed by ADAM-10 Western blot as shown in figure 8b. ADAM-10 is only recovered in the bead fraction after labelling with **ML22** and no unlabeled ADAM-10 binds to the streptavidin beads. The amount of recovered ADAM-10 was very low, when compared to alternative analysis methods where more than half the ADAM-10 is actually in the catalytically active isoform (As determined by activity-based enrichment, see chapter 4). This may be an indication that the crosslinking yield is relatively low, leaving most active ADAM-10 unreacted, and invisible to detection.

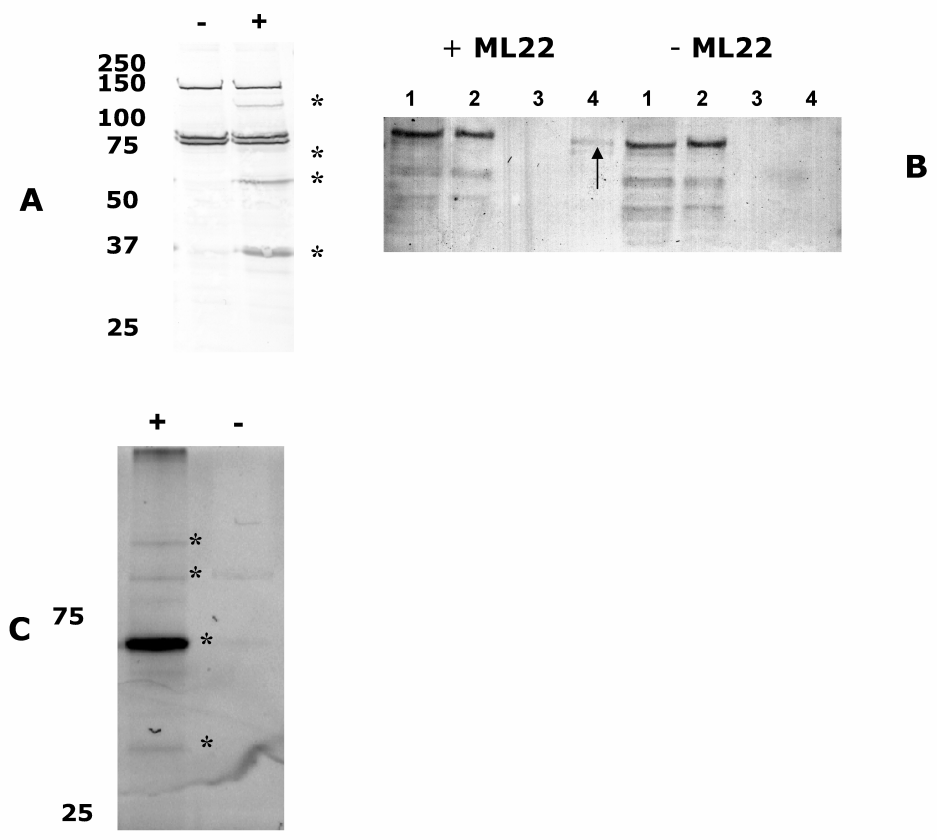


Figure 8:
A) *A lysate of A549 cells stimulated with phorbol 12-myristate 13-acetate (PMA) was labelled with (+) and without (-) the photoactivatable probe* ***ML22****. Biotinylated bands were visualized by Western blotting with alkaline phosphatase conjugated streptavidin staining. Labelled bands are marked with an asterisk.*
B) *Lysates of PMA stimulated A549 cells were labelled with (left lanes) and without (right lanes) the photoactivatable probe* ***ML22*** *and a pull-down was performed with streptavidin-conjugated Sepharose beads. Proteins were eluted from the beads by boiling in non-reducing SDS sample buffer. 1: original lysate; 2: supernatant after incubation with streptavidin beads; 3: final wash fraction; 4: supernatant obtained after boiling of the beads. ADAM-10 was visualized by immunoblotting with a monoclonal anti-ADAM10 antibody and alkaline phosphatase staining only after labelling with* ***ML22*** *(see arrow).*
C) *Fluorescence scan of an SDS-PAGE gel of a lysate of PMA stimulated A549 cells after photolabelling with and without 1 µM of the BODIPY-TMR conjugated inhibitor probe* ***ML28****. The gel was scanned using a fluorescence imager (λ_{ex} 532, λ_{em} 555 nm long pass).*

The labelling of several proteins by **ML22** was confirmed by the results from an experiment where an A549 lysate was labelled with 1 μM **ML28**, a structurally identical counterpart of **ML22**, except for the conjugated fluorescent dye BODIPY-TMR as reporter molecule instead of biotin (see figure 1 for structure). Figure 8c shows four distinct fluorescent bands in the sample incubated with **ML28**. The very prominent band around 70 kDa was not visible in the anti-Biotin Western blot (figure 8a) most likely since it was shielded by the presence of intense bands from endogenously biotinylated proteins. This 70 kDa band is selectively labelled since the binding can be outcompeted with both EDTA (indicating the process is metal-dependent) as well as by an excess of control probe **ML29** (data not shown).
The labelling pattern obtained with **ML28** is somewhat different than with **ML22**. two higher molecular weight bands are visible, one of which may correspond to the upper band in Fig. 8A at about 125 kDa, while the lower band at 50-60 kDa seems to have disappeared. This effect may be due to a subtle difference in selectivity due to the presence of the rather bulky fluorophore in the molecule, although it is not possible to compare the two results exactly due to the use of different molecular weight markers.

Labelling of proteins in complex proteomes with probe PPG3.

To investigate if the improved labelling yield of the PPG series of probes towards MMPs could also be demonstrated in a complex biological sample, CHAPS-based lysates of basal and PMA stimulated A549 cells were incubated with 1 μM of PPG3 and subsequently analysed by SDS-PAGE and biotin Western blot.

One interesting observation from figure 9 is the presence of many more naturally biotinylated proteins, but absence of the differentially detected, labelled bands in the higher MW region that were labelled with **ML22**. The band around 37 kDa that was also observed in the **ML22** labelled lysate was most prominent in this case. When increasing the contrast of the lower molecular weight region several less intense bands became visible in the sample treated with **PPG3**. An interesting fact about this experiment is the stronger labelling of the 37 kDa protein by treatment of the cells with PMA indicating upregulation or activation. Phorbol esters are known to activate PKCδ, and experiments in another type of epithelial cells (16HBE) have shown that this activation leads to an increased release of TNF-α from the cell membrane (a process that was not observed in A549 cells under identical conditions). Since this process is strongly dependent on ADAM-17 (and to a lesser extent ADAM-10) it was hypothesized that PMA treatment could lead to an activation of metalloproteases in the cell, but this was not observed on the protein level in either cell line. Our result based on activity-dependent photoaffinity labelling may shed more light on the mechanism of TNF-α release.

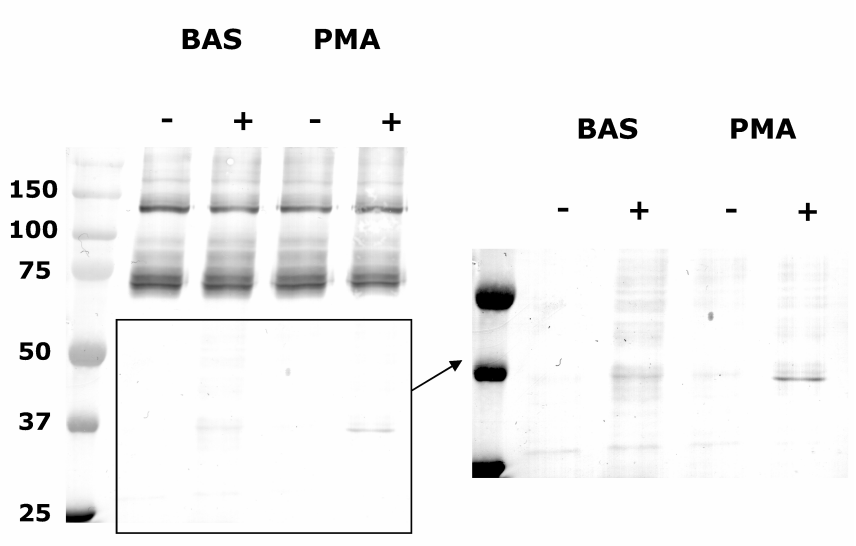


Figure 9:
*Biotin blot of total cell lysates of basal (BAS) and PMA stimulated A549 cells after incubation with (+) and without (-) 1 µM photoactivatable inhibitor probe **PPG3**. The higher contrast zoom of the lower molecular weight region shows several bands visible in the labelled lysate.*

The task of identifying the 37 kDa protein has so far not been completed and is again complicated by the presence of large amounts of naturally biotinylated proteins. Several experiments were carried out to remove naturally biotinylated proteins from the cell lysates prior to analysis, but for unknown reasons complete depletion of biotinylated protein using immobilized streptavidin was impossible (as judged from anti-biotin staining of Western blotted samples prior to and after preclearing).

To investigate if the novel probe **PPG3** could be useful in clinical samples, a preliminary experiment was carried out using fresh urine of proteinuria patients with various diseases where metalloprotease activity could be implicated (with patient consent, kindly provided by Wynand Melenhorst, Dept. of Pathology, University Medical Center Groningen). Figure 10 shows the labelling obtained in the urine of a patient visiting the clinic for a check-up after kidney transplantation. The blot shows labelling of 2 distinct bands around 70 kDa, the anticipated molecular size of most active full length ADAM proteases. Identification of the labelled protein was in this case not complicated by interference of naturally biotinylated protein, but the total amount of the protease in the sample was probably too low since no database identification was achieved. This experiment showed, however, that labelling of protein in clinical samples is possible, and may be a good starting point for further studies.

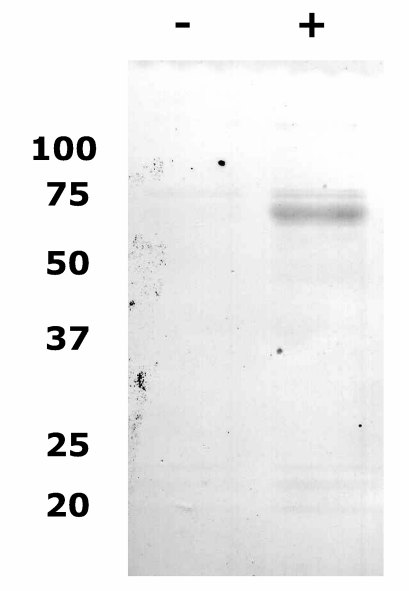


Figure 10:
*Biotin blot showing labelling of proteins in urine of a kidney-transplant patient with 1 μM **PPG3**. Biotinylated bands were visualized by Western blotting with alkaline phosphatase conjugated streptavidin staining.*

Conclusions

Functional proteomic tools can provide valuable additional information that complements results obtained with generic proteomics techniques. In this chapter two novel activity-based, photoactivatable inhibitor probes that label MMP and ADAM proteases were characterised.
Placing the photoactivatable trifluoroazirine group in the P'1 position of the inhibitor **PPG3** proved to enhance labelling efficiency significantly relative to the first generation inhibitor **ML22** (trifluoroazirine group in the P'2 position), especially with respect to the labelling of MMPs. This second generation probe selectively labels catalytically active members of both enzyme families and may therefore provide a valuable tool for development of activity-based methods for the analysis of these enzymes in biological samples.
The results obtained with both probes show that there is little correlation between measured IC_{50} values and the suitability of a given probe to be an effective photoaffinity label. Further experiments to study the actual kinetics of enzyme-inhibitor interactions may prove useful to optimize probes, since it likely that a slow off-rate is beneficial to efficient photoaffinity labelling. This in combination with the developed library synthesis approach (see chapter 4) holds promise to arrive at further improved photoactivatable inhibitors.

The biotin moiety incorporated in the probe provides not only the possibility for visualization but also for enrichment of labelled ADAMs through pull-down with immobilized streptavidin or avidin beads. Although far from trivial, this opens the way to the identification of labelled proteins by proteolytic digestion and mass spectrometry. The problem of interference of naturally biotinylated proteins in the sample still poses a major hurdle.

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Chapter 4

Activity-based enrichment of metalloproteases using immobilized inhibitor probes

Part of this chapter was published as

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"A peptide hydroxamate library for enrichment of metalloproteinases: towards an affinity-based metalloproteinase profiling protocol" Org Biomol Chem. 2008 Apr 7;6(7):1244-50

* equal contribution

Summary
A compound library of 96 enantiopure N-terminal succinyl hydroxamate functionalized peptides was synthesized on solid phase. All compounds were tested for their inhibitory potential towards MMP-9, MMP-12 and ADAM-17 which led to the identification of both broad spectrum inhibitors and metalloproteinase selective ones. Eight potent and less potent inhibitors were immobilized on Sepharose beads and evaluated in solid-phase enrichment of active MMP-9, MMP-12 and ADAM-17. In addition, one of these inhibitors was used for solid-phase enrichment of endogenous ADAM-17 from a complex proteome (a lysate prepared from cultured A549 cells)

Introduction

Matrix metalloproteinases (MMPs) are involved in numerous biological processes such as cell migration, wound repair and tissue remodeling. MMPs exert their role in these processes by the processing of extracellular matrix proteins including gelatin, elastin, and collagen and the release of growth factors. ADAMs (a disintegrin and metalloproteinase) are metalloproteinases that contain a membrane-spanning and a disintegrin (integrin-binding) domain. These membrane-bound enzymes are involved in membrane fusion, cytokine and growth factor shedding, cell migration, muscle development, fertilization, cellular differentiation, cell-cell interactions and cell-matrix interactions.[1-3] The best known ADAM is ADAM-17, also known as TACE or tumor necrosis factor α (TNFα) converting enzyme, which was discovered based on its sheddase activity with respect to membrane-bound TNFα.[4,5]

The expression of MMPs and ADAMs is regulated by transcription factors and activity is controlled by natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Disturbances in these regulatory mechanisms are believed to cause, or be involved in, a wide range of pathological states. These include cancer metastasis, rheumatoid arthritis and autoimmune diseases.[6,7] Deregulation of ADAM expression or activity has also been linked to asthma, Alzheimer's disease, bacterial lung infections and allergies of the airways.[1,8] Both academic and industrial research groups are therefore actively involved in the study of MMP and ADAM inhibitors to modulate activity in an effort to develop novel therapeutic agents based on a better insight into the role of MMPs and ADAMs in disease etiology and progression. As a result a large number of MMP and ADAM inhibitors have been described in the literature,[7] some of which have been evaluated in clinical trials.[9-11]

MMPs and ADAMs contain a Zn^{2+} ion in their active site, which forms a complex with the carbonyl group of the scissile amide bond, thereby enhancing its reactivity towards nucleophilic attack of the water molecule that is present in the active site.[7,12] As a result, a requirement for potent MMP or ADAM inhibitors is that they contain a good zinc binding group (ZBG). A large number of MMP and ADAM inhibitors that have appeared in the literature consist of an oligopeptide sequence that is equipped with a hydroxamate moiety at either the C- or the N-terminus. In these structures the oligopeptide portion ensures recognition by the metalloproteases, whereas the hydroxamate acts as zinc chelator. Indeed, of the different zinc binding moieties known, hydroxamates have proven to be the most versatile.

C-terminal peptide hydroxamic acids are readily available through modified solid phase peptide synthesis (SPPS) protocols.[13-18] In contrast, there are very few synthetic procedures towards N-terminal peptide hydroxamates,[19-21] which obviate a non-SPPS step during synthesis.[22-26] The preparation of compound libraries containing N-terminal peptide hydroxamates would be greatly facilitated by the existence of suitable, complete SPPS methods. With this aim in mind, we recently reported[27] the synthesis of an enantiomerically pure *N*,*O*-diprotected succinyl hydroxamate building block **1** (Figure 1) and demonstrated that *N*-terminal peptide hydroxamates can be prepared by SPPS using compound **1** in the penultimate step, prior to acid cleavage and deprotection. Here we

report the application of building block **1** in the preparation of a library containing 96 enantiopure peptide hydroxamates **2** (Figure 1).

The compound library consists of various amino acid residues at positions R^1 and R^2, and a lysine residue at the C-terminus, enabling future modifications with for instance biotin, fluorophores or for coupling to a matrix for solid phase extraction (SPE) procedures.[28,29] The synthesis and characterization of the compound library as well as the inhibitory potential against recombinant MMP-9, MMP-12 and ADAM-17 are reported. A selected number of inhibitors have been immobilized for SPE enrichment of active metalloproteases.

