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Functional Proteomics on Zinc-Dependent Metalloproteinases using Inhibitor Probes

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Metzincins are a family of zinc(II)-dependent metalloproteinases with well known members such as the matrix metalloproteinases (MMPs) and A disintegrin and metalloproteinases (ADAMs). Metzincins are largely responsible for the modulation and regulation of the extracellular matrix by proteolytic degradation of extracellular matrix (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 members of this superfamily that have been described most fully are the matrix metalloproteinases (MMPs) and A disintegrin and metalloproteinases (ADAMs).

The main physiological role of MMPs lies in the regulation of the extracellular matrix (ECM) by promoting turnover through direct proteolytic degradation of ECM proteins, such as collagens, or by the production of bioactive signaling proteins through proteolytic activation; MMP biological function has been extensively reviewed in the literature, for instance in Ref. [1]. ADAMs have a bimodal function; they mediate cell–cell and cell–matrix interaction through integrin binding to their disintegrin domain. More than half the known human ADAMs contain a catalytic domain that enables modulation of the cellular environment by activation of signaling pathways through proteolytic release of bioactive proteins from their membrane anchored pro-form. This ectodomain shedding is well described for many ADAMs 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 ADAMs see Ref. [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 center 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 center 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 osteoarthritis 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 remodeling in heart failure and gastrointestinal diseases linked to TNF (such as inflammatory bowel syndrome). ADAM-33 has been implicated in airway diseases for example, asthma and emphysema, although it is not yet known if this is due to an altered proteolytic activity.

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Activity-Based Metalloproteinase Analysis

The majority of hypotheses associating metzincins to disease states have been focused 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 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 1980s. Table 1 gives an overview of the available "traditional" methods for measuring metalloproteinase activity. The earliest methods (preceding the identification and nomenclature of the MMPs) were first described in the 1950s and 1960s, and are based 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 incor-

Table 1. Overview of analytical methods to determine metzincin activity.

	Technique	Advantages	Limitations
Monitoring of substrate conversion.[5]	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.
Gelatine/ collagen zymography.[6]	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.
Conversion of biotinylated gelatine.[4]	After degradation by gelatinases monobiotinylated 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 accessible for most labs.	Limited by substrate specificity. Gives only the total gelatinase activity in the sample.
Fluorogenic substrate conversion.[7]	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.
Activity-based ELISA assays.[8,9]	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 individual MMPs. Limited profiling possibilities.
MMP/TIMP ratio.[10,11]	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 proteinase 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.

porated in an SDS-PAGE gel, which after visualization 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 monobiotinylated fragments after proteolytic degradation that can be distinguished from the original polybiotinylated substrate in a rather elegant two-step capture–labeling procedure)[4] or small synthetic peptide sequences with internally quenched fluorophores. These substrates are readily available for most MMPs and some of the better studied ADAMs, and give the opportunity for the 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 proteinases.

Finally activity-based analysis of metalloproteinases may be based on the hypothesis that metzincin-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 found within an aqueous sample do not necessarily reflect the situation within the tissue. Therefore, parallel studies are often performed using tissue samples and histological staining techniques. This problem is particularly important in the case of ADAMs, since many of the ADAMs are cell membrane anchored molecules that are not easily released into biological fluids. Another problem is the lack of specificity of TIMPs for individual metzincins, 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 therapeutic targets, attempts at producing synthetic MMP inhibitors have been made, especially in the field of oncology. Several compounds have entered clinical trials, but ultimately failed to reach the clinic due to undesirable side effects or a lack of efficacy in phase II/III. This failure rate can be largely attributed to the strong homology between catalytic sites within the MMP and ADAM families. The majority of the first generation inhibitors lacked selectivity. This lack of selectivity was probably responsible for the observed side effects and the lack in clinical efficacy, since it has become clear that some metalloproteinases, for instance MMP-8, exert a protective effect against development and metastasis of tumors.[12]

Most MMP inhibitors synthesized to date are based on a zinc(II)-chelating group fitted onto a peptide or peptidomimetic backbone mimicking the endogenous protein substrate of the proteinase. Following the Schechter and Berger nomenclature,[13] three inhibitor types can be distinguished: inhibitors with a peptide sequence N-terminal (P_1–P_3) or C-terminal (P'_1–P'_3) to 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 flanking backbone.

