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Mechanism-Based Profiling of MMPs

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Abstract

The recognition that the successful clinical use of MMP inhibitors will require quantitative correlation of MMP activity with disease type, and to disease progression, has stimulated intensive effort toward the development of sensitive assay methods, improved analytical methods for the determination of the structural profile for MMP-sub-type inhibition, and the development of new methods for the determination – in both quantitative and qualitative terms – of MMP activity. This chapter reviews recent progress toward these objectives, with particular emphasis on the quantitative and qualitative profiling of MMP activity in cells and tissues. Quantitative determination of MMP activity is made from the concentration of the MMP from the tissue, using immobilization of a broad-spectrum MMP inhibitor on a chromatography resin. Active MMP, to the exclusion of MMP zymogens and endogenous TIMP-inhibited MMPs, is retained on the column. Characterization of the MMP sub-type(s) follows from appropriate analysis of the active MMP eluted from the resin. Qualitative determination of MMP involvement in disease can be made using an MMP sub-type-selective inhibitor. The proof of principle, with respect to this qualitative determination of the disease involvement of the gelatinase MMP-2 and MMP-9 sub-types, is provided by the class of thiirane-based MMP mechanism-based inhibitors (SB-3CT as the prototype). Positive outcomes in animal models of disease having MMP-2 and/or -9 dependency follow administration of this MMP inhibitor, whereas this inhibitor is inactive in disease models where other MMPs (such as MMP-14) are involved.

Keywords

Activity-based protein profiling (ABPP); hydroxamate small molecule microarrays; affinity chromatography resin

1. Overview

The optimism that non-selective inhibitors of the MMP family would possess clinical antitumor and antimetastatic activity (1–9) has given way to the sober realization that the relationships among the expression of MMP sub-type, the different roles of the extracellular matrix as an MMP substrate, the inhibitor structure, and the temporal and spatial evolution of the cancer are extraordinarily complex (10–20). As this realization has developed over the past decade, experimental enquiry concerning the MMP family has increasingly addressed the *selectivity* and *specificity* aspects of this complexity: Which MMP sub-types are culpable, and can this information be used for diagnosis? What sub-type selectivity should an MMP inhibitor possess? For which cancers, and at what times during the clinical progression of these cancers, is intervention with an MMP inhibitor therapeutically useful? In no circumstance are the answers yet complete. In many circumstances, however, the

inchoate answers affirm the opinion that the MMPs remain valid therapeutic targets for the amelioration of disease (not just cancer, but also including inflammation, atherosclerosis, CNS, and cardiovascular diseases). For example, the involvement of MMP-2 and -9 (both gelatinases) and MMP-7 (matrilysin) in colorectal cancers (21) indicates these MMP activities as possible biomarkers for disease progression (22–26). Moreover, Massagué et al. have validated (by RNA interference) the cooperative action of an EGFR ligand, COX-2, MMP-1, and MMP-2 in human breast cell metastasis using implanted tumors in mice (27) and have replicated interference of this cooperation using a combination of EGFR and COX-2 inhibitors (28). The added value (to the paired EGFR and COX-2 inhibitors) of a broad-spectrum MMP inhibitor was less evident in this study (28), possibly due to the simultaneous antagonism of MMP-dependent inhibition of metastasis and of MMP-promotion of metastasis, by the broad-spectrum inhibitor (ilomastat, GM6001) that was used. *It is precisely this dilemma – a lack of specificity in the available MMP reagent – that is a stimulus to the current research continuing to examine the value of the MMPs as therapeutic targets.*

The experimental evaluation of enzyme selectivity may be made in terms of substrate or inhibitor profiling, coupled to an experimental method for assessment of the enzyme–substrate or enzyme–inhibitor recognition. The variety of approaches – both in terms of reagent and method of analysis – reported for the MMPs is astonishing. Among the questions addressed by these approaches were the following: How can substrate or inhibitor arrays be used to characterize the MMP sub-type? How can advanced analytical methods characterize the endogenous substrates recognized by the MMPs within cells? What new methods are available to quantify the amount of active MMP in the cell? In different but complementary ways, these approaches are efforts toward the improved and specific profiling of the presence and the catalytic character of MMP sub-types.