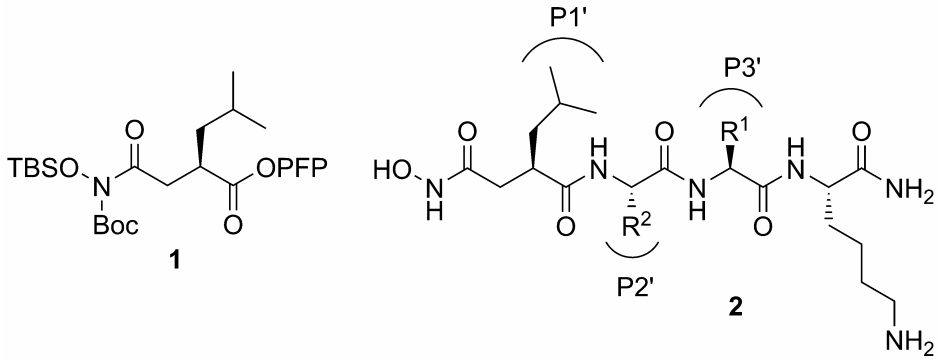
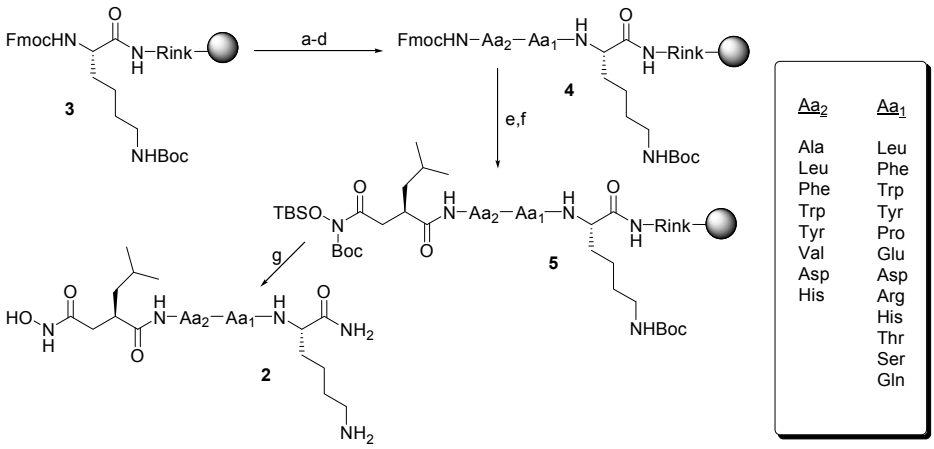


Figure 1: *Structure of the chiral succinylhydroxamate building block **1** for SPPS of a library of 96 compounds with the general structure **2**. P1', P2' and P3' refer to the binding pockets in the metalloproteinase. Boc: tert-butyloxycarbonyl, TBS: tert-butyldimethylsilyl, PFP: pentafluorophenyl. R^1 and R^2 represent amino acid side chains.*

Results and discussion

The preparation of the target compound library (see Scheme 1) commenced with α-NHFmoc-, ε-NHBoc-protected lysine on Rink amide resin **3**. After removal of the Fmoc protecting group the first set of amino acids (Aa^1) was coupled in a parallel fashion under standard SPPS coupling conditions giving 12 different peptides. These resin bound peptides were divided into 8 equal portions. Removal of the Fmoc group and coupling of the second amino acid (Aa^2) gave 96 immobilized peptides with the general structure **4**. Final Fmoc deprotection and condensation with building block **1** (see Figure 1) in the presence of 2 equivalents of DIPEA resulted in the immobilized and fully protected peptide hydroxamates **5**.[27] Acidic cleavage from the resin and concomitant deprotection of the TBS and Boc protecting groups resulted in a 96-membered library of crude compounds **2**, which were purified by HPLC. The yields of the pure peptides based on **3** (purity >95% as

determined by LC-MS analysis) varied between 3% and 40%. The amount of side products formed differed considerably between the compounds. Hydrolysis of the hydroxamic acid to the carboxylic acid in the final step appeared in most cases to be the major side reaction. The formation of this side product was apparent from the LC-MS analyses of the crude mixtures by a 15 Da decrease in molecular weight. In some cases condensation with the activated hydroxamate ester was incomplete. In general, the best results in terms of yield and side product formation were obtained for compounds containing an amino acid with an aliphatic side chain at the Aa^2 position. Representative LC-MS analyses of crude peptides with and without high levels of side products together with their HPLC-purified counterparts are shown in the supporting information.



Scheme 1. *Solid phase synthesis of a succinylhydroxamate library containing 96 compounds: a) 20% piperidine/DMF, 15 min.; b) Fmoc-Aa^1-OH (5 equiv.), HCTU (5 equiv.), DIPEA (10 equiv.), NMP, 1h; c) 20% piperidine/DMF, 15 min.; d) Fmoc-Aa^2-OH (5 equiv.), HCTU (5 equiv.), DIPEA (10 equiv.), NMP, 1h; e) 20% piperidine/DMF, 15 min.; f) compound* ***1*** *(5 equiv.), DIPEA (2 equiv.), NMP, 2h; g) 95% TFA/H_2O, 1h (2.5h for Aa^1 = Arg(Pmc)). Aa^1 = D, E, F, H, L, P, Q, R, S, T, W or Y; Aa^2 = A, D, F, H, L, V, W or Y.*

The results of the inhibitory potential of the 96 compounds against MMP-9, MMP-12 and ADAM-17 are depicted in Figure 2 (heat map representation, the compounds were screened at 100 nM). We performed this initial screen to obtain qualitative insight in the difference in inhibitory potential of the 96 peptide hydroxamates. It is apparent that the efficacy of the inhibitors towards MMP-12 is generally higher than for the other two enzymes. Introduction of a proline residue at the Aa_1-position greatly decreases the activity of the inhibitor with respect to both MMPs. This effect appears to be strongest for MMP-12 but inhibition of ADAM-17 appears to be less affected. This observation can be explained by

the fact that MMPs contain a straight horizontal cleft and therefore a proline would result in a large steric hindrance within the active site. ADAMs, however, do not contain such a rigid cleft and the inhibitor activity is thus less affected by proline.[30,31] It is also obvious that the presence of acidic residues (D and E) in either position greatly reduces the efficacy of the inhibitors for MMP-9 and ADAM-17 and to a somewhat lesser extend for MMP-12. The inhibitors with the highest efficacy towards MMP-12 are those with the aromatic amino acids phenylalanine, tryptophan or tyrosine in either position. These results are in line with earlier observations by Lang and co-workers.[31]

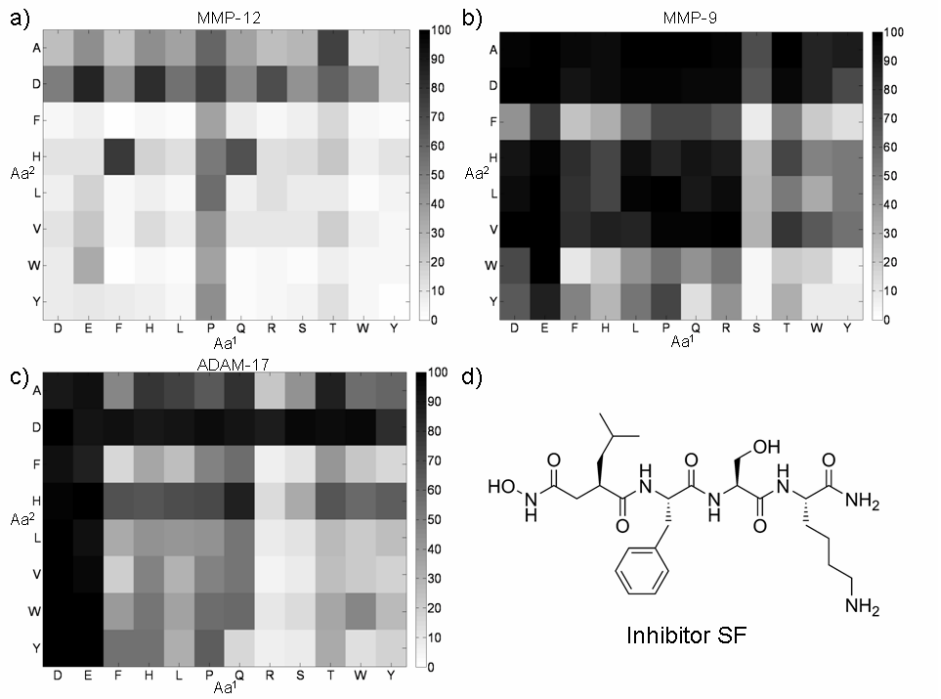


Figure 2

Remaining enzymatic activity of three recombinant metalloproteinases (5 ng) at 100 nM of inhibitor (black: no inhibition; white: complete inhibition). Each value is the average of three individual experiments: a) MMP-12 (0.25 pmol); b) MMP-9 (0.25 pmol); c) ADAM-17 (66 fmol).

The beneficial effect of incorporating aromatic moieties also holds true for MMP-9, especially if both positions are occupied by phenylalanine, tryptophan or tyrosine. Interestingly, a serine residue in the Aa^1 position yields very active MMP-9 inhibitors, whereas threonine at Aa^1 has a much less beneficial effect. MMP-12 and ADAM-17 show a similar, albeit not so strong tendency. The presence of an aliphatic amino acid (A, L or V) in the Aa^2 position decreases the efficacy against MMP-9 in a more pronounced way than

for the other two enzymes. Netzel-Arnett and co-workers reported an extensive study on the substrate preference of MMP-9 towards a set of oligopeptides.[32] Their findings corroborate our results with respect to a positive effect of aromatic moieties in both positions (Aa^1 and Aa^2) or a serine residue at Aa^1 on inhibitory potential towards MMP-9.

Table 1. *IC_{50} values (nM) of eight selected inhibitors for MMP-9, MMP-12 and ADAM-17. Each value represents the mean of three independent inhibition curves (standard deviation in brackets). [a] Activity of enzyme greater than 50% at 10 μM inhibitor.*

	MMP-9	MMP-12	ADAM-17
DV	905 (221)	10.5 (4.0)	2241 (250)
FF	23.2 (3.9)	0.92 (0.22)	16.0 (6.4)
FW	6.69 (0.66)	2.57 (0.80)	29.6 (9.1)
PD	>10,000[a]	2788 (392)	5998 (2555)
PL	3624 (328)	147 (12)	92.1 (28)
QY	9.92 (0.79)	0.85 (0.020)	18.9 (2.0)
SF	9.93 (1.3)	7.70 (1.3)	11.1 (2.3)
YW	6.71 (0.96)	4.03 (0.95)	36.0 (3.4)

Interestingly, our results are in disagreement with their findings that leucine, and to a lesser extent alanine, at Aa^2 has a beneficial effect on inhibitory potential, since we observe a detrimental effect for both residues at this position. Incorporation of arginine in position Aa^1 improves efficacy towards MMP-12 and ADAM-17 but highly reduces the efficacy towards MMP-9, as was also shown by Netzel-Arnett and co-workers.[32] The positive effect of aromatic amino acids also holds true for ADAM-17 but to a lesser extent than for the tested MMPs. In addition it is found that heteroaryl moieties (His and Trp) or a serine at the Aa^1 position improves the potency towards ADAM-17. These observations are consistent to what is reported in literature.[7, 33]

To assess the applicability of the novel inhibitors for activity-based extraction[34] eight inhibitors were selected for further experiments. The IC_{50} values (see Table 1) of these inhibitors for the target enzymes span the entire range from sub-nanomolar to over 10 µM (see for instance FF with PD) and some of them show considerable selectivity towards one or two of the three tested enzymes (for example YW towards both MMPs and PL towards ADAM-17). Activity-based extraction of the three metalloproteases was performed at both 5 and 0.5 nM enzyme concentrations.[39] Especially at lower concentrations of active enzyme, highly efficient interaction of the immobilized ligand with metalloproteases in the sample is likely critical for efficient extraction. Next to achieving high affinity, it is important to minimize non-specific binding to the carrier material, especially at low enzyme concentrations. Since finding a carrier material that exhibits no non-specific interaction with proteins in the sample is practically impossible, good controls are required. For these experiments two control materials were used: a) NHS-Sepharose that was reacted with ethanolamine instead of the inhibitors to study non-specific interaction with the Sepharose itself, and b) a low-affinity inhibitor (PD) was immobilized to assess the importance of a good fit with the enzyme's active site.

Table 2 shows the results of extraction studies with the eight selected inhibitors. Quantitative extraction of ADAM-17 proves much more challenging than extraction of the MMPs. MMP-9 and MMP-12 can be extracted by a number of inhibitors with yields above 99% at both 5 and 0.5 nM, but only inhibitors QY, SF and FF show enrichment of ADAM-17 at 5 nM enzyme concentration (extraction yields of 70% or higher). The efficiency drops significantly at 0.5 nM for inhibitors QY and SF. The negative control with immobilized ethanolamine shows no detectable non-specific binding at both concentrations which is also the case for the low-affinity inhibitor PD.

The results (summarized in Table 2) show that it is difficult to classify immobilized inhibitors according to their inhibition efficacy by affinity-SPE and that it may be misleading to assess inhibitor selectivity in this manner.[28] One interesting inhibitor in this respect is DV, which gives extraction yields of >99% for MMP-12 at both concentrations while its IC_{50} value is 10.5 nM. This is higher than, for example, inhibitor YW, which extracts between 96% and 99%. Inhibitor DV also looses its selectivity towards MMP-12 after immobilization, since both MMP-12 and MMP-9 were almost completely extracted even at low concentration. In contrast ADAM-17 is not extracted at all although the IC_{50} values for MMP-9 and ADAM-17 are in the same range.

Table 2. *Extraction yield (%) of three recombinant active metalloproteases at two concentrations of enzyme by using the immobilized inhibitors as affinity ligand (standard deviation in brackets). EA: ethanolamine.*

	MMP-9		MMP-12		ADAM-17	
	5 nM	0,5 nM	5 nM	0,5 nM	5 nM	0,5 nM
DV	98.7 (1.2)	>99	>99	>99	<1	<1
FF	98.9 (0.85)	98.8 (0.99)	98.9 (0.92)	>99	72.9 (1.8)	73.7 (9.8)
FW	99.0 (0.78)	>99	95.1 (0.14)	>99	35.7 (24)	43.3 (10)
PD	7.2 (9.4)	31.7 (24)	96.6 (0.071)	75.7 (11)	<1	<1
PL	98.8 (1.1)	96.9 (0.92)	97.8 (0.49)	80.8 (20)	65.8 (0.85)	<1
QY	98.4 (0.57)	98.6 (1.3)	>99	>99	88.2 (12)	4.60 (5.8)
SF	>99	98.5 (0.21)	98.9 (0.92)	>99	85.7 (1.3)	49.6 (22)
YW	>99	>99	97.1 (1.3)	96.0 (5.0)	70.0 (6.4)	2.15 (2.3)
EA	<1	<1	<1	<1	<1	<1

Figure 3 shows activity-dependent enrichment of ADAM-17 from a complex proteome (a lysate prepared from cultured A549 cells) using immobilized inhibitor FF (see Scheme 1). The extraction was almost complete, with no loss of mature 70 kDa ADAM-17 in the flow-through fraction (FT) and minor loss in the wash fractions. Active ADAM-17 could be eluted with a competitive inhibitor (SF; see Scheme 1) further confirming that the interaction was inhibitor-mediated. We have shown in previous work that inhibitor-metalloprotease interactions are strictly dependent on a functional active site and that enzyme-inhibitor complexes or pro-enzymes are not bound.[29] The minimal losses in the flow-through and washing fractions indicate that there is little inactive ADAM-17 in non-stimulated A549 cells and that the interaction is relatively tight. We anticipate that enrichment of active ADAM-17 from larger sample volumes is possible allowing detection

of low levels active ADAM-17. Extraction of the same sample on a control material that was derivatized with ethanolamine (EA cartridge) did not result in any enriched ADAM-17.

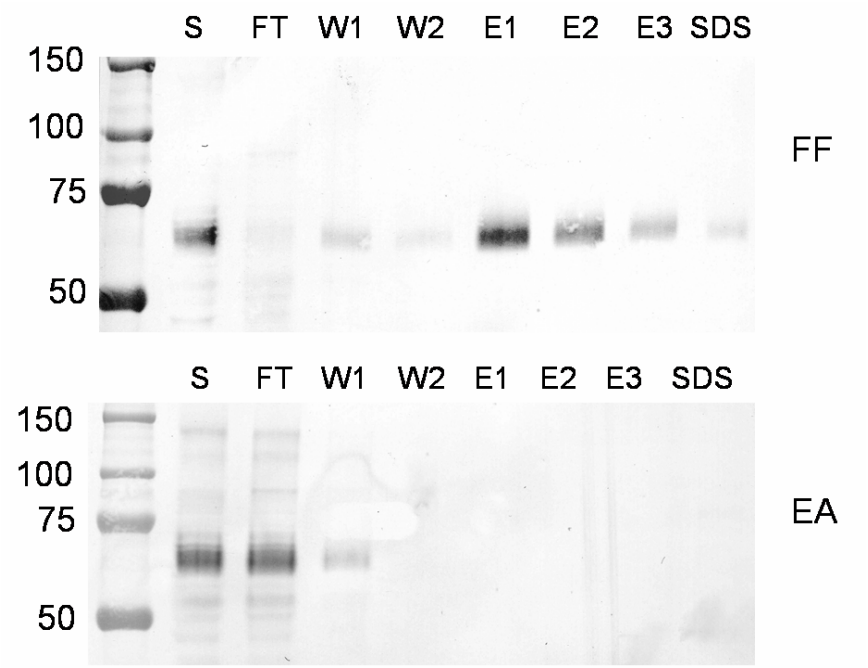


Figure 3. *Activity-dependent enrichment of ADAM-17 from a cell lysate of non-stimulated cultured A549 cells using immobilized inhibitor (FF) and control ethanolamine-Sepharose (EA) cartridges. S: original lysate, FT: flow-through, W: wash fractions, E: elution with 100 µM competitive inhibitor SF, SDS: final elution with 1% sodium dodecyl sulfate. Fractions were analyzed by electrophoresis on 8% polyacrylamide gels and transferred to PVDF membranes. ADAM-17 was detected by Western blotting.*

Phorbol esters have been described to enhance ADAM-mediated shedding of membrane anchored compounds.[37] It is therefore assumed that ADAM-17 becomes activated upon PMA (phorbol-12-myristate-13-acetate) stimulation. Figure 4 shows the effect of PMA on A549 cells with respect to extraction of active ADAM-17. Interestingly, short-term exposure (30min) to PMA results in appearance of a non-active form of ADAM-17 with an apparent molecular weight corresponding to the 93 kDa pro-ADAM-17 zymogen (marked with *). Extraction efficiency decreases as shown by the recovery of a substantial fraction of ADAM-17 in the flow-through and wash fractions, although the major portion still binds the immobilized inhibitor. Decreased extraction might be explained by mobilization of

endogenous inhibitors or by inactivation of ADAM-17 by another, unknown mechanism. After two hours of exposure the expression level of ADAM-17 has decreased significantly (Figure 4, lower panel). Down-regulation of ADAM-17 after cell stimulation has been described.[38] Our results show that this down-regulation does not cause complete disappearance of active ADAM-17 from the proteome.