The "N-terminal" approach has proved less than 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 center of the molecule are being investigated,[14] the most popular approach has been the synthesis of inhibitors that interact at the "C-terminal" side of the catalytic zinc ion.

Several functionalities can be used as effective ZBGs, for instance carboxylic acid derivatives, thiol groups and, phosphonates, and can also be used to modulate selectivity.[15] However, 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 atom of the highly conserved alanine residue (Ala 182 in MMP-1).[16]

The strong interaction of the hydroxamate group with the catalytic Zn^{2+} ion, combined with the homology of the catalytic center 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 Ref. [17]). Although variation at other positions can also lead to differences in inhibition specificity,[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 essential to develop analytical methods to profile the activity of MMPs and ADAMs on a family-wide scale in clinical samples so that their respective roles in disease can be assessed, and to follow the activity of individual members during therapy.

Activity-Based Probes (ABPs) 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. 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. Should

an inhibitor have affinity for (almost) all known metzincin proteinases in the human body, it may be used as a ligand for selective analysis of active metzincin proteinases. This approach, termed activity-based or chemical proteomics, was pioneered by Bogyo, Ploegh, and Cravatt, developing probes for cysteine proteinases,[20] the proteasome[21] and serine hydrolases,[22] respectively. 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 visualization and enrichment of labeled, active proteinases from complex proteomes (Figure 1).

Considering that the catalytic mechanism of metalloproteinases is somewhat different to 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 ADAMs[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 photo-cross-linking 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.

Sieber and Cravatt recently succeeded in coupling this approach to mass spectrometric identification of a labeled proteinase by enriching ABPP-labeled 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 cross-linked agarose beads) can give rise to loss of protein due to nonspecific interaction (unpublished observations). An additional problem with photo-cross-linking probes is the fact that the cross-linking step is not a quantitative process, and may be influenced by many experimental factors. This limits the use of this technique to semiquantitative 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, the potential for their use in creating a platform to enrich active metalloproteinases can be envisaged. 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 an 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 led to the analysis of active gelatinases in synovial fluid from a rheumatoid arthritis patient[29] and the finding that membrane-type 1 MMP (or MMP-14) was present in its active form in breast carcinoma tissue but not in tissue from benign breast tumors.[30] Figure 2 shows a schematic representation of the process of activity-dependant enrichment using immobilized reversible inhibitors.

Since this technique is essentially 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 in-