As the first three of the above four questions are more completely addressed in the companion chapters within this volume, we here only cite the recent advances in these areas. The ability to construct extraordinarily large peptide arrays for the determination of protease specificity (29) has been used by Thomas et al. (30) to validate an endopeptidase profiling library with MMP-12 and MMP-13. Overall et al. (31, 32) and Fields et al. (33–36) have examined MMP recognition of collagen as a triple helical peptide substrate. These concepts have been applied to new MMP inhibitor design (37). The methodologies for MMP assay (38) have expanded to include increasingly sophisticated methods for cell, tissue, and animal imaging (39–44). Among the recent methods reported for improved evaluation of MMP activity in vitro and in vivo are zymographic (45–47), Raman (48), IR (49–51), MRI (52–55), xenon NMR (56), radiochemical (57–62), PET (63–67), MMP-triggered magnetic nanoparticle self-assembly (68), and luminescent (40, 69–76) analyses. Increasingly refined MMP sub-type-selective substrates (for MMPs-1, -2, -3, -7, -8, -9, -10, -12, -13, and -14) for FRET fluorescent assay are commercially available (77). Using porous silicon photonic film overlaid (spin-coated) with label-free gelatin as an MMP-2 substrate, Gao et al. (78) described a highly sensitive and dose-responsive (detecting 0.1 to 1000 ng mL⁻¹ MMP in μ L drops) assay, which they estimated to be 100-fold more sensitive than standard MMP zymography and to fulfill the practical requirements for diagnostic MMP detection (rapid, simple, dose responsive, and inexpensive). With respect to the identification of endogenous

MMP substrates – a matter of increasing relevance, given the new appreciation of the MMPs as having both anti- and pro-angiogenic activity – Overall et al. have developed mass spectrometry methods to evaluate the cellular substrates (the MMP degradome) recognized by the MMPs (79–84).

2. Methods for MMP Activity Profiling

The final question is that of MMP profiling by the use of inhibitors. The foundational principle to this approach is the use of functional groups that target the catalytic zinc of these enzymes (ZBG, a zinc-binding group) placed into a peptidomimetic structure biased toward selective MMP recognition. The key aspect of this approach is the use of a ZBG-containing MMP inhibitor with intrinsic specificity. While considerable progress has been made toward the structure-based optimization of MMP inhibitor structure (85–89), the creation of MMP sub-type-selective structure is extraordinarily challenging and still remains largely vested in empirical experiment. For this reason, and also recognizing that one of the most powerful of the ZBGs (the hydroxamate) is easily incorporated by the standard methods of peptide array synthesis, large peptidomimetic inhibitor libraries (arrays) have been prepared for MMP profiling.

Yao et al. have developed hydroxamate inhibitor microarrays (small molecule microarrays, SMM) for rapid in vitro metalloprotease profiling (“fingerprinting”) (90–92). Their initial 1400-member library used a diversified tripeptide motif having an ilomastat-type β -substituted succinyl hydroxamate C terminus and a biotinylated N terminus to allow streptavidin capture. Comparative SAR (activity, specificity, potency, hierarchical clustering) was assessed with respect to inhibition of bacterial collagenase, carboxypeptidase, thermolysin, and anthrax lethal factor as enzyme targets. Detailed and complete protocols for the implementation of a 400-member biotinylated P_1' -leucine sub-set of this library for MMP-7 profiling (including a full description of the synthesis of the β -isopropyl-substituted succinyl hydroxamate warhead, the split-pool solid-phase peptide synthesis of the library, and the use of either microplate or microarray analysis) are described by the Yao group (93). A companion protocol (exploiting the P_1' -leucine-based succinyl hydroxamate warhead, but using Click-derived triazole diversification) is also described (94, 95). In this latter protocol, the β -substituted succinyl hydroxamate is coupled with propargylamine to generate the terminal alkyne necessary for subsequent Click diversification using azide-containing secondary binders. Direct screening against the target enzyme is done by microplate assay. Complementary hydroxamate inhibitor array efforts are described by Flipo et al. for screening against neutral aminopeptidase (96) and by Johnson et al. (97) for screening against anthrax lethal factor. Vegas et al. (98) describe the use of fluororous methodology for hydroxamate screening against histone deacetylase.