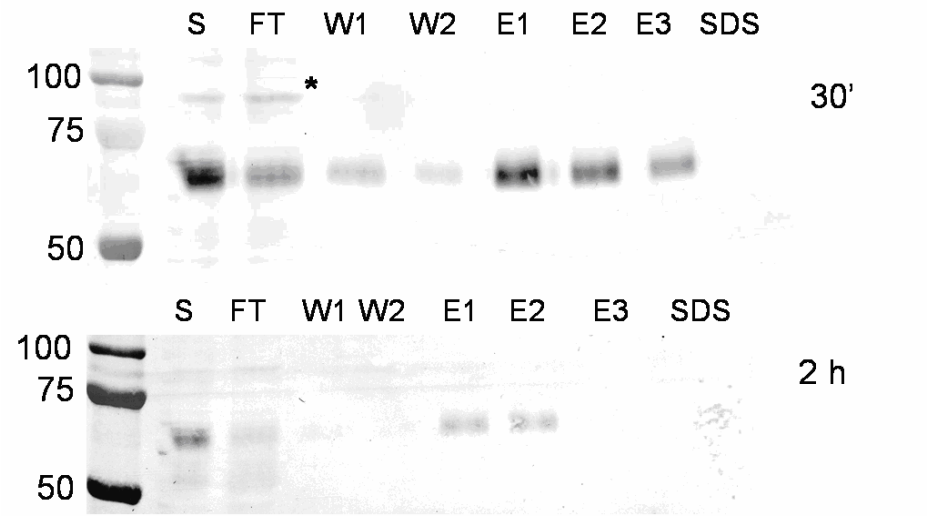


Figure 4. *Western blots of extracted cell lysate of PMA-stimulated cultured A549 lung carcinoma cell line on immobilized inhibitor FF beads. S: original lysate, FT: cartridge flowthrough, W: wash fraction, E: elution with 100 µM competative inhibitor SF, SDS: final elution step with 1% sodium dodecyl sulfate. Fractions were analyzed by electrophoresis on 8% polyacrylamide gels and transferred to PVDF membrane. ADAM-17 was detected by Western blotting*

In summary we prepared a library of 96 enantiopure peptide hydroxamates which were tested on their inhibitory efficacy towards three metalloproteases.[40] Our results show that different amino acids at both positions (Aa^1 and Aa^2) have a great influence on the inhibitory capacity towards MMPs. This is in contrast to what Whittaker *et al.* report by stating that an amino acid at the Aa^2 position sticks out of the enzyme and therefore has 'a modest effect' on the potency.[7]

Several potent inhibitors were immobilized on Sepharose beads and evaluated for use in solid phase enrichment of active metalloproteases. Experiments showed complete enrichment of recombinant MMP-9 and MMP-12 even with lower-affinity inhibitors. Enrichment of active ADAM-17 proved more challenging, but two of the tested inhibitors were successful. Our results may be the first step towards an MMP-selective enrichment material, and furthermore shows that the correlation between affinity (i.e. IC_{50} value or Ki) of free inhibitor and suitability for activity-based enrichment may be less clear than could be expected. One of the inhibitors was used for enrichment from a complex biological sample, showing for the first time activity based enrichment of endogenous ADAM-17.

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Chapter 5

An improved integrated analysis system for online activity-based analysis of MMPs

Manuscript in preparation

Introduction

The previous chapter described the use of small synthetic metalloprotease inhibitors as affinity ligands for activity-dependent solid phase extraction (SPE). Although the technique seems to be robust and applicable to the analysis of both MMPs and ADAMs in biological and clinical samples it suffers from being rather labour-intensive, especially when dealing with larger numbers of samples.
To overcome this problem attempts have been made to incorporate this activity-based enrichment step into a platform for on-line SPE, which enables automated extraction of analytes from aqueous samples and in-line coupling to analysis techniques such as liquid chromatography[1].
A prototype of an integrated analysis system (figure 1) was described recently[2] and consisted of a PROSPEKT-2 automated SPE module fitted with an in-house prepared cartridge packed with Sepharose conjugated with the commercially available inhibitor TAPI-2 and an in-line trypsin reactor packed with modified immobilized trypsin beads (Porozyme)[3] coupled to an LC-(nano)ESI platform with an ion trap for mass spectrometric detection of the extracted and digested proteases.
Although this is a working system, it required high femtomol levels of the model enzyme MMP-12 and lacked in robustness due to instability of the nano-LC-ESI interface. The analytical column was a prototype 50 μm ID capillary filled with a C18 reversed phase monolith (Merck), production of which was discontinued, and the ESI interface was basically a gold-coated nano-emitter that was sleeve connected to the capillary HPLC column and fitted in a casing for electrical contact. Although this setup did work relatively well, it was prone to analytical variation due to the limited lifetime of the emitter tips, and the fact that the tips have to be cut manually to the correct size, which was a rather difficult procedure in itself. Introducing a new tip after a previous one had “run out” led to different electrospray conditions, and possibly changes in peak shape due to variation in post-column dead volume.

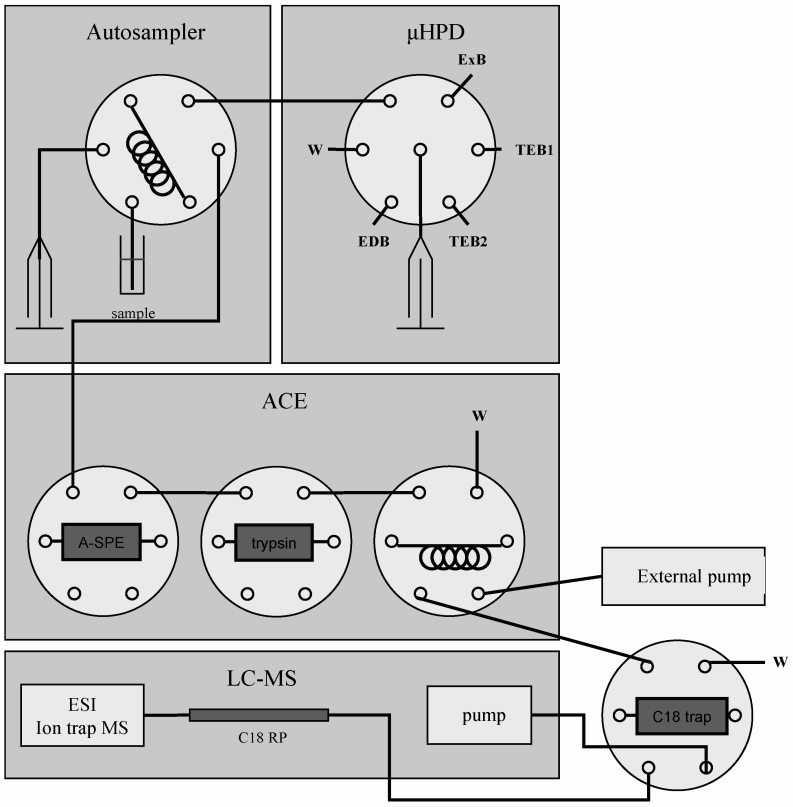


Figure 1:
Schematic representation of the first online platform for activity-dependent analysis of MMPs. The system consists of a PROSPEKT-2 module with a Triathlon autosampler for automated introduction of sample, a µ-high pressure dispenser, which is capable of delivering four individual solvents (ExB: extraction buffer; TEB1-2: trypsin equilibration buffer 1-2; EDB: elution-digestion buffer) at µL/min flow rates, and an automated cartridge exchange system (ACE) with three 6-port switching valves. The first valve is fitted with a 2 mm ID SPE cartridge filled with TAPI-2 conjugated Sepharose, the second valve is fitted with an immobilized trypsin reactor and the third valve is fitted with a collection loop for transfer of the digested proteins to the nano-LC system.

To improve the robustness of the system, alongside with the ease of use, a new version of the analysis platform, where the nano-LC-ESI interface is replaced by a chip-based microfluidic device is described in this chapter.

Experimental

Chemicals

Recombinant human MMP-12 catalytic domain (18.8 kDa [4]), stable isotope labelled ^{15}N-MMP-12 catalytic domain and recombinant human MMP-9 catalytic domain (without fibronectin type II inserts, 18.4 kDa [5]) were a kind gift from AstraZeneca R&D (Lund & Moelndal, Sweden).
MMP inhibitor ML5 was synthesized as described earlier[6].
N-hydroxy succinimide (NHS)-activated Sepharose was from Amersham Bioscience (Uppsala, Sweden), TAPI-2 (*N*-(*R*)-(2-(Hydroxyaminocarbonyl)Methyl)-4-Methylpentanoyl-l-t-Butyl-Glycine-L-Alanine 2-Aminoethyl Amide) was from Calbiochem (La Jolla, CA, USA). Calcium chloride (>99% pure), ethanolamine (>98% pure) and Tris(hydroxymethyl)aminomethane (Tris, >99.5% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (>99.5% pure) and EDTA (Ethylenediaminetetraacetic acid, >99% pure) were from Fluka (Seelze, Germany). Brij-35 was from ICN Biomedicals (Zoetermeer, The Netherlands), Polyethyleneglycol-400-monooctylether (C_8-PEG) was from Acros Organics (Geel, Belgium), and dodecyl-β-D-maltoside was from MP Biomedicals (Solon OH, USA).
Acetic acid N-hydroxysuccinimide (NHS) ester (AANHS) was from ICN Biomedicals (Zoetermeer, The Netherlands); Poroszyme® immobilized-trypsin beads were from Applied Biosystems (Foster city CA, USA).
Acetonitrile (Supragradient grade) was from Biosolve (Valkenswaard, The Netherlands); formic acid (Suprapure grade) was obtained from Merck (Darmstadt, Germany). Ultra-pure water, produced in-house by an Elga purification system, was used for all buffer and mobile phase preparations, which were filtered (0.22 μm pore size) before use.
The term combi surfactant is used for a mixture of equal concentrations of C_8-PEG and dodecyl-β-D-maltoside.

Preparation of cartridges

The immobilized inhibitor cartridge was prepared as described before [7] by incubating NHS-activated Sepharose with an equal volume of 2 mM MMP inhibitor (either TAPI-2 or ML5) in phosphate buffer pH 7.5 for 2 hours at room temperature. The remaining activated ester groups were reacted by incubation with 0.5 M ethanolamine for 1 hour. Control beads were prepared by immediately incubating the NHS-activated Sepharose with ethanolamine. The beads were stored in 20% ethanol at 4ºC until use. Before extraction the beads were slurry packed in empty 2 mm ID x 10 mm PROSPEKT cartridges fitted with a stainless steel filter on one side.
The immobilized inhibitor cartridges were tested for efficacy in an extraction protocol of 10 ng MMP-12 catalytic domain with detection of active enzyme in the flowthrough and washing fractions using an enzyme assay (as described in chapter 4).

The modified trypsin reactor was prepared as described before[3] by packing Poroszyme immobilized trypsin beads in a clean 2 mm ID x 10 mm PROSPEKT cartridge fitted on one side with a stainless steel frit. The cartridge was fitted in a stainless steel clamp (Spark Holland, Emmen, the Netherlands) for further use. The trypsin cartridge was acetylated by pumping 25 mM AANHS in phosphate buffer pH 8.0 over the cartridge using a syringe pump and incubating at stopped flow for 20 minutes. The clamp with the modified trypsin reactor was stored at 4ºC until use.

Chip interface

Agilent Technologies (Palo Alto, CA, USA) has developed a commercially available microfluidics device specifically geared towards peptide analysis in proteomics[8]. This Chipcube (figure 2) combines an analytical C18 microbore column (75 µm ID) with an ESI emitter and a 40 nL C18 trapping column in one device, eliminating dead volumes introduced by nut-connections and theoretically reducing the analytical variation. The flow paths on the chip are regulated by an external six-port rotor and stator that are pressed onto the chip surface when loaded into the interface.

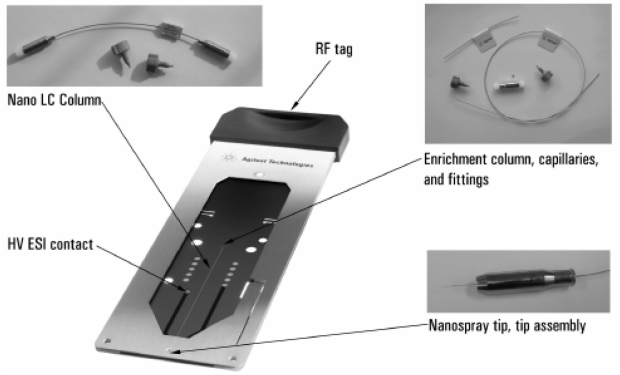


Figure 2:
Comparison of the Agilent HPLC Chip to conventional nano-LC-ESI components.

Early experiments with this interface showed sub-femtomol sensitivity for a standard bovine albumin digest sample, indicating that this system is well suited for the (anticipated) picomolar concentrations of active MMPs in biological or clinical samples.

One problem that arose during the incorporation of the chip interface into the integrated system was the small dimensions of the enrichment column and flow paths on the chip. The earlier version described by Freije *et al* contained an external 400 nL trapping column allowing loading of the digested proteins from the trapping loop on the right-most valve on the ACE (see figure 1) at a flow rate of 20 µL/min, while the chip only tolerates a flow rate of 2-3 µL/min without exceeding the pressure limits of the (relatively) pressure sensitive polyimide chip (maximum pressure 150 bar).
To overcome this problem a third enrichment column was introduced into the system design by replacing the trapping loop on valve 3 of the ACE with a cartridge packed with strong anion exchange (SAX) material (trimethylaminopropyl modified HySphere silica, 40-70 µm particles, Spark Holland)

Instrumental setup with a chipLC-MS interface

Figure 3 shows a schematic representation of the integrated analysis system with the chip interface. The system consists of a PROSPEKT II module with a Triathlon autosampler, a µHPD pump for delivering solvents and an automated cartridge exchange module with three 6-port switching valves (all Spark Holland). The autosampler is fitted with a 1 mL syringe to accommodate introduction of larger sample volumes.

The µHPD is connected to four solvents (all prepared with UP water and passed through a 0.22 µm filter before use): Extraction buffer (ExB: 25 mM Tris pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$ and 0,01% combi surfactant); Trypsin equilibration buffer (TEB: 25 mM Tris pH 8.5, 1 mM $CaCl_2$, 3% ACN); Elution digestion buffer (EDB: 25 mM Tris pH 8.7, 1 mM EDTA, 3% ACN, 0.005% combi surfactant (w/v); Trypsin wash buffer (TWB: 25 mM Tris pH 8.2, 1 mM CaCl2, 45 % v/v ACN).
The PROSPEKT II platform is connected with a 75 µm ID PEEK coated fused silica capillary to an Agilent 1100 series HPLC-MS system with a nano HPLC pump, a capillary HPLC pump, a Chipcube LC-ESI interface and an SL ion trap mass spectrometer.

