Published in final edited form as:

Methods Mol Biol. 2017 ; 1626: 79–102. doi:10.1007/978-1-4939-7111-4_8.

Zymography as a Research Tool in the Study of Matrix Metalloproteinase Inhibitors

Zongli Ren, Juanjuan Chen, and Raouf A. Khalil

Vascular Surgery Research Laboratory, Division of Vascular and Endovascular Surgery, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Abstract

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade various components of the extracellular matrix (ECM) and play a role in tissue remodeling. Changes in MMPs have been observed in cancer, connective tissue disorders and vascular disease, and both endogenous tissue inhibitors of MMPs (TIMPs) and synthetic MMP inhibitors (MMPIs) have been evaluated as modulators of MMP activity in various biological systems. Zymography is a simple technique that is commonly used to assess MMP activity and the efficacy of MMPIs. Also, reverse zymography is a modified technique to study the activity of endogenous TIMPs. However, problems are often encountered during the zymography procedure, which could interfere with accurate assessment of MMP activity in control specimens, and thus make it difficult to determine the pathological changes in MMPs and their responsiveness to MMPIs. Simplified protocols for preparation of experimental solutions