Cravatt et al. (99–102) describe the creation of inhibitor arrays for the activity-based enzyme profiling (ABPP) of metalloproteases. The objectives of the ABPP approach are the identification of an enzyme-selective inhibitor within the array and subsequent quantitative evaluation of the activity in tissue (crude proteomes) using the inhibitor. Absolute selectivity toward a particular enzyme is not necessary, as will be evident from this summary of the ABPP method. The array is based around an inhibitor structure that confers a structural bias

(such as a ZBG for the MMPs) for recognition as an inhibitor by the target enzyme. The ZBG is diversified by the addition of secondary (an amino acid) or tertiary (a dipeptide) structure. The probe structure is completed by the addition of a photoaffinity label to enable covalent linkage of the inhibitor to the target enzyme(s) and a biotin tag to enable recovery of the enzyme–inhibitor pair(s) from the crude proteome. Identification of the enzyme(s) is done by tandem mass spectrometry (MS/MS) assignment of the tryptic peptides obtained from the recovered enzyme. The power of this method was proven by MMP profiling (103–105). Specifically, Saghatelian et al. (103) identified GM6001 (ilomastat) – one of the early MMP inhibitors clinically evaluated for anticancer activity – as not merely a broad-spectrum MMP inhibitor but as an inhibitor within the neprilysin, aminopeptidase, and dipeptidylpeptidase metalloprotease families. Using β -monosubstituted and α,β -disubstituted succinyl hydroxamates as the zinc-binding group (ZBG), and benzophenone photoaffinity labeling, Sieber et al. (106) have compared relative expression of several subfamilies of the zinc metalloprotease superfamily (107, 108) in an invasive (MUM-2B) and in a non-invasive (MUM-2C) human melanoma cancer cell line. By using an alkyne-functionalized terminus in the inhibitor structure, to allow post-photoaffinity labeling addition of the biotin tag by Click derivatization, two enzymes of the zinc metalloprotease superfamily (alanyl aminopeptidase and neprilysin) were seen to be expressed in significantly greater amounts in the invasive cell line. The sensitivity of this assay for MMPs, determined by progressive addition of MMP into a constant background of proteome, was approximately 0.25–2.5 μg MMP per milligram of proteome using gel-based detection. LC/MS-MS detection of the MMP improved the sensitivity of MMP detection (compared to the gel assay method) by 5–50-fold. Sieber et al. (106) make several important observations concerning the implementation of affinity probes for proteome analysis. Separate steps for photoaffinity labeling of the enzyme–inhibitor complex, and subsequent incorporation of the functional group to be used for detection (such as biotin or a fluorophore), gave probes with better performance than did probes having the detection group pre-incorporated into the structure. The reason for this is the significant modification to the inhibitor structure by large mass of the detection group itself, as opposed to the small and (otherwise) unreactive alkyne terminus used for Click-based addition of the detection group in the two-step tag incorporation method. Moreover, Cravatt et al. emphasize the necessity of the control experiment to establish the proteome background (non-specific protein binders). For Click-based incorporation of the detection group, the control used is the cognate probe structure, wherein the alkyne functional group is replaced by an alkane functional group. The alkane-substituted probe will bind to the target, but cannot participate in the Click functionalization, and hence the target enzyme should not appear in avidin pull-down. For example, in the assay of the MMPs, one MMP sub-type – MMP-14 – was found to be problematic for its non-specific appearance. The Cravatt laboratory has given detailed protocols describing the further refinement of this activity-based protein profiling method, wherein the N-terminal biotinylated tag is separated from its C-terminal azide by an octapeptide spacer (109). This octapeptide encodes the unusual Gln-Gly cleavage site of the tobacco etch virus (TEV) protease. This method – termed tandem orthogonal proteolysis (TOP)-ABPP – involves photoaffinity labeling of the enzyme by the probe, biotin tagging of the probe–enzyme complex by Click azide–alkyne cycloaddition, avidin capture, trypsin digestion, and final TEV release of the covalently labeled tryptic peptide (by the