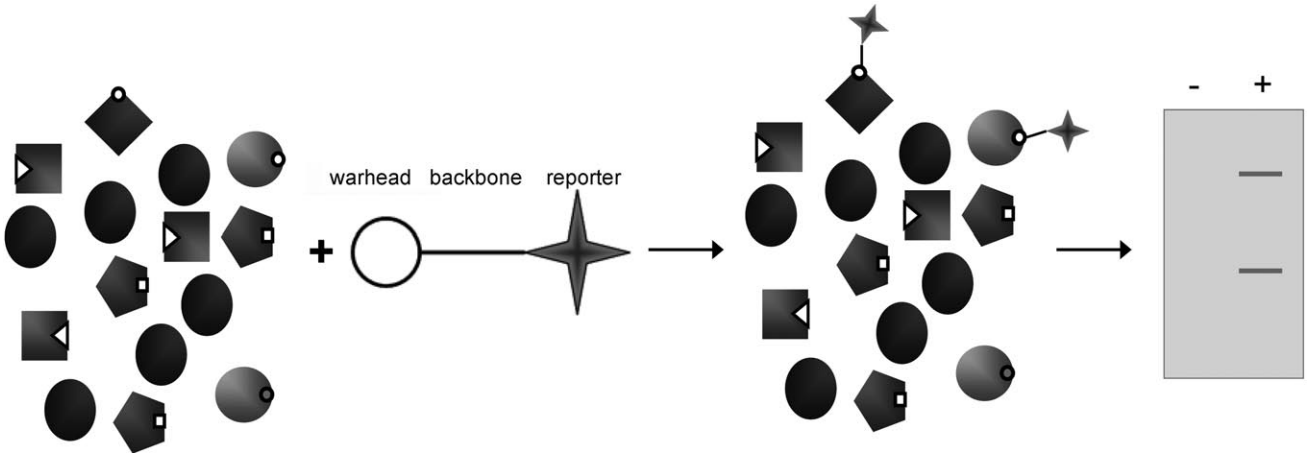
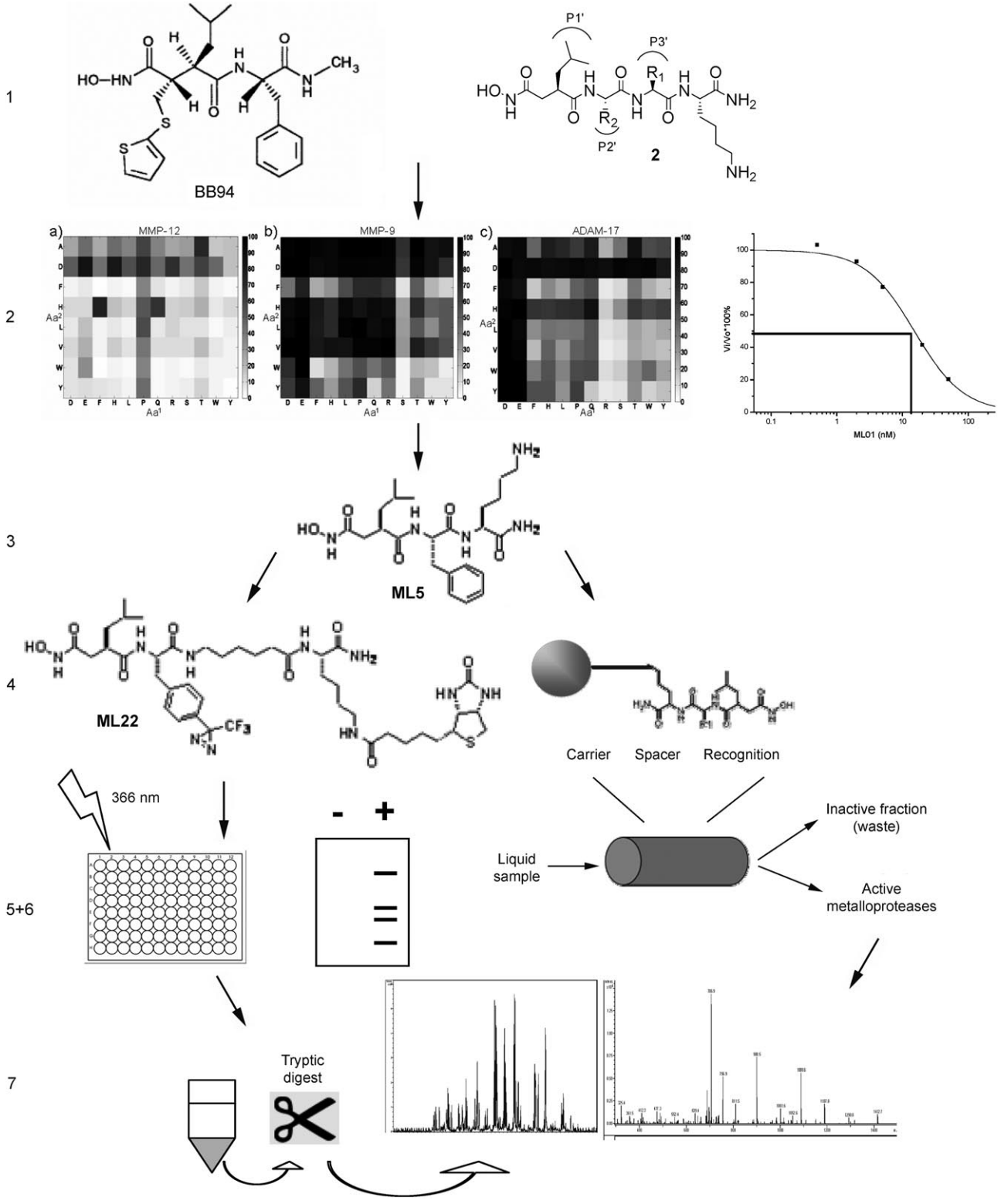