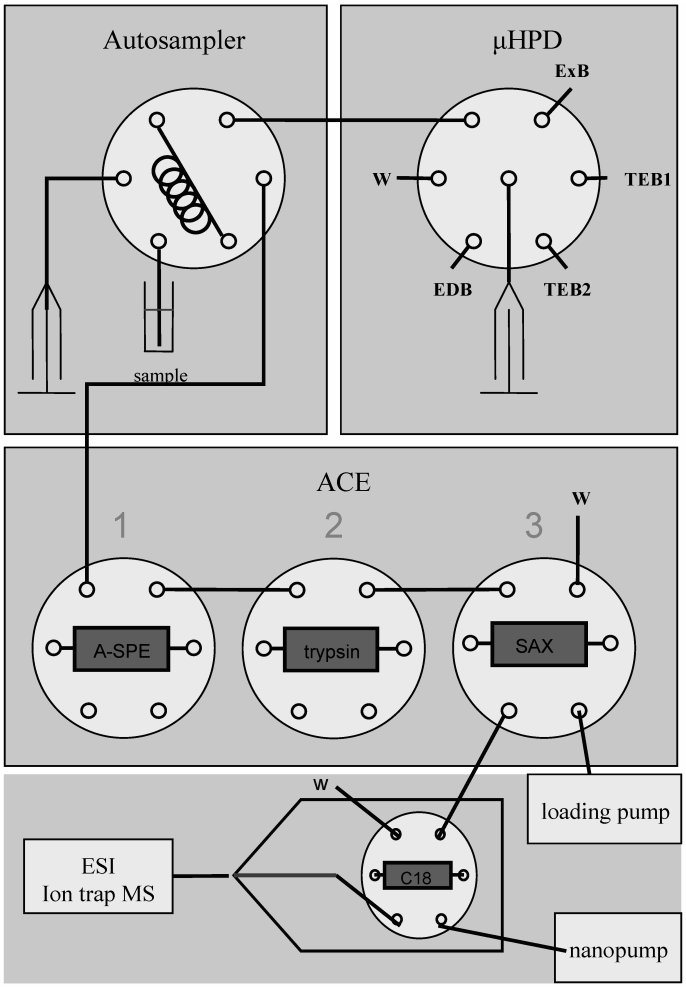


Figure 3:
Instrumental setup of an integrated system for the activity-based analysis of MMPs. The trapping loop on valve 3 of the ACE has been exchanged for a 1 mm ID x 10mm strong anion exchange (SAX) cartridge that can be eluted using the capillary LC pump of the LC-Chip-MS system (loading pump). After elution to the C18 enrichment column on the chip the peptides can be resolved and identified by LC-MS(/MS) using the separating column.

PROSPEKT II method

Table 1 gives an overview of a programme used in the automated extraction method for an injection volume of 100 µL. This method will be discussed further in the results and discussion section.

Table 1: *Step-by-step software programming of the extraction protocol for the PROSPEKT-2 system with duration of each step in minutes. The total analysis time for this method is 1h:40min.*

Step + time	Description	HPD	ACE	AS User Programme
1. Wash lines ExB 1:00	Wash tubing with ExB	Pump 500 µl ExB, 1 ml/min	AUX1 off = high AUX2 off, AUX4 off Cartridge exchange left LCV1-2, ISS1:1-2, ISS2:1-2	1 WFI high 2 Valve: load position 3 Wash 750 ul 4 AUX1 AS on = low
2. Equil SPE ExB 3:51	Equilibrate SPE cartridge with TEB Load loop AS with sample	Pump 350 ul ExB, 100 µl/min	StepExCond:input 1 ACEon=low AUX1 on=low LCV6-1	5 WFI Low 6 Aspirate 170 µl from sample, 7 speed 2, height 1 mm 8 AUX1 AS off = high
3. Inject + wash 15:45	Inject sample on SPE cartridge, wash with ExB	Pump 750 ul ExB, 50 µl/min	StepExCond:input 1 ACEoff=high AUX1 off=high	9 WFI high 10 Valve: inject position
4. Wash lines TWB 0:30	Wash tubing with TWB	Pump 250 ul TWB, 1 ml/min	AUX1 on=low LCV1-2	11 WFI low 12 Valve: load position
5. Wash tryp TWB 3:00	Wash trypsin cartridge with TWB	Pump 500 µl TWB, 200 µl/min	ISS1:6-1	
6. Equil tryp TEB 2:00	Equilibrate trypsin cartridge with TEB	Pump 400 µl TEB, 200 µl/min		
7. Fill lines EDB 0:30	Fill tubing with EDB for elution step 8	Pump 250 µl EDB, 1 ml/min	ISS1:1-2	

8. Equil SAX 5:15	Equilibrate SAX with EDB	Pump 250 µl EDB, 50 µl/min	ISS2: 6-1	
9. Elute-dig EDB 21:05	Elute digest MMPs, load peptides on SAX	Pump 250 µl EDB, 12 µl/min	LCV6-1, ISS1:6-1	
10: Start LC-MS	Start LC-MS		AUX2 on, AUX4 on	
11. Elute SAX 2:00	Delay SAX switch-in to prevent contact with acidic mobile phase		StepExCond: stage start delay 2 minutes ISS2:1-2	
12. Wash SPE 5:15	Elute/wash A-SPE	Pump 250 µl EDB, 50 µl/min	ISS1:1-2	
13. Fill lines TWB 29:45		Pump 250 µl EDB, 500 µl/min	StepExCond: stage start delay 28 minutes LCV1-2	
14. Wash trypsin + SAX 2:31	Wash SAX and trypsin TWB	Pump 250 µl TWB 100 µl/min	ISS1:6-1 ISS2:6-1	
15. Wash SPE TWB 10:30	Wash SPE with TWB	Pump 500 TWB, 50 µl/min	AUX1 off=high LCV 6-1	13 WFI high 14 Valve: inject position 15 End

LC-MS/MS method

The LC-MS acquisition method is started by an auxiliary signal from the PROSPEKT workstation. The mobile phases connected to the HPLC are as follows:
Nano LC solvent A: water, formic acid 0.1% (v/v); solvent B: acetonitril/water 90/10, formic acid 0.1% (v/v).
Capillary (loading) pump solvent A: water/acetonitril 97/3, formic acid 0.1 % (v/v); solvent B: ammonium acetate 200 mM pH 5.0/acetonitrile 97/3.

Table 2: *gradient for nano-LC pump (flow rate 300 nL/min)*

Time (min)	% B
33	3
74	40
79	95
87	95
92	3

Table 3: *gradient for capillary LC pump (flow rate 3 µL/min)*

Time	% B
0	100
32	100
33	0

Table 4: *valve switching scheme for the Chipcube interface.*

Time	position
0	Enrichment
33	Analysis
94	Enrichment

Tables 2 and 3 show the gradients used during elution of the bound peptides from the SAX cartridge on valve 3 of the ACE to the chip, and chromatographic separation of the peptides. The method is further explained in the results section.
The electrospray conditions were as follows: capillary voltage 1800-2000V (adjusted for spray stability, the lowest value where a stable spray current of 50-100 nA was obtained at 97% phase A was used), drying gas (N_2) flow 4 L/min, drying gas temperature 300ºC.
Mass spectrometric data was acquired in profile mode using MS^2 with data-dependent precursor selection with an absolute minimal threshold for precursor ions of 5000. Monoisotopic m/z values for ubiquitous background ions (polysiloxanes) were excluded from fragmentation. The scan range was 300-1500 m/z, and ICC target was set to 30,000 ions. The maximum accumulation time in the trap was set to 15 ms.

The first 33 minutes of each run was stored only in centroid mode to save storage space, since during this period no analyte is expected to elute from the chip.

Database identification of proteins

The data from each run was extracted using the Bruker Daltonik DataAnalysis software (version 3.2 build 121). The intensity threshold for exported fragment m/z values was set to 5000 counts by using the export AutoMSn function in the software. The peak lists were analyzed using the Phenyx (https://phenyxonline.genebio.com) and Mascot (http://www.matrixscience.com) software platforms. Database search settings for both algorithms were as follows:

Phenyx:
Database: uniprot_sprot (version 55.3 29-april-2008)
Taxonomy: mammalian
Instrument type: ESI-ion trap
Scoring Model: LCQ
Default Parent Charge: 1,2,3,4
Trust Parent Charge: medium
Modifications: Oxidation_M (+16Da), variable
Enzyme: Trypsin_KR_noP
Missed Cleavage: 2
Cleavage Mode: normal
Parent Error Tolerance: 1000 ppm
Turbo scoring (fragment tolerance)
* tolerance 0.7 Da
* coverage > 0.1
* series b ; b++ ; y ; y++
Acceptance parameters:
* peptide length 5
* score 5.0
* p-value individual peptides 0.05

Mascot
Exported spectra in the autoMSn spectra list were limited to 300 due to upload limitations on the online version of Mascot (http://www.matrixscience.com).

Database: Swissprot (version 56.0)
Taxonomy: mammalian
Enzyme: Trypsin
Allow up to 2 missed cleavages
Variable modifications: oxidation (M)
Peptide tolerance: 0.1%

MS/MS tolerance: 0.7 Da
Peptide charge 1+, 2+ and 3+
Instrument: ESI-TRAP

After database search only peptides with an individual score corresponding to a significance level of $p < 0.05$ or higher are included. Redundant hits were manually examined.

Results and discussion

Introduction of strong anion exchange enrichment step

To overcome the large volume difference between the eluate coming from the immobilized trypsin cartridge (at least 100 µL) and the dimensions on the HPLC chip (40 nL enrichment column), an additional trapping or enrichment step was introduced. The use of ion exchange in this case seemed logical, since this technique is orthogonal to the other separation methods used in the system (affinity and reversed phase chromatography). Traditionally strong cation exchange (SCX) is preferred as a complementary separation technique in proteomics, and is well described in literature (for instance the MuDPIT 2D LC-MS platform[9]) but since (most) peptides can only be efficiently trapped on a cation exchange column at acidic pH (leading to positively charged peptides) this phase is not be ideal, since the eluate coming from the trypsin reactor (the elution digestion buffer) is at pH 8.7. Since most acidic residues in the peptides will be deprotonated at this pH and the primary amines only partially positively charged, the choice for anion exchange was made. An additional advantage of using ion exchange material for the trapping step is the fact that the non-ionic surfactants used in the extraction, and that are essential in keeping the active protease and the larger peptides in solution, will not be retained in this step. This should result in a cleaner sample being sent to the mass spectrometric step and reduced risk of ion suppression due to the surfactant in the electrospray ionization process.
Preliminary experiments incorporating a strong anion exchange trapping step between the trypsin reactor and the Chipcube interface were carried out by simply incorporating an 8 µL polymer-based monolithic high capacity SAX nano trap column (Styros HQ, Orachrom, Woburn, MA, USA). After trapping of the peptides in the flowthrough of the trypsin reactor the rightmost valve on the ACE (see figure 1) was switched to elute the trapped peptides using the capillary (loading) pump of the 1100 HPLC system. This approach seemed the "cleanest" option, since theoretically the pH switch from the elution digestion buffer (pH 8.7) to the mobile phase of the capillary HPLC (water/acetonitril 97/3 with 0.1 % (v/v) formic acid, pH ~2.7) should be sufficient to introduce positive charge on the peptide leading to elution from the SAX cartridge. Using this approach the necessity to introduce additional solvent flows as make-up solvents could be avoided. Although the use of SAX in peptide analysis is rather rare, there are examples of this approach[10,11].

Experiments using this setup to perform an automated analysis of 5 pmol (injected) of MMP-12 catalytic domain yielded somewhat disappointing results as compared to the original system setup. Only two peptides ($(I^{55}\text{-}R^{63})^{2+}$ and $(I^{19}\text{-}R^{36})^{3+}$ (see figure 4 for protein sequence) were detected, but yielded no significant database hits in either Mascot (Mowse scores resp. 27 and 23 with 43 corresponding to a significant hit at p=0.05) or Phenyx.

The earlier described total analysis system[12], on the contrary, was capable of yielding 70-80% sequence coverage, although identification of the peptides was done manually by creating extracted ion chromatograms from LC-MS data of the peptides observed earlier in standard analyses of MMP-12 digests instead of automatically by MS^2 with data-dependent precursor selection. Manual investigation of the data showed poor signal intensity for the two observed MMP-12 tryptic peptides (see figure 5) compared to earlier results with the first generation platform. Another problem was the high variability in the obtained results (observed peptides and intensity per run) after the introduction of the SAX cartridge into the system.

The peptide masses from your sequence are:

[Theoretical pI: 6.12 / Mw (average mass): 18795.02 / Mw (monoisotopic mass): 18783.23]

mass	position	#MC	peptide sequence
6950.2430	67-134	1	GAHGDFHAFDGKGGILAHAF GPGSGIGGDAHFDEDEFWTT HSGGTNLFLTAVHEIGHSLG LGHSSDPK
6648.1740	79-142	1	GGILAHAFGPGSGIGGDAHF DEDEFWTTHSGGTNLFLTAV HEIGHSLGLGHSSDPKAVMF PTYK
5710.7009	79-134	0	GGILAHAFGPGSGIGGDAHF DEDEFWTTHSGGTNLFLTAV HEIGHSLGLGHSSDPK
2759.3721	53-78	1	INTGMADILVVFARGAHGDF HAFDGK
2199.0135	19-36	1	INNYTPDMNREDVDYAIR
2070.9814	13-28	1	HYITYRINNYTPDMNR
1964.9938	135-150	1	AVMFPTYKYVDINTFR
1882.0255	50-66	1	FSKINTGMADILVVFAR
1847.9497	151-167	1	LSADDIRGIQSLYGDPK
1797.9129	143-157	1	YVDINTFRLSADDIR
1751.9479	38-52	1	AFQVWSNVTPLKFSK
1519.8301	53-66	0	INTGMADILVVFAR
1517.8474	37-49	1	KAFQVWSNVTPLK
1389.7525	38-49	0	AFQVWSNVTPLK
1331.6677	1-11	1	FREMPGGPVWR
1320.6430	158-169	1	GIQSLYGDPKEN
1258.5599	67-78	0	GAHGDFHAFDGK
1237.5630	19-28	0	INNYTPDMNR
1156.5931	3-12	1	EMPGGPVWRK
1108.5633	29-37	1	EDVDYAIRK
1077.5575	158-167	0	GIQSLYGDPK
1028.4982	3-11	0	EMPGGPVWR
1027.5207	143-150	0	YVDINTFR
980.5312	12-18	1	KHYITYR
980.4683	29-36	0	EDVDYAIR
956.4910	135-142	0	AVMFPTYK
852.4362	13-18	0	HYITYR
789.4101	151-157	0	LSADDIR

100.0% of sequence covered (you may modify the input parameters to display also peptides < 500 Da):

```
        10         20         30         40         50         60
FREMPGGPVW RKHYITYRIN NYTPDMNRED VDYAIRKAFQ VWSNVTPLKF SKINTGMADI

        70         80         90        100        110        120
LVVFARGAHG DFHAFDGKGG ILAHAFGPGS GIGGDAHFDE DEFWTTHSGG TNLFLTAVHE

       130        140        150        160
IGHSLGLGHS SDPKAVMFPT YKYVDINTFR LSADDIRGIQ SLYGDPKEN
```

Figure 4:
Amino acid sequence and theoretical tryptic peptide map of recombinant MMP-12 catalytic domain ($F^{100}\text{-}N^{268}$ in swissprot entry P39900) obtained from PeptideMass at http://www.expasy.org/tools/peptide-mass.html).

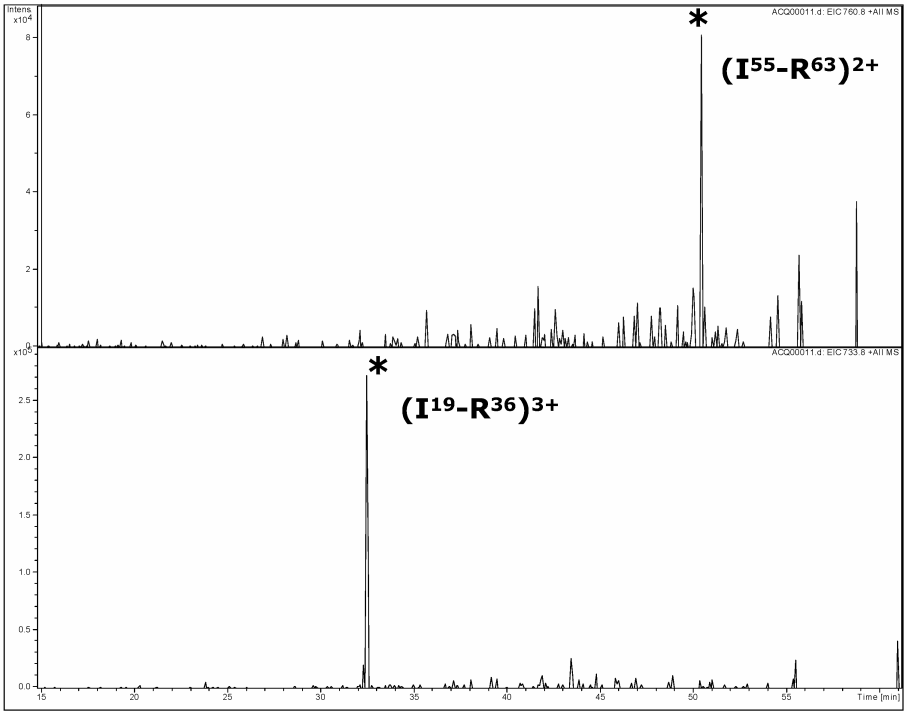


Figure 5:
Extracted ion chromatograms of the two detected peptides from MMP-12 in an automated PROSPEKT-LC-MS^2 analysis of 5 pmol MMP-12 in 100 µL extraction buffer after the incorporation of an 8 µL polymer-monolithic SAX trapping column into the system.