photoaffinity label) for LC/MS-MS identification (109). The implementation of TOP-ABPP has not yet been applied to the MMPs.

Overkleeft et al. (110) have independently reported the solid-phase synthesis of MMP hydroxamate inhibitors for use in MMP profiling. Complete synthetic methods are given for the synthesis of the P₁'-leucine-based succinyl hydroxamate library, the incorporation of a trifluoromethyldiazirene photoaffinity label, and the addition to the N terminus of either a boratriazaindacene fluorophore or a biotin tag. In vitro validation (successful photoaffinity labeling) of the most potent inhibitor using MMP-12 (IC₅₀ = 4 nM) and ADAM-17 (IC₅₀ = 21 nM) was shown by strong streptavidin labeling of the ADAM-10 band in the SDS-PAGE gel.

A conceptually identical, but experimentally quite different, approach to the determination of active MMP is that the cell or the tissue uses an MMP inhibitor covalently attached to an insoluble support (resin). The objective – the determination of the MMP sub-type and the total activity of that sub-type present in the cell – is identical to that of the ABPP method. The advantage of covalent immobilization of the inhibitor is its ability to capture *and* concentrate the MMP, enabling the use of different assay methods for the characterization of the MMP (111–114). Two implementations of this approach have recently been described for the MMPs. Hesek et al. prepared an MMP capture resin based on the attachment of a structurally modified, broad-spectrum hydroxamate MMP inhibitor to a Sepharose resin (115). Examination of the structure of the MMP–batimastat complex indicated solvent exposure of the thienyl ring. Replacement of this thiophene with a cysteine-like thiomethyl substituent enabled attachment of the modified inhibitor to epoxy–Sepharose, using the thiolate of the modified inhibitor to open the epoxide functional group of the resin. The structure of batimastat and the batimastat-type affinity resin is shown in Fig. 27.1a. This resin has high capacity (0.4 μmol of ligand per gram of dry resin). Extensive in vitro validation of this resin indicated specificity for recovery of active MMP-2 and MMP-9. Neither the zymogen form of these enzymes nor the TIMP complex of these enzymes is retained by the resin. The flow-through of active MMPs from the resin is negligible, and the resin-bound active enzyme is stable to extensive column washing. Release of the enzyme from the resin is accomplished with reducing SDS sample buffer, or with buffer containing marimastat (also a broad-spectrum hydroxamate MMP inhibitor). Validation of the resin with biological samples, using tissue extracts prepared from human breast and laryngeal carcinomas, recovered both MMP-2 and MMP-14 from the extracts (whether additional MMPs were present was not determined). The successful application of this resin to the recovery of active MMP-2 present in breast and laryngeal carcinomas has been thoroughly described, using gelatin zymography as a readout of the free MMP activity in these tissues (116). Additionally, this same resin captured the TIMP-free MMP-14 present in detergent-free breast carcinoma extracts (117). Zucker and Cao (114) provide an excellent perspective on the importance of the selective determination of active MMP in tumor tissue, using MMP inhibitor-tethered resins, to the understanding of the role of the MMPs in the tumor microenvironment and to the development of improved MMP inhibitor therapy.