Figure 1. Schematic representation of labeling of active proteinases in a complex proteome by activity-based probes. The probe selectively recognizes the active site of the proteinase and forms a covalent bond with the active enzyme. By incorporation of a reporter molecule, such as a fluorescent dye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



Scheme 1. Schematic protocol for functional proteomics of metalloproteinases using inhibitor probes. 1) Selection of a suitable available inhibitor (e.g. BB94), or synthesis of novel inhibitors (e.g. compound **2**). 2) Screening of the inhibitory profile using a panel of recombinant metalloproteinases. 3) Selection of the most appropriate inhibitor (e.g. for profiling or selective tagging/enrichment). 4) Conversion of inhibitor to a suitable structure for activity-based proteomics. a) Synthesis of activity-based probe based on the selected inhibitor by introducing photo-cross-linker and a reporter molecule. b) Immobilization 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 proteinases. a) Labeling of samples of interest with the activity-based probe and visualization of active metalloproteinases after gel electrophoresis. b) Extraction of samples of interest with the immobilized inhibitor and visualization of enriched active metalloproteinases by Western blot. 6) Isolation and enrichment of active proteinases from biological samples. a) Enrichment of photo-labeled proteins with immobilized streptavidin. b) Extraction of active proteinases from a biological sample by activity-based solid phase extraction. 7) Tryptic digestion of enriched proteins and mass spectrometric identification.

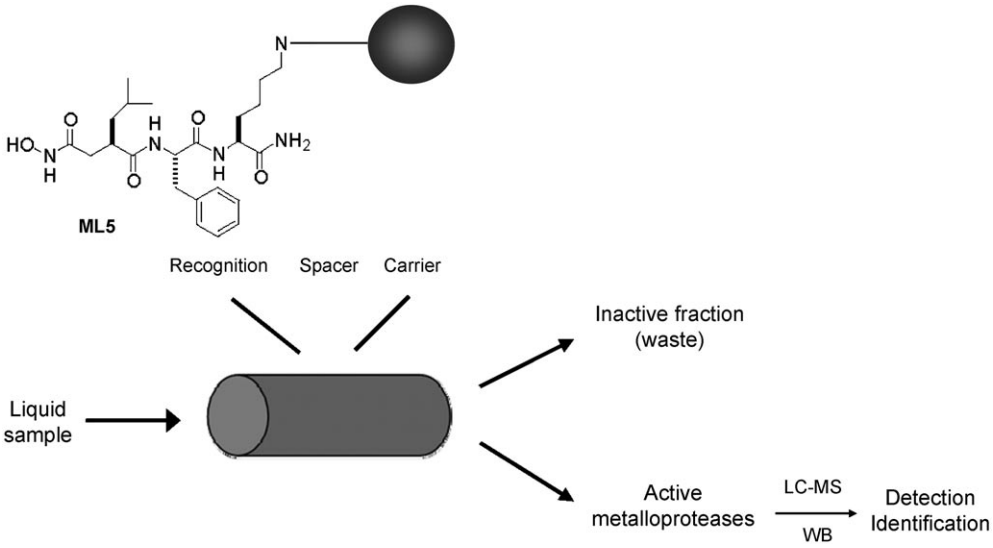


Figure 2. Schematic representation of activity-based solid-phase extraction using immobilized inhibitors as affinity ligands. A suitable inhibitor is immobilized on a solid support (e.g. Sepharose) and packed into a column. Aqueous samples containing active metalloproteinases are pumped over the column, 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 analyzed by immunological techniques (Western blot; WB), or processed for protein identification by liquid chromatography–mass spectrometry (LC–MS).

hibitor, 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 immobilization. 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]

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 fluorescence-labeled MMPs was evaluated leading to better optimization of inhibitor selectivity. By evaluating the binding profiles of catalytically active MMPs and inactivated enzymes, the specific interaction of the inhibitor with the enzyme can be determined and distinguished from non-specific binding.

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 Scheme 1, 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.

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