To evaluate to performance of the SAX column in trapping tryptic peptides, off-line extraction experiments were carried out using a tryptic digest of bovine serum albumin in elution-digestion buffer as a standard sample. The results of trapping and elution experiments revealed that the trapping itself was successful, although one peptide with a high pI of 8.75 (K_{437}-S_{451} in BSA swissprot entry P02769) did break through during washing steps. However, elution using only a pH switch was not very efficient. Using formic acid, trifluoroacetic acid or acetic acid at different concentrations (0.1-1% (v/v)) elution of the bound peptides from the SAX cartridge was not observed . This strong retention was probably based on ionic and not a non-specific hydrophobic interactions, since increasing the acetonitrile content of the elution buffer did not improve elution. Only when elution was performed using 1 M acetic acid, or when the column was seemingly saturated with BSA peptides detection of most BSA peptides was possible. This finding is in line with the irreproducible identification of the observed MMP-12 peptides in the first experiments using the new setup of the online system.

Traditionally ion exchange chromatography uses salt gradients to elute bound analytes, but this introduces the risk of precipitation and blockage, especially when analysing the samples with techniques that use small dimension capillaries and connections, such as nano- or chip-LC. Two volatile salts (ammonium acetate and ammonium formate) were tested for their efficacy in eluting bound peptides from a strong ion exchange column. To increase system compatibility the Orachrom column was replaced by a 1 mm ID (bed volume 8 µL) SAX (quartenairy ammonium, silica based trimethylaminopropyl phase) PROSPEKT cartridge by Spark housed in a stainless steel clamp. When using a 0.2 M solution of either salt adjusted to pH 5 elution of bound peptides was readily observed, with ammonium acetate resulting in a slightly better yield. This phenomenon may be explained by the higher ionic strength of acetate as compared to formate counter ions[13].

Automated analysis method

Table 1 gives a point-by-point overview of the individual steps of the PROSPEKT method for the automated extraction-digestion-trapping of MMPs. The Triathlon autosampler is programmed separately from the ACE and HPD, and synchronisation is ensured by a series of cross-talking auxiliary signals (aux 1 of ACE and aux in autosampler programme).
In step 1, the system is flushed with extraction buffer (ExB) with all cartridges offline. Flushing with the Sepharose-inhibitor cartridge not included in the flowpath enables higher flow rates without risk of damaging the pressure sensitive Sepharose material. Simultaneously, the autosampler performs a wash cycle to reduce carry-over between runs. In the second programme step the inhibitor cartridge is included in the flowpath by switching valve 1 into the 6-1 position. The cartridge is equilibrated with ExB and prepared for injection of the sample. At the same time, an aliquot of sample is aspirated from the sample vial. This volume can be modified depending on the desired injection volume. In step 3 the sample is loaded onto the inhibitor cartridge by switching the autosampler valve to the inject position while the µHPD delivers a flow of ExB to carry the sample to the cartridge. After washing the inhibitor cartridge with ExB (step 4) the system is filled with trypsin wash buffer (TWB) with all cartridges off-line in step 5. The next step is the washing of the trypsin reactor with TWB to ensure a clean trypsin cartridge before the elution step. After the cleaning step the system is filled with Trypsin equilibration buffer (TEB) and the trypsin reactor is conditioned with TEB to maximize trypsin activity. In steps 8 and 9 the system lines are filled with Elution digestion buffer (EDB), and the SAX cartridge on valve 3 of the ACE is equilibrated to pH 8.7 with EDB. This last step was included since in all previous steps the SAX column is in contact with the acidic mobile phase of the capillary LC pump. In step 10 both the inhibitor cartridge and the trypsin reactor are switched in the flowpath and elution of bound proteins is performed with EDB. This buffer contains EDTA which is capable of chelating the Zn^{2+} ions in the catalytic site of the MMPs and thereby eluting proteins that are bound via metal-chelation from the immobilized inhibitor cartridge. The eluate from the inhibitor cartridge is pumped over the trypsin reactor where digestion occurs. This step is carried out at a flow rate of 12 µL/min

(the lowest flow that the μHPD can deliver in this setup) to maximize digestion time to ~ 1 min. Previously published results have shown that a contact time of 4 seconds is sufficient for tryptic digestion of cytochrome C at this pH[3]. The peptides resulting from the proteolytic cleavage in the trypsin reactor are subsequently trapped on the SAX cartridge which is situated on valve 3 of the ACE. After elution and digestion are complete, the ACE gives separate auxiliary signals to both the HPLC and the mass spectrometer. The signal from the ACE starts the HPLC methods described in tables 2-4 and the MS acquisition. The capillary loading pump switches from the standard acidic mobile phase to the SAX elution solvent B (200 mM ammonium acetate pH 5.0 with 3% acetonitrile). There is a 2 minute delay in the ACE method (step 12) to ensure a stable flow of solvent B and to avoid contact of the SAX column with the acidic mobile phase that is still present in the tubing connecting the pump to valve 3 on the ACE. The next steps comprise several washing steps of the injector loop, Sepharose-inhibitor cartridge and trypsin reactor to reduce carry-over between runs. Step 14 contains a delay to ensure synchronisation between the PROSPEKT method and the LC-MS analysis. By making the PROSPEKT method longer than the LC-MS method, samples can be analysed in parallel, meaning that while the first sample is analyzed the next sample can already be introduced into the PROSPEKT system. The total analysis time for this method is 1h:40min per sample meaning that with a continuously running system on average 14 samples may be profiled in 24 hours. The overall analysis time is, however, dependent on the injection volume. An injection of 500 μL instead of 100 μL under the same conditions will take 6 minutes longer.

Figure 6 shows a representative chromatogram resulting from analysis of 2.9 pmol MMP-12 catalytic domain in 100 μL of extraction buffer. Marked by arrows are the five peaks that correspond to MMP-12 derived tryptic peptides as identified by database search against the exported spectra of this run (Auto-MS[2]). This result demonstrates that the system is capable of performing fully automated activity-based analysis of MMP-12 at the low nM concentration level and that the inclusion of the anion exchange trapping step did not affect sensitivity and system performance adversely. The chromatogram in Fig. 6 (right panel) shows the result of an experiment where the SAX cartridge was taken out of the system, and the eluate from the immobilized trypsin reactor was directly loaded onto the chip enrichment column. The large peak that appears in the chromatogram is the result of the high surfactant load in the extraction and elution digestion buffer on the chip in this experiment and shows an additional positive effect of the inclusion of the SAX step. Because the surfactants that are present in the extraction and the elution-digestion buffer are non-ionic in nature their retention on the anion exchange material will be limited and the eluate is cleaned up before sending it to the LC-MS system. Surfactant addition to the extraction and elution buffer was found to be necessary at low concentration of target protease to avoid non-linear response and poor system sensitivity due to loss caused by non-sepcific adsorption of protein and peptide to system tubing[12].

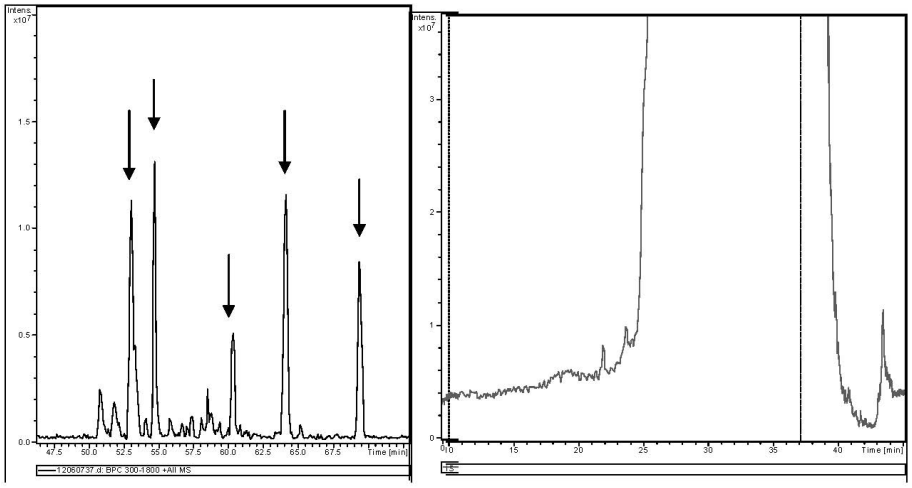


Figure 6:
Left: LC-MS chromatogram obtained with 2.9 pmol MMP-12 catalytic domain in 100 µL ExB on the PROSPEKT-LC-MS/MS system with a SAX trapping step. MMP-12 peptides are indicated by arrows. Right: LC-MS chromatogram of 5.4 pmol MMP-12 catalytic domain in 100 µL ExB analysed on the PROSPEKT-LC-MS/MS system without a SAX trapping step. The eluate of the trypsin reactor was directly loaded onto the chip enrichment column.

Figure 7 shows a similar experiment where recombinant MMP-9 was used as standard protease, and spiked into extraction buffer. From an injected amount of 5.4 pmol MMP-9 catalytic domain in 100 µL extraction buffer two peptides were identified as resulting from MMP-9 by automatic precursor ion selection MS^2. This result shows that the sensitivity of the system towards MMP-9 is slightly lower but that especially the F319-R334 peptide still gives a signal with good intensity. This is an interesting finding since MMP-9 as a gelatinase is a very well studied protease (along with its sister gelatinase MMP-2) and has been implicated in various disease states. MMP-9 has been described to be present at relatively high concentration in urine of bladder cancer patients and synovial fluid of people suffering from rheumatoid arthritis[14].
One interesting observation is that although the SAX step removes the majority of surfactant present in the EDB, there is still a significant signal at the end of the chromatogram. This may be a cause for concern since this detergent load could lead to breakthrough of (more hydrophilic) peptides on the chip enrichment column due to saturation, but unfortunately the addition of surfactants is unavoidable when analysing proteases at low concentrations due to non-specific adsorption. Contrary to the direct loading step, the detergent peak does no longer overlap the peptide elution region, so effects such as surfactant-mediated ion suppression are eliminated.

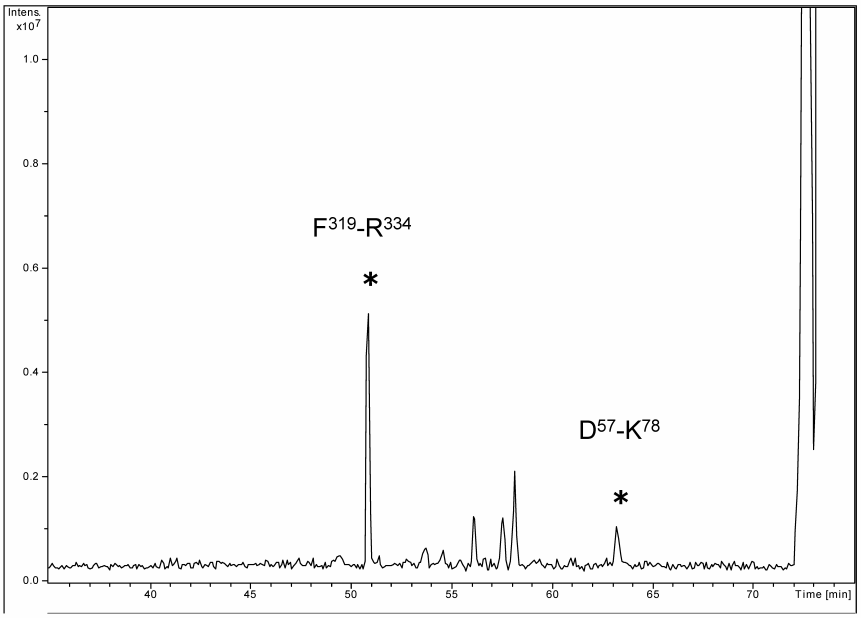


Figure 7:
LC-MS chromatogram obtained after injection of 5.4 pmol MMP-9 catalytic domain in 100 µL extraction buffer on the PROSPEKT-LC-MS/MS system. Marked are two MMP-9-derived tryptic peptides that were identified by MS/MS.

Incorporation of stable isotope labelled ^{15}N-MMP-12 catalytic domain as internal standard

When using mass spectrometry for quantitative analysis inclusion of an internal standard is advisable to correct for analytical variation between different analyses. Especially when analysing complex biological samples (prone to ionisation suppression) or when using nanoLC hyphenated to mass spectrometry between-run variation can complicate interpretation of the results, especially considering the multiple steps in the system described here, all contributing to total analytical variation. Stable isotope labelled compounds are preferred as internal standards for mass spectrometry, since the chromatographic behaviour of the analyte and the deuterated or ^{13}C/^{15}N labelled will be very similar or identical, thus correcting for variation in, for instance, ionisation efficiency.
In the case of the activity-based analysis of proteases such as MMP-12 this is less obvious. To act as a good internal standard the target protease (MMP-12 in this case) and the internal standard need to be very similar and preferably be included in the original sample to correct for losses during the entire analytical process. The stable-isotope-labelled standard therefore needs to be the active metalloprotease itself. In the case of MMP-12 catalytic

domain the internal standard was created by metabolic stable isotope labelling, where the genetically modified organism (*E. coli*) is cultured in medium with an exclusively ^{15}N-containing nitrogen source. This process ensures (almost) complete incorporation of ^{15}N into all proteins the organism produces yielding a proteolytically active, ^{15}N-labeled version of the catalytic domain of MMP-12. The labelled protein is thus co-extracted on the inhibitor cartridge and follows the rest of the analytical steps of the automated system. The co-eluting peptides can be easily distinguished by mass spectrometry, since the mass difference between the natural and the stable isotope labelled peptides will be at least one mass unit per amino acid depending on the number of nitrogen atoms.

Table 5:
Relative standard deviation (in %; n=3) of the peak area of the three MMP-12 peptides with the highest intensity with and without inclusion of ^{15}N-MMP-12 as internal standard (injection volume: 100 µL).

	With IS correction			**Without IS correction**		
MMP12 (pmol)	**514 m/z**	**734 m/z**	**760 m/z**	**514 m/z**	**734 m/z**	**760 m/z**
0,15	29,9	22,4	21,5	52,4	57,0	44,4
0,29	18,7	16,2	31,4	36,2	33,1	64,8
0,59	15,7	21,1	4,4	59,4	115,3	48,1
1,47	3,1	7,3	8,0	36,1	79,7	29,4
2,94	3,1	26,8	7,8	30,0	56,7	34,3
4,41	4,6	3,4	12,3	17,2	31,1	31,7
5,88	9,9	12,9	11,8	14,5	24,4	35,0
average RSD	12,1	15,7	13,9	35,1	56,8	41,1

Table 5 shows the data obtained for calibration curves of MMP-12 catalytic domain in the integrated system. The results clearly show the beneficial effect of including the internal standard as opposed to "label-free" analysis, where the RSD may be as high as 100%. After correction of the peak area for the internal standard, variation is well below an RSD of 20%, which is acceptable considering the complexity of the entire system.
One problem that was encountered in the earlier version of the integrated system was the non-linear response at lower (sub pmol injected or low- to sub-nanomolar concentration) analyte concentrations. This effect was ascribed to losses due to non-specific adsorption to system components and could not be completely reversed by addition of the surfactant mixture to the extraction and elution-digestion buffer. This greatly limited the sensitivity of the system, since analysis of samples having sub-nM concentrations was very difficult.
The addition of a relatively high amount of internal standard (2.9 pmol injected) probably helped to overcome this problem, since the sensitivity of the method improved notably with the addition of ^{15}N-MMP-12 to the samples. Figure 8 shows a calibration curve of MMP-12

catalytic domain (with internal standard correction) in extraction buffer. The linearity of the response ratio is good over the investigated range. The amount of MMP-12 needed to yield a positive database hit in Mascot was found to be around 300 fmol injected (3 nM from 100 µL). A similar curve was obtained when spiking MMP-12 CD in urine from a healthy human volunteer. When increasing the injected volume to 500 µL the required amount remained the same, indicating that the system is capable of identifying MMP-12 at sub-nanomolar concentration in aqueous samples (0.8 nM) emphasizing its capability to enrich low amounts of active MMP-12 from larger sample volumes.