The enrichment of active MMP from biological samples is also described by Bischoff et al., also using Sepharose immobilization of an alternative broad-spectrum hydroxamate

(TAPI-2) inhibitor of the MMPs (118). A free amine at the N terminus forms a stable amide bond with NHS–Sepharose to give the affinity resin (Fig. 27.1b). Elution of the MMP from the resin uses an EDTA-containing elution buffer. Subsequent reports from the Bischoff laboratory provide extensive validation of their TAPI-2 resin (119, 120). Control experiments indicate that this resin efficiently captures and concentrates MMPs-1, -7, -8, -10, -12, and -13 with extraction yields exceeding 96%. Otherwise undetectable quantities of MMP-9 in synovial fluid, obtained from a patient with rheumatoid arthritis, were enriched sufficiently to allow facile gelatin zymography detection of the MMP-9 activity (119). The interface of the TAPI-2 column with an immobilized tryptic reactor (thus allowing online tryptic digestion) enabled MS detection and analysis of the tryptic peptides of recombinant MMP-12-spiked urine (120). The performance of this resin indicates high concentrative ability of the MMP from a biological fluid and high sensitivity of the MMPs (using MMP-12 as the analyte). MMP-12 at picomole levels was detected easily (using 0.5 mL urine containing 8 nM MMP-12) (120). This result indicates the likelihood that this method could be developed for MMP-12 detection as a possible biomarker for malignant bladder cancer.

The methods for the chemical synthesis of both the Heseckmodified batimastat resin and the Bischoff TAPI-2 resins are fully described. Moreover, detailed protocols for the liquid-phase chemical synthesis of very closely related inhibitors are given by Yao (93, 95) and for solid-phase synthesis are given by Overkleeft et al. (110). Nonetheless, all of these syntheses are labor intensive. The Heseck resin uses a neutral thioether functional group for the attachment of its ligand to the resin. The use of a neutral (uncharged) linker is known to minimize the non-specific capture of proteins by the resin itself acting as an ion-exchange resin. Preliminary experiments with a Heseck resin that uses the same structure but with a carboxylate ZBG, instead of a hydroxamate ZBG, are equally successful toward MMP capture from tissue. While the carboxylate ZBG typically gives less potent inhibitors than does the hydroxamate ZBG, its use as an affinity ligand may further improve MMP selectivity during these extractions, by suppression of non-specific enzyme capture. The TAPI-2 structure used in the Bischoff resin is commercially available but is very expensive. TAPI-2 attaches to NHS-activated Sepharose, which has a non-neutral (albeit weakly basic) linker between the caproate NHS active ester and the Sepharose. Hence, appreciable limitations remain for both the ABPP and the affinity resin approaches to MMP profiling. The former method requires access to a mass spectrometer capable of peptide MS/MS analysis. The latter method requires extensive up-front organic synthesis for resin preparation.

A final method for MMP activity profiling is the use of the MMP-selective inhibitor. All of the methods discussed thus far use a hydroxamate ZBG within a peptidomimetic motif that confers selectivity, but by no means specificity, for the MMP zinc metalloproteases. The use of a non-selective inhibitor [such as ilomastat, as is used in the Cravatt ABPP (106); batimastat, as is used for the Heseck resin (116); or TAPI-2, as is used for the Bischoff resin (118, 120)] has the advantage of the simultaneous profiling of many MMP sub-types. There can also be no doubt that extension of the ABPP method to determine the total MMP inventory (active *and* inactive MMP) of a cell is well within the power of the ABPP methods, albeit with the requirement for sophisticated MS analysis. Whilst this analysis will

greatly increase our understanding of the MMP proteome (101) and complement our understanding of the MMP degradome (84, 121), this increased understanding does not directly connect to a chemical strategy for the selective inhibition of the MMP activity contributing to the disease. The power of the hydroxamate ZBG is its potency for zinc chelation and the ease of its incorporation by solid-phase synthesis into diverse peptidomimetic inhibitors of the MMPs. The limitation of the hydroxamate is its inadequacy as a functional group for drug development. As greater appreciation of the shortcomings of the hydroxamate as a ZBG has followed the abandonment of early generation hydroxamate MMP inhibitors as clinical candidates (5, 7), the search for more drug-compatible ZBGs has coincided with diminished interest in drug development against the MMPs. Nonetheless, there is an outstanding example of the power of a selective inhibitor for MMP activity profiling. The MMP inhibitor SB-3CT uses a thiirane (a three-membered, sulfur-containing ring), and not a hydroxamate, as a latent ZBG. Mechanism-based activation of the thiirane is accomplished only by the gelatinase (MMP-2 and MMP-9) MMP subclass (122). Moreover, the synthesis of members of the SB-3CT class is very straightforward (123). The gelatinase specificity of SB-3CT has allowed it to implicate gelatinase involvement in a number of animal models of human disease, including apoptosis following transient focal cerebral ischemia (124), T-cell lymphoma metastasis to the lung (125), retinal ganglion cell axon guidance (126), MMP-2 mediation of ethanol-induced invasion of mammary epithelial cells (127), β -adrenergic receptor-stimulated apoptosis in myocytes (128), prostate cancer metastasis to the bone (129, 130), and $A\beta_{(1-40)}$ -induced secretion of MMP-9 (131). Conversely, the ineffectiveness of SB-3CT in a model of collagen I invasion by ovarian cancer cell implicated MMP-14 involvement in this metastatic event (132).

A second non-hydroxamate ZBG has also been evaluated for possible MMP sub-type selectivity. Using a novel phosphinate insert, Dive et al. (133–137) have prepared peptidomimetic inhibitors with MMP-11 and MMP-12 selectivity. Using a high specific activity (8 Ci mmol^{-1}) ^3H -radiolabel, phosphinate peptidomimetics with an aryl azide photoaffinity label were synthesized. Following UV irradiation, 1D SDS-PAGE autoradiography identified the threshold detection quantity of MMP-12 to be 50 pg (2.5 fmol of MMP-12 at 100 pM concentration) (138). Among the other MMPs with similar active sites as MMP-12, MMP-2, -12, -13, and -14 were comparably labeled, while the labeling of MMP-3, -8, -9, and -11 was several-fold poorer (138). These data indicate that the level of MMP sub-type selectivity necessary for sub-type profiling has yet to be attained with this phosphinate insert.

3. Summary

The challenge of MMP activity profiling is being addressed by a convergence of improved synthetic methodology and increasingly more sophisticated analytical methods, with an increasingly better understanding of the complex roles of the MMPs in disease. The power of these methods, and the quality of the instructions to implement the methods, is evident. These methods are robust. They are not, however, routine. The enabling investment – whether in synthetic chemistry or in instrumentation – to perform MMP activity profiling is substantial. A substantial investment is always required to implement new technologies. The extraordinary breadth of the recent approaches, and the vigor of the inquiry, indicates

recognition of the importance that these technologies advance to a level of robustness *and* routine. Whether by the perseverance of the single laboratory or by multi-laboratory collaboration, the methods cited in this chapter will further elucidate the MMP activity profile. The value of this information for disease diagnostics, and as guidance to rekindle medicinal chemistry and pharmacological interest in MMP inhibition for the treatment of disease, cannot be underestimated.

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Fig. 27.1.

a. Structure of batimastat (*left*) and the structurally modified analog of batimastat (*right*) that is attached, as a broad-spectrum MMP affinity inhibitor, to epoxy-Sepharose as described by Heseck et al. (115, 116). **b.** Structure of marimastat (*left*) and the related TAPI-2 structure (*right*) attached to NHS-Sepharose as described by Freije and Bischoff (118).