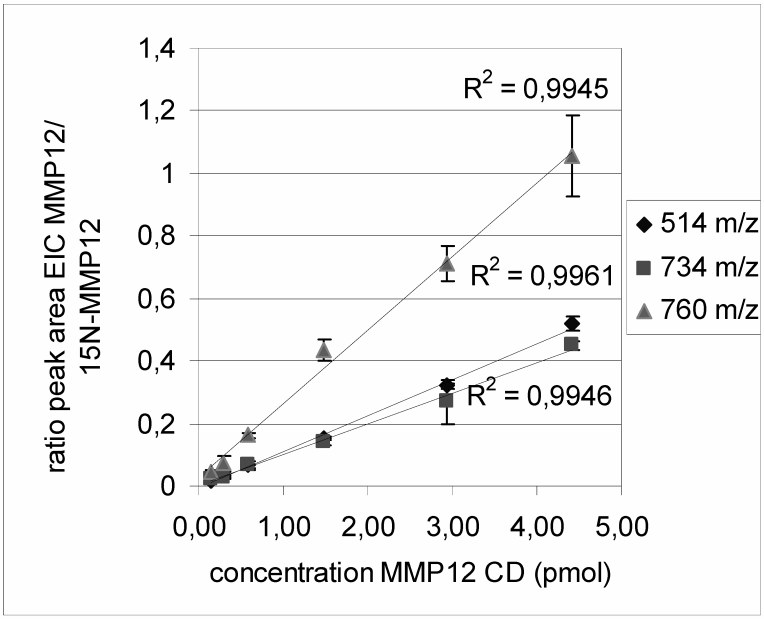
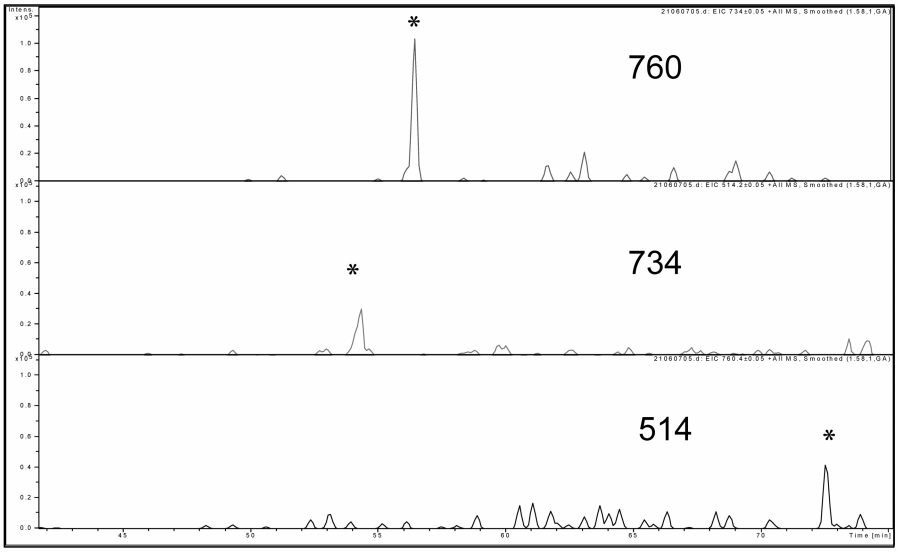


Figure 8:
Calibration curve of MMP-12 catalytic domain in extraction buffer (n=3 for each sample). The ratio of the area of the three peptides with the highest intensity of MMP-12 and ^{15}N-MMP-12 is plotted against the amount of MMP-12 injected. The amount of ^{15}N-MMP-12 added to each sample was 2.9 pmol.

Figure 9 shows smoothed EIC traces of the three peptides observed with the highest intensity after an injection of 59 fmol MMP-12 catalytic domain in 100 µL ExB on the online system. The sample was extracted on an inhibitor cartridge with TAPI-2 as a ligand, and although no database hit could be obtained by automatic MS/MS the peaks can still be identified and quantified after smoothing of the background. This indicates that this system may be used for targeted analysis of MMP-12 at concentrations of 0.6 nM using an ion trap mass analyzer in profile mode. It is likely that even lower concentrations can be reached

when using triple quadrupole mass analyzers in the SIR (Selective Ion Recording), SRM



(selective reaction monitoring) or MRM (Multiple Reaction Monitoring) modes.

Figure 9:
Extracted ion chromatograms (smoothed) of three MMP-12 peptides with the highest intensity obtained upon analysis of 59 fmol MMP-12 catalytic domain spiked into in 100 µL human urine with 2.9 pmol ^{15}N-MMP12 as internal standard.

Analysis of active endogenous metalloproteases

Since spiking experiments metalloproteases in biological matrices such as urine, total cell lysates and broncheo-alveolary lavage fluid (BALF) showed the possibility of analyzing active proteases in complex biological samples when resorting to Western blotting (see chapters 4 and 6), we performed multiple experiments for proving the applicability of the integrated analysis system to such complex samples. The main problem that was encountered in these experiments was the detection and identification of a large number of highly abundant non-metalloenzyme proteins that presumably interacted non-specifically with the inhibitor cartridge. When performing offline experiments, as described in the previous chapter, this non-specific binding was presumed to be low, since gel electrophoresis of the eluting fractions of several extraction experiments of biological samples showed almost no detectable protein. The high sensitivity of nanoLC-MS was, however, able to pick these proteins up, as shown in tables 6a and b and the chromatograms in figure 10. This table shows the identified proteins in a representative run of nephropathic rat urine (courtesy of Dr. Rob Henning, university of Groningen) on an inhibitor cartridge

with inhibitor ML5. Figure 10 shows the corresponding LC-MS and LC-MS/MS total ion chromatograms.

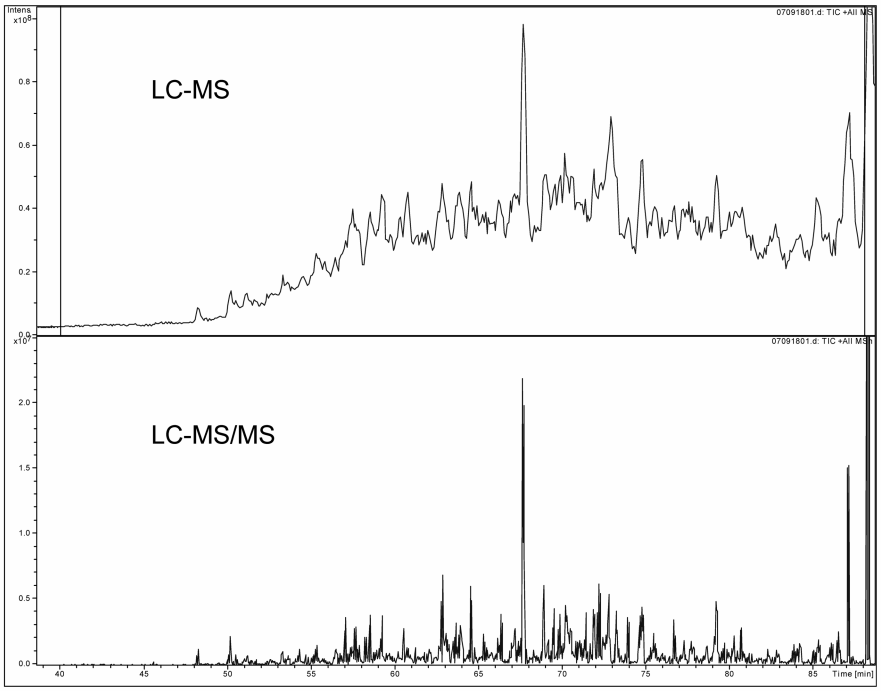


Figure 10:
TIC chromatograms obtained with a total analysis system run of 100 µL rat urine, using ML5 as immobilized inhibitor. The top trace shows the single MS signal, while the bottom trace shows the fragmentation signal obtained with data-dependent precursor selection.

Table 6a:
Top 30 proteins identified using the Phenyx algorithm in an extraction performed on 100 µL rat urine

Protein	Protein ID	score	unique peptides
Alpha-1-antiproteinase precursor	A1AT_RAT	43,6	5
Plasminogen precursor	PLMN_RAT	42,5	5
Serine protease inhibitor A3N precursor	SPA3N_RAT	36,3	4
Alpha-1-antitrypsin precursor	A1AT_HUMAN	34,1	4

Ceruloplasmin precursor	CERU_RAT	33,3	4
Serotransferrin precursor	TRFE_RAT	33,1	4
Ig gamma-1 chain C region.	IGHG1_HUMAN	24,5	3
Complement C3	CO3_RAT	23,5	3
Serum albumin precursor	ALBU_RAT	23,5	3
Serine protease inhibitor A3L precursor	SPA3L_RAT	20,0	3
Complement C3 precursor	CO3_HUMAN	19,8	2
Pigment epithelium-derived factor precursor (PEDF)	PEDF_MOUSE	19,4	2
Ceruloplasmin precursor	CERU_HUMAN	16,8	2
Ceruloplasmin precursor	CERU_SHEEP	16,4	2
Serine protease inhibitor A3K precursor	SPA3K_RAT	15,4	2
Actin, cytoplasmic 1	ACTB_CERPY	15,1	2
Apolipoprotein A-IV precursor	APOA4_RAT	14,4	2
Macrophage metalloelastase precursor	MMP12_HUMAN	14,4	2
Fibrinogen beta chain precursor	FIBB_RAT	14,0	2
Ig kappa chain C region.	KAC_HUMAN	11,4	2
Complement component C9 precursor.	CO9_RAT	10,1	1
Ig gamma-2 chain C region.	IGHG2_HUMAN	9,3	1
Liver carboxylesterase 1 precursor	EST2_RAT	9,1	1
Ig alpha-2 chain C region.	IGHA2_HUMAN	9,0	1
Hibernation-specific plasma protein HP-55 precursor	HP55_TAMSI	8,8	1
Fetuin-B precursor	FETUB_RAT	8,7	1
Ig heavy chain V region MOPC 47A.	HVM17_MOUSE	7,7	1
Apolipoprotein A-II precursor	APOA2_RAT	7,5	1
NMDA receptor-regulated 1-like protein	NARGL_HUMAN	7,0	1
Fibrinogen gamma chain precursor	FIBG_MOUSE	6,9	1

Although the amount of high-confidence hits (when excluding "one-hit wonders" and interspecies duplicates) is relatively low, the total intensity on non-specific binding is rather high when evaluating the chromatogram in figure 10. One interesting observation is the presence of 2 MMP-12 peptides, which is likely the result of carry-over from previous experiments. This underlines the importance of system washing between runs, which has to be further optimized to avoid making false conclusions about the presence of MMPs in biological samples.

Table 6b:
Proteins identified using the Mascot algorithm in an extraction performed on 100 µL rat urine

Protein	Protein ID	score	unique peptides
Ig gamma-1 chain C region	IGHG1_human	144	4
Alpha-1-antiproteinase	A1At_rat	135	4
Complement C3	CO3_rat	133	4
Ig gamma-2 chain C region	IGHG2_human	109	2
Plasminogen	PLMN_rat	107	4
Alpha-1-antitrypsin	A 1AT_human	103	3
Serine protease inhibitor	SPA3N_rat	90	1
Apolipoprotein A-IV	APOA4_rat	83	3
Serum albumin	ALBU_rat	81	2
Serotransferrin	TRFE_rat	69	2
Pigment epithelium-derived factor	PEDF_human	61	1
Serine protease inhibitor A3L	SPA3L_rat	61	2
Complement component C9	CO9_rat	61	1
Ceruloplasmin	CERU_human	58	1
Ig heavy chain V region MOPC 47A	HVM17_mouse	56	1
Ig alpha-1 chain C region	IGHA1_human	55	1
Complement C3	CO3_human	53	1
Fibrinogen beta chain	FIBB_rat	50	1
Annexin A2	ANXA2_human	50	1

Actin, aortic smooth muscle	ACTA_rat	49	1
Glyceraldehyde-3-phosphate dehydrogenase	G3P_human	48	1
Liver carboxylesterase 1	EST2_rat	47	1
Transthyretin	TTHY_rat	47	1
Macrophage metalloelastase	MMP12_human	46	1
Ig lambda chain C regions	LAC_human	45	1
Ig kappa chain C region	IGKC_human	43	1
Fibrinogen gamma chain	FIBG_rat	42	1

There are several options to reduce interference: A) Analyse the same sample on both an immobilized inhibitor cartridge as well as an (ethanolamine-inactivated) control cartridge and to use statistical classifiers to discriminate between peptides that are exclusively present or significantly increased in the inhibitor extracted sample. B) Optimize the loading and washing conditions to minimize non-specific binding. Earlier optimizations had the goal to maximize the binding of MMPs but this may have also lead to binding of non-related proteins. The choice for Sepharose as carrier material for the affinity SPE step was based on the fact that this material showed the lowest non-specific interaction with biomolecules when compared to a range of silica-based or polymeric materials.
The occurrence of this non-specific binding that is visualized by mass spectrometry and the fact that Sepharose as a non-pressure stable material is not ideal for online SPE has been the initiator for a search for pressure-stable materials with comparable properties to Sepharose with respect to non-specific interactions with proteins. Several supports were tested, but all displayed higher non-specific binding than Sepharose. One material, as described in offline experiments described in the previous chapter (ResQ aldehyde functionalized polymer with a hydrophilic coating from ResQ labs, Nijeveen, The Netherlands) seems to have favourable properties and may be a promising candidate for further testing.

Another factor in MMP analysis that has surfaced as an important limitation when applying functional proteomics to clinical and other biological samples is the importance of proper handling and storage[15]. Since the described method depends on the presence of functional active protease, in contrast to earlier activity-based techniques such as zymography that also work when no activity is left in the sample because of the renaturation and reactivation step in the procedure, it is critical that all steps between the collection of the sample and the analysis are optimized with respect to maintaining enzyme activity. Alternatively, it may be best to collect a given sample directly on immobilized inhibitor beads to avoid further inactivation or degradation. Experiments to evaluate these options are presently underway.

Conclusions

The integrated system for activity-based analysis of MMPs described in this chapter is an improved version of the system developed by Freije *et al.*[12]. The robustness and ease of use of the LC-MS component of the system was improved by replacing the capillary monolithic HPLC column and the in-house modified nano-ESI interface by a standardized microfluidic HPLC chip. Incorporation of a strong anion exchange column prior to the chip not only enabled transfer of a rather large volume of trypsin-digested proteins to the chip enrichment column, but also to remove surfactants that are necessary to keep the proteases in solution during the initial steps of the analysis. The improved automated analytical system showed a concentration sensitivity for MMP-12 catalytic domain that is within the expected biological high picomolar to low nanomolar range. Sluijter *et al* have for instance demonstrated that the level of MMP-2, -8 and -9 in atherosclerotic plaques lies around 5-10 ng/mg plaque biopt which would correspond to around 75-150 fmol/mg[16] and Demedts *et al* found up to 200 ng/mL (equals 10 nM of the fully processed form) of MMP-12 in induced sputum from patients suffering from COPD[17]. The question that remains to be answered is of course which fraction of this total protein amount is in fact the proteolytically active form, but the literature on quantitative analysis of active metalloproteases is very limited at best.
The introduction of automated LC-MS^2 with data-dependent precursor ion selection opens the possibility of profiling unknown active metalloproteases.
Incorporation of a stable isotope labelled enzyme as internal standard demonstrated that the system gives quantitative results with a reproducibility of +/- 20% relative standard deviation in peak area. This technique is of course dependent on the availability of a suitable stable isotope labelled enzyme, but it remains to be investigated whether using one or two internal standards is sufficient to quantify a wide range of metalloproteases, provided that the stable isotope labelled peptides correct for variations along the entire length of the chromatogram.

A factor that limits the use of this complicated, integrated analytical system on a routine basis at this stage of development is its limited robustness and the high level of expertise that is needed in its daily operation. While the start-up time is relatively long, the system is able to run at least 14 samples per day in an automated fashion, making this a viable approach for small clinical studies and target discovery. An advantage is that the method is not targeted or biased towards one metalloprotease when using broad-spectrum inhibitors such as TAPI-2 or ML5. It may thus lead to new insights into the roles of relatively unknown MMPs in a given biological process or pathology. The major challenge that needs to be overcome to prove the apllicability of this approach to clinical samples is the selection of suitable samples. While many clinical samples are available for analysis, conservation of proteolytic activity is often not taken into account in long-term storage of patient samples. Collaboration in early phases, and setup of clinical samples seems pivotal for future research. The uncertainty whether the concentration sensitivity reached in this study is

sufficient to identify active metalloproteases as marker for a given disease can only be solved by testing the system on diseases where the concentration of MMP in a certain biofluid is known to be rather high, combined with a high likelyhood of involvement of MMP activity in the disease pathology. The desired sensitivity may still lie around a factor 10 lower than at present for widescale application, the suggested cut-off value for MMP-9 in urine as a marker for bladder cancer for instance is reported to be 0.51 ng/mL, which would correspond to 6 pM for the mature form[18]. By continuing condensation of the analysis system on microfluidic devices (for instance inclusion of the trypsin reactor on-chip) the relative loss of analyte that is inevitable in bioanalysis may be reduced. A further step that may be taken is selective mass spectrometric detection of the analyte when the MMP of interest is identified. Targetted MS/MS analysis or multi reaction monitoring is capable of maximazing the signal for a certain peptide. The application of suitable internal standards for profiling of multiple MMPs is a further issue that has to be considered. While it is theoretically possible to produce stable isotope labeled versions of any MMP, in practise this would amount to a disproportionate amount of work, and severely complicate profiling analyses. Approaches generally applied in proteomics like ICAT and iTRAQ labeling procedures for semi-quantitative work is likely not suitable for functional proteomics since the labeling procedure itself may have a detrimental effect of proteolytic activity, making enrichment by inhibitor-affinity difficult. Perhaps taking the middle road is advisable, by taking one or two stable isotope labeled active MMPs which are know to yield tryptic peptides that span the chromatographic range and use those peptides to correct for peptides resulting from all MMPs.

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Chapter 6

Conclusions and future perspectives

This thesis describes the development of novel methods for activity-based proteomics on zinc-dependent metalloproteases. The functional proteomics field can roughly be divided into two fields. The first approach uses labelling of active enzymes with functionalized probes (usually based on small synthetic inhibitor of the protease of interest) followed by detection or visualization via a fluorescent dye, radioactivity or a biotin moiety on the probe. The second approach focuses on selective enrichment of active proteinases by using immobilized protease inhibitors which can be used to fish out the protease of interest from an aqueous sample followed by detection or identification by Western blot or mass spectrometry. Both approaches were explored within this thesis.

The development and testing of two novel photoactivatable probes for activity-dependent labelling of metalloproteases is described in chapter 3. The structure of the probes was based on optimization of the peptide-like backbone, yielding a novel inhibitor with a high inhibition efficacy even at low nanomolar concentrations (the basic structure for the probes was reversible inhibitor ML5). Addition of a trifluoroazirine photoreactive group for covalent crosslinking of the probe to the active site of the metalloprotease did decrease the affinity towards the tested metalloproteases (as tested by IC_{50} determination) but the inhibition constants were still within the nanomolar range. The photocrosslinking approach was chosen since, contrary to for instance serine and cysteine proteases the proteolytic cleavage is not mediated by an amino acid residue in the active site, but by an activated water-zinc (II) complex which is coordinated in the active site by three histidine residues. This makes labelling of the metalloproteases more difficult as is the case for the other protease classes, which are readily labelled by probes that attack the intramolecular nucleophilic group that is part of the active site residue (serine or cysteine). The latter approach has yielded several highly successful probes that are now at the point of being used in clinical research (see chapter 2 for references). The photocrosslinking approach is somewhat controversial since the labelling process is not initiated by actual proteolytic activity of the enzyme, but rather by irradiation with UV light. This makes the reaction possibly less efficient and less selective, and some investigators consider the technique not to be truly activity-based. On the other hand, since the labelling of both MMPs and ADAMs using the two novel probes has been demonstrated to be inhibited by TIMPs in a concentration dependent manner gives an indication that the interaction is at least dependent on the availability or accessibility of the catalytic cleft. This may be considered as a measure of activity since the catalytic pocket is not accessible in active metzincins, as it is shielded by the prodomain in the inactive zymogen, and by TIMP in the inhibited form. Preincubation of the enzymes with non-functionalized versions of the probes revealed that even a moderate excess was sufficient to inhibit labelling which is an indication that the labelling is site specific. Since the UV-mediated activation of the trifluoroazirine moiety and subsequent covalent attachment to the protein is by nature non-selective this efficient competition shows that there is at least a structural interaction which positions the probe in a certain part of the enzyme prior to UV irradiation, effectively limiting the possible binding sites. To assess the actual amino acid residue where the probe binds some experiments were performed to determine the labelling site by mass spectrometry.

Recombinant enzymes were labelled with the photoactivatable probe ML22, subsequently digested with trypsin and the tryptic peptides were analysed with LC-MS/MS. One problem that arose during the first attempts was the excess presence of unlabeled peptides, which seemed to indicate a low labelling efficiency. Experiments were further complicated by the primary structure of the proteases themselves, since the tested MMPs and ADAMs have a very low abundance of lysine and argentine residues in the catalytic region the resulting tryptic peptides containing the putative binding site are very large. This makes identification and sequencing by mass spectrometry challenging. The actual binding site has not been identified to date, but experiments are ongoing.
Some attempts have been made to create an actual mechanism-based probe for the MMPs using the structure of the N-terminal generic MMP cleavage sequence Pro-Leu-Gly with a crosslinking group that is activated by cleavage and yields a highly reactive quinolimine methide group after proteolysis. The first generation inhibitor did unfortunately not show any covalent labelling of MMP-12 (data not shown).

Although the first probe ML22 performed well in labelling ADAM proteases, and was actually the first ABP described in literature capable of labelling ADAMs, labelling of MMPs was considerably less efficient. Since an ideal probe for profiling purposes has a broad selectivity attempts were made to improve the labelling of MMPs by transfer of the photocrosslinking moiety from the P'2 site to the P'1 site. The resulting inhibitor PPG3 demonstrated a family-wide selectivity in labelling both MMPs and ADAMs and labelling of the latter was even more efficient than for ML22. Attempt to identify the labelling site with mass spectrometry using the novel inhibitor probe are ongoing.
Since in both probes the labelling of ADAM-10 was exceptionally strong and active ADAM-10 is known to be present in A549 cellular lysate (see figure 1 where the activity-based extraction method was used, followed by Western blot detection of ADAM-10) labelling of endogenous ADAM-10 followed by pull down of biotinylated (endogenous or labelled) proteins with immobilized streptavidin beads was performed. Western blot analysis of the resulting extract showed that in the labelled fraction of the lysate a small extracted band was visible, corresponding to the molecular size of active ADAM-10, while in the non-incubated control lysate no extraction was observed. As observed in the results of ADAM-10 extraction by inhibitor ligand pull down the extraction of the mature form of ADAM-10 from cellular lysates is never complete (compare figure 1, considering the enrichment effect in the elution fractions by a factor of~8). This may imply that a considerable amount of ADAM-10 is present in an inactive, presumably TIMP-inactivated form. This finding is in line with the anticipated role of ADAM-10 in constitutative shedding events leading to a continuous turn-over of the protease. The low labelling efficiency in the lysate by the photoactivatable probe may be explained by this.

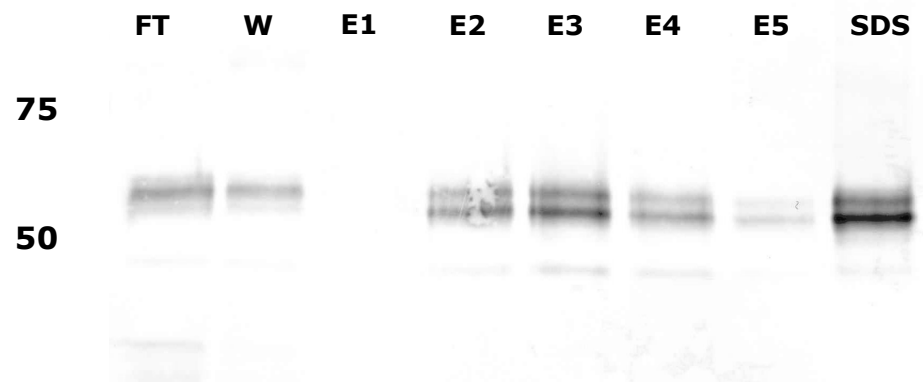

Figure 1:
Western blot of cartridge-extraction of an A549 lysate ($2.5x10^6$ cells) on an immobilized ML5 cartridge FT: flowthrough; W: wash fraction; E1-3: 30 µL elution fractions 10 µM ML05; E4-5: 30 µL elution fractions with 10 mM EDTA; SDS: final wash with SDS-PAGE sample buffer; 8% SDS-PAGE, immunoblotted with anti-ADAM10 ectodomain antibody (R&D systems).

As demonstrated in chapter 4, the activity-based enrichment technique can be used for extraction of active ADAM-17 from a complex proteome (lung carcinoma cell lysate). This report was the first example of application of this technique to ADAM proteases, with earlier being demonstrated for MMPs (recombinant MMP-12 and endogenous gelatinases in synovial fluid). The synthetic work on the first generation reversible inhibitors in Leiden resulted in the availability of an enantiomerically pure building block and the possibility to generate a large library of reversible hydroxamate-based inhibitors with variable amino acid residues in the peptide backbone. The amino acid residue on both the P'2 and P'3 position were demonstrated to have a substantial effect on the inhibition potential of the tested MMPs (MMP-9 and -12) and ADAM-17, with many inhibitors having a strong inhibitory effect on MMP-12, while some inhibitors had a preference for the gelatinase MMP-9 and one was found to exhibit the strongest inhibition towards ADAM-17. The introduction of a primary amine group attached to a spacer or linker enables the immobilization to a solid support by simple chemistry and the use of the novel material for activity-based solid phase extraction of metalloproteases from aqueous samples. The sample chosen for proof of principle was the lung carcinoma cell line A549, and inhibitor FF was selected since optimization experiments demonstrated that the extraction yield of ADAM-17 was still high even at lower concentrations of enzyme. By applying this screening technique, suitable inhibitors for immobilization may be selected and tested prior to application to biological samples, and may improve the extraction results (screening procedure is discussed in chapter 2).
The SPE approach coupled to immunochemical detection by Western blot has proven to be applicable to analysis of endogenous metalloproteases, and attempts have been made to apply the technique to analysis of clinical samples. Sputum samples from an incidental

cigarette smoke exposure study (courtesy of Dr. N. ten Hacken, University Medical Center Groningen) were analyzed for the presence of active MMP-12. Although the sputum of some volunteers contained considerable amount of MMP-12, and the putative active 22 kDa isoform was detected on Western blot, no detectable extracted amount was observed in EDTA elution fractions. This may indicate that the majority of the MMP-12 is in fact complex with one of the endogenous inhibitors (TIMPs) or that prolonged storage of the samples has deteriorated the active protease. Analysis of the samples for MMP-9 revealed a strong presence of both the zymogen as the mature isoform of this protease in the original sample, but only a very minor extracted amount, which was unexpected since the original study [1] revealed an excess of MMP-9 over TIMP-1 in some of the selected samples.
The issue of sample storage on MMP activity has just begun to be explored. First reports indicate that even storage at -80ºC under normal circumstances can have a detrimental effect after time[2]. This implies that for preservation of (dilute) samples containing metalloprotease activity care has to be taken. Addition of immobilized inhibitor beads immediately after sample acquisition may help to trap and enrich the active proteases for further storage. This approach may also overcome the problem of autoproteolytic degradation of MMPs as observed for e.g. MMP-9. Experimental work on this procedure is underway at present.
The online analysis system described in chapter 5 is still a work in progress. Although the system has performed rather well in term of reproducibility and sensitivity for MMP-12 the identification of endogenous active MMPs has proven difficult. Several issues appear to have to be dissolved. Firstly the level of non-specific interaction that, although low when considering the high protein load on the extraction cartridge, is still at a level that makes identification and detection of very diluted active proteases difficult. Secondly sample handling has to be strictly regulated to conserve MMP activity prior to analysis. At present a study is underway where rat urine samples were incubated with the immobilized inhibitor beads immediately after sampling. A further project is planned focusing at use of alternative solid supports for the inhibitor. Preliminary results show that non-specific binding of proteins (e.g. albumin) in a novel functionalized solid support (ResQ) is comparable to Sepharose, but the pressure stability and solvent tolerance may lead to an improved washing procedure.

When ADAM activity is to be analyzed some additional problems have to be faced. Since the majority of ADAMs is membrane-anchored in their mature, active form they require solubilisation prior to analysis by, for instance activity-based SPE. The usual method to achieve this is the use of (non-denaturing) surfactants in the lysis buffer, an approach that has been used throughout total proteome preparation within this thesis. Although many surfactants are reported to be non-denaturing, they may have an effect on protease activity as demonstrated by a decreased proteolytic activity of recombinant ADAM-17 in the presence of deoxycholate and Brij-35 (data not shown). Negative effects of surfactant in analysis are also described in chapter 3, where the photocrosslinking efficiency of probe ML22 in the presence of the commonly used detergent Triton X-100 was found to be

dramatically reduced. Using CHAPS in stead of Triton was demonstrated to be sufficient to overcome this problem.
When preparing total cell lysates, besides the obvious precautions like storing the samples on ice, addition of a protease inhibitor cocktail is advisable to avoid proteolytic degradation of the proteome by intracellular endopeptidases. Commercially available inhibitor cocktails usually contain inhibitors of all major subclasses of mammalian endopeptidases (serine, cysteine and aspartic proteases, aminopeptidases and depending on the cocktail a broad-range metalloprotease inhibitor such as EDTA). The cocktail we used in early development of the activity-based extraction method in preparation of A549 lysate was from Merck, and when used at the recommended dilution contained the inhibitors in the concentration described in table 1. It was observed that addition of this cocktail had a negative effect on enrichment of active ADAM-17 from A549 lysate, and some experiments were carried out to assess the effect of the individual protease inhibitors on. One observation that is immediately clear from table 1 is that the concentration of the individual inhibitors at the recommended dilution is far higher than the minimal effective concentration, possibly giving rise to non-specific inhibition effects. As shown in an assay evaluating the effect of the used concentration of each individual inhibitor on the activity of recombinant ADAM-17 and the total proTNFα convertase activity in A549 lysate (measured by conversion of the standard "ADAM-17"-fluorogenic substrate from R&D systems) most protease inhibitors have a small effect of proteolytic activity of the recombinant enzyme, but the cleavage of the substrate is almost completely abolished in lysate containing the recommended concentration of the serine protease inhibitor AEBSF (see figure 2).

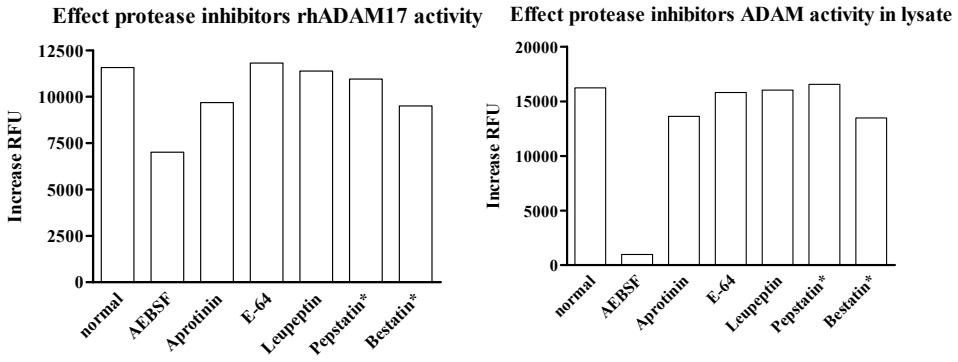

Figure 2:
*Residual proteolytic activity of rhADAM17 and endogenous metalloproteinase activity in an A549 lysate in the presence of individual components of a standard protease inhibitor cocktail for mammalian cells (Merck). Concentration protease inhibitors: AEBSF 4 mM; Aprotinin 3.2 μM; E-64 50 μM; Leupeptin 80 μM; Pepstatin 60 μM; Bestatin 160 μM. *: corrected for inhibition caused by solvent methanol.*

Table 1:
Composition of the Merck protease inhibitor cocktail for mammalian cells without EDTA. For comparison the effective concentrations described in literature are included.

Protease inhibitor	Target protease class	Concentration in cocktail	Effective concentration
AEBSF	Serine	4 mM	< 1 mM
Aprotinin	Serine	3.2 μM	0.09 – 0.3 μM
Bestatin	Metallo-aminopeptidase	160 μM	1 – 10 μM
E-64	Cysteine	50 μM	1 – 10 μM
Leupeptin	Serine & Cysteine	80 μM	10 – 100 μM
Pepstatin	Aspartic	60 μM	1 μM

The inhibitory effect of AEBSF on total ADAM activity was reduced to around 20% at 0.5 mM, and to evaluate whether this decreased activity in cell lysate is caused by inhibition of post-lysis activation of ADAM zymogens by proprotein convertases like furin two proprotein convertase inhibitors were tested. The irreversible proprotein convertase inhibitor decanoyl-RVKR-chloromethylketone and the competitive furin inhibitor hexa-D-arginine were shown to both inhibit the activity of recombinant TACE directly, but had a stronger effect on the proteolytic activity in cell lysate. These results illustrate the complexity of the biological system regulating ADAMs that has to be considered, and effort may be required to further investigate if the contribution of post-lysis zymogen conversion is existent and has a significant effect on levels of active metalloproteases.

The experiments described in this thesis have demonstrated that activity-based profiling of ADAM proteases is possible, but practical limitations are obvious. Since in-situ ADAM activity is likely to have a better correlation with disease development and progression several groups have attempted to develop probes for in vivo imaging using near infrared fluorescence technology (recently reviewed in[3]). We evaluated the base inhibitor structure of ML5 as a potential probe for in vivo imaging by linking of 99m-technetium as a radiolabel, resulting in probe ML23. The freshly prepared ^{99m}Tc-inhibitor structure was evaluated in a binding assay to the human bronchial epithelial cell line 16HBE 14o$^-$ (a kind gift from Dr. D.C. Gruenert, University of California, San Francisco) and the effect of a competitive inhibitor (ML5) on the binding of the probe. 16HBE cells release TNFα upon stimulation which is an indication for ADAM activity on the cell surface, and this release can be (partially) blocked by 10 μM inhibitor ML5, indicating this inhibitor is suitable for competition regarding ADAM binding on living cells (figure 3). Figure 4 shows that the binding of the radioactively labelled ML23 to the cells is reversible by ML5, indicating that the interaction is selective. Further work on this probe is of course needed to demonstrate suitability for in vivo imaging (like pharmacokinetic profiling in animals) but these

preliminary results show this approach may be worthwhile pursuing, as is being planned in future work.

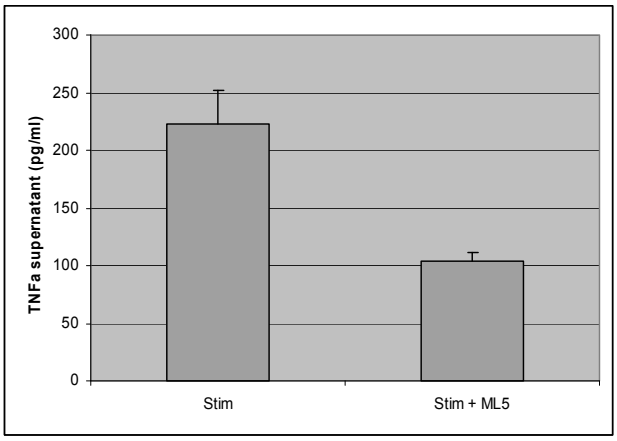

Figure 3:
TNFα production by 16HBE after combined stimulation with PMA and LPS and the inhibitory effect of metalloprotease inhibitor ML5. TNFα is measured in the supernatant of the cells by ELISA (Sanquin).

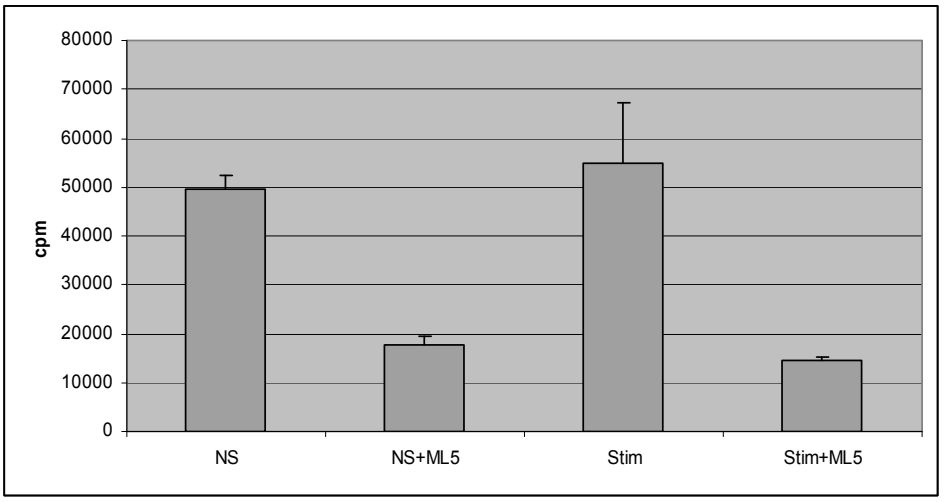

Figure 4:
Radioactivity on 16HBE cells after incubation with ^{99m}Tc-ML23

On interesting question that was attempted to be tackled by using activity-based SPE was whether ADAM-33, a protein that is likely involved in asthma pathology, is present in its active form in lung epithelia cells. Cartridge extraction of A549 total cell lysates on an immobilized TAPI-2 cartridge followed by Western blot detection revealed a staining pattern similar to that observed by Powell in fibroblasts[4], with selective enrichment of some of the lower molecular size bands on the TAPI-2 cartridge as opposed to the ethanolamine control cartridge. The reproducibility of these results with other columns and immobilized inhibitors was poor, leading to an impossibility of drawing distinct conclusion, but these findings may be worth further investigation, especially since the biological role of ADAM-33 involvement in asthma and bronchial hyperresponsiveness may involve disturbed cellular adhesion by mutations in the disintegrin- or cysteine-rich domains but also dysfunctional activity of the metalloprotease domain.
In conclusion the work described in this thesis shows that functional proteomics on zinc-dependent proteases is a challenging field, an observation that is corroborated by the very limited amount of literature that has been published on this subject to date. The activity-based SPE method seems to have crystallized to a point that it may be applied to the first clinical studies, but care has to be taken with respect to sample handling and storage. Gaining actual functional insight in metalloprotease activity in diseases may be essential in evaluating the role of these enzymes in development and progression of disease, and profiling results may aid pharmacological intervention by identifying (novel) targets and follow up of the effects of therapy.

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Nederlandse samenvatting

Metalloproteinases zijn proteolytisch actieve enzymen die voor hun werking afhankelijk zijn van een metaalion in de actieve site. Dit proefschrift richt zich op de analyse van een subfamilie van zink (II) bevattende proteinases, de metzincins. Deze familie bestaat uit vier subgroepen, de matrix metalloproteinases (MMPs), de membraangebonden *A Distintegrin And Metalloproteinases* (ADAMs), de ADAMs met een thrombospodine motief (ADAMTSs) en een restgroep die proteinases zoals de meprins en de bacteriële serralysines bevat.

De belangrijkste biologische functie van de metzincins is het reguleren van de cellulaire omgeving, de extracellulaire matrix (ECM). Dit gebeurt enerzijds direct door proteolytische afbraak van ECM eiwitten zoals collageen, en anderzijds door het vrijmaken van biologisch actieve eiwitcomponenten, een proces dat *shedding* wordt genoemd. Het bekendste voorbeeld van dit laatste proces is het vrijmaken van de oplosbare vorm van de cytokine TNFα uit de membraangebonden pro-vorm door ADAM-17 of *TNF alpha converting enzyme* (TACE).

De proteolytische functie van de metzincins wordt sterk gereguleerd *in vivo*. De eiwitten komen tot expressie als inactieve enzymen (zymogenen) en worden pas functioneel na activatie door autoproteolytische activatie, of activatie door andere proteinases zoals furine. Naast deze transcriptionele regulatie heeft het lichaam de beschikking over een viertal endogene remmers, de *tissue inhibitors of metalloproteinases* (TIMPs) die ongewenste actieve MMPs en ADAMs inactiveren.

Bij verstoring van deze protease-remmer balans kan de excessieve protease activiteit zorgen voor pathologische processen zoals destructie van weefsels, ontstekingsprocessen, ongewenste remodelling van weefsels en ontstaan en uitzaaiing van tumoren.

Door de hoge mate van regulatie kan een eenvoudige analyse van de expressie van een metalloprotease (bijvoorbeeld met kwantitatieve PCR) of een bepaling van de totale hoeveelheid eiwit een vertekend beeld geven. Om dit probleem op te lossen zijn diverse analytische methoden ontwikkeld om selectief actieve proteinases te detecteren (zie **hoofdstuk 2**). Veel van deze methoden zijn gebaseerd op het monitoren van de omzetting van een endogeen of synthetisch substraat van het proteinase waarnaar onderzoek gedaan wordt. Een veelgebruikte variatie op dit thema is de zymografie; een substraat voor de proteinase wordt in een SDS-PAGE gel gegoten waardoor na electroforese en renaturatie van de eiwitten een proteolytische afbraak optreedt op de plaat waar de protease zich bevindt. Ook zijn activiteitsafhankelijke ELISA assays opgezet waar de proteinase uit het monster gevist wordt met een antilichaam waarna de activiteit kan worden bepaald door een geschikt substraat toe te voegen.

Alle technieken die gebaseerd zijn op substraatomzetting zijn enerzijds elegant omdat de daadwerkelijke activiteit van de proteinases gemeten wordt, maar voor profileringsdoeleinden is deze aanpak minder geschikt. Aangezien er aanzienlijke overlap in substraatspecificiteit bestaat toont men in deze assays alleen de totale activiteit van een subfamilie aan, en is identificatie van de individuele enzymen niet mogelijk. Bij de

activiteitsafhankelijke ELISA is weliswaar het doeleiwit bekend, maar wordt ook slechts een enkele proteinase gedetecteerd.
Om een daadwerkelijke profilering van de actieve proteinases in een monster te kunnen bereiken is begin deze eeuw begonnen met het ontwikkelen van de *activity-based proteomics* (ABP) waarbij de specifieke interactie van synthetische remmermoleculen met de katalytische site van een actief enzym wordt gebruikt om onderscheid te kunnen maken tussen actief en niet-actief (zymogeen en geremde actieve vorm).
Deze aanpak bleek succesvol voor diverse proteasefamilies zoals de cysteine- en serineproteases, maar door het afwijkende proteolytische mechanisme (gemedieerd door het metaal ion dat een watermolecuul activeert) was voor de metalloproteinases nog geen succes behaald.
Een mogelijke aanpak voor de activiteitsafhankelijke analyse van metalloproteinases wordt geschreven in **hoofdstuk 3**. Een nieuwe synthetische MMP-remmer, gebaseerd op een peptidenstructuur gekoppeld aan een zinkbindende hydroxamaat groep, werd uitgerust met een lichtreactie groep die zorgt voor een covalente binding van de remmer aan het enzym na bestraling met UV licht. Doordat de remmerstructuur ook een biotine groep bevat kan het gelabelde eiwit zichtbaar worden gemaakt met behulp van een biotine-streptavidine conjugatie. Deze zelfde interactie kan eveneens worden gebruikt om de gelabelde eiwitten uit het monster te vissen en na verrijking verder te analyseren.
De resultaten beschreven in hoofdstuk 3 laten zien dat de eerste generatie probe een goede labeling laat zien van recombinant actief ADAM-9, -10 en -17, maar de labeling van de geteste MMPs bleef achter. De interactie tussen de probe ML22 en de ADAMs is selectief voor de actieve vorm van de proteinases, aangezien na denaturatie of preïncubatie met de endogene remmer TIMP-3 geen labeling meer optreedt. Ook preïncubatie met een kleine overmaat niet-gebiotinyleerde remmer laat een concentratieafhankelijke afname van de labeling zien, een indicatie dat de covalente crosslinking op een specifieke plaats in het eiwit plaats vindt.
Labeling van eiwitten in een complex proteoom zoals een cellysaat van gekweekte longkankercellen laat een beperking zien van deze techniek. Aangezien de detectie is gebaseerd op visualisatie van biotine worden ook alle endogeen gebiotinyleerde eiwitten zichtbaar die in veel hogere concentratie aanwezig schijnen te zijn. Door een vergelijkbare probestructuur uit te rusten met een fluorescent (BODIPY-TMR) label te gebruiken kon dit probleem worden vermeden, maar de gevoeligheid van detectie was lager, iets dat problemen kan opleveren bij de vermoedelijk zeer lage concentraties waarin de metalloproteases aanwezig zijn.
Om een probe te verkrijgen die zowel MMPs als ADAMs labelt is de lichtreactieve groep op een andere plaats in de remmerstructuur geplaatst. De probe met de crosslinker op de P’1 positie in plaats van de P’2 positie bleek een goede labeling te geven van zowel recombinante MMPs als ADAMs en de labeling lijkt effectiever te verlopen bij deze nieuwe probe (PPG3). Competitie-experimenten door preïncubatie met TIMP-1 en de niet-gebiotinyleerde remmer laten zien dat ook de labeling van MMPs (zoals getest voor MMP-9 en MMP-12) selectief is voor de actieve vorm van het eiwit, en op een specifieke positie plaatsvindt.

Hoofdstuk 4 beschrijft een tweede mogelijke aanpak voor activiteitsafhankelijke proteomics van metalloproteases. Door reversibele remmermoleculen met een zeer hoge affiniteit te immobiliseren op een dragermateriaal kan een ligandmateriaal dat selectief is voor actieve proteinases gecreëerd worden. Dit materiaal kan vervolgens worden gebruikt om de actieve fractie niet alleen te scheiden van de niet-actieve eiwitten, maar ook om de actieve metalloproteinases te verrijken. Deze verrijking lijkt noodzakelijk voor detectie en vooral kwantificering omdat de concentratie aan actief proteinase in veel gevallen uitzonderlijk laag zal blijken te zijn.
In dit hoofdstuk wordt de evaluatie van een set van 96 nieuwe remmers beschreven. Door de aminozuren in de peptidenstructuur van de remmer te variëren kunnen de remmers worden geoptimaliseerd voor een hoge affiniteit ten opzichte van de proteinases waarin men geïnteresseerd is. Een aantal interessante remmers is verder geëvalueerd waarbij enkele structuren een zeer goede remmingefficiëntie bleken te hebben met IC_{50} waarden onder de nM. Na immobilisatie bleek dat een lage remmingsconstante niet per definitie een garantie is voor een goede extractieopbrengst, waaruit te concluderen valt dat voor een efficiënte verrijking van de actieve metalloproteases optimalisatie op het niveau van de daadwerkelijke extractie, en dus de geïmmobiliseerde remmerstructuur wenselijk is.
Eén geselecteerde remmer is gebruikt voor de extractie van endogeen ADAM-17 uit een complex proteoom, een cellysaat van A549 long carcinoma cellen. Na detectie door middel van Western blot liet de remmerextractie een verrijkte band van de actieve vorm van ADAM-17 zien terwijl een controlemateriaal zonder geïmmobiliseerde remmerligand geen extractie gaf. Door een stimulatie van de A549 cellen met een phorbolester te volgen met extracties op verschillende tijdspunten konden we laten zien dat de activatie van ADAM-17 niet veroorzaakt wordt door een verandering van de hoeveelheid eiwit, maar waarschijnlijk een translocatie van actief enzym dat in een pool aanwezig is in rustende cellen. Na enkele uren stimulatie scheen de hoeveelheid actief ADAM-17 weer af te nemen, een observatie die bekend is uit de literatuur voor de totale hoeveelheid ADAM-17.

In **hoofdstuk 5** wordt de automatisering van de in het vorige hoofdstuk beschreven activiteitsafhankelijke extractie beschreven. Door het geïmmobiliseerde remmermateriaal in een cartridge te gieten kan het worden gebruikt in een systeem voor automatische *solid phase* extractie (PROSPEKT II). Door deze extractie te koppelen aan een in-line digestie van de geëxtraheerde proteinases met een trypsine reactor kan een volledig gesloten monstervoorbewerkingssysteem gecreëerd worden dat gekoppeld kan worden aan een geschikte detectiemethode. In dit hoofdstuk wordt de koppeling van het extractieplatform aan een vloeistofchromatografiechip met electrospray ionisatie gekoppeld waardoor koppeling aan een maasspectrometer en daarmee identificatie van de geëxtraheerde eiwitten, mogelijk is. Door de introductie van een verrijkingskolom met een anionenwisselaar kunnen de tryptische peptiden die uit de trypsine reactor komen opgevangen worden terwijl storende componenten zoals oppervlakteactieve stoffen uit het monster verwijderd worden. Elutie met een geconcentreerde oplossing van een vluchtig

zout (ammoniumacetaat) gecombineerd met een pH switch van basisch naar zuur bleek een goede elutie te geven van de anionenwisselaar naar de HPLC chip.
De model MMP, MMP-12 is gebruikt voor het evalueren van het analysesysteem, en om te compenseren voor variatie is als interne standaard ^{15}N-gelabeld MMP-12 toegevoegd aan elk monster. Door deze toevoeging kon een goede lineariteit worden bereikt en werd een detectielimiet onder de nM mogelijk. Het spiken van recombinant MMP-12 in diverse biologische monsters zoals urine (menselijk en rat) en long lavage vloeistof liet zien dat er geen storende matrix effecten optreden in het MMP-12 signaal, een indicatie dat de monstervoorbewerking effectief is.
De applicatie van dit systeem op klinische en biologische monsters heeft tot op heden geen identificatie van endogene metalloprotease opgeleverd. Dit wordt enerzijds veroorzaakt door de aanwezigheid van sterk geconcentreerde niet-metalloprotease eiwitten die ondanks de sterke verrijking van de actieve metalloproteinases nog steeds een sterk signaal geven in de massaspectrometer en laat anderzijds het belang zien van de behandeling van de monsters voor de analyse. Zaken als vries-dooi cycli, monsters tijdelijk bewaren bij kamertemperatuur en de lage endogene concentratie kunnen de aanwezigheid van actieve metalloproteinases op den duur zeer negatief beïnvloeden.
Hoofdstuk 6 beschrijft een aantal projecten waar preliminair werk aan is verricht, en wat in een volgend project verder uitgediept zou kunnen worden. De grootste beperking van de probes zoals beschreven in de voorgaande hoofdstukken is het feit dat er alleen een indirect beeld ontstaat van de situatie in het lichaam. Door vergelijkbare remmermoleculen met een radioactief (bv. 99m-technetium) label te synthetiseren kunnen de probes eventueel gebruikt worden voor *in-vivo* beeldvorming van metalloprotease activiteit en nuttig zijn om bijvoorbeeld actieve tumoren op te sporen.
Dit hoofdstuk beschrijft verder een aantal experimenten die aantonen dat de monsterbehandeling cruciaal is in het correct analyseren van de actieve metalloproteinases in biologische monsters. De standaard proteaseremmer cocktail die wordt toegevoegd aan cellysaten om degradatie van eiwitten tegen te gaan blijkt een negatief effect te hebben op de activiteit en ABP analyse van ADAM-17, ondanks de afwezigheid van metalloproteaseremmers. Ook de keuze voor het detergens dat wordt gebruikt om de membraangebonden eiwitten in oplossing te krijgen tijdens cellysis bleek een groot effect te hebben op de activiteit van ADAM-17, waarbij Triton X-100 als een veilige keuze gevonden werd in concentraties onder de 1%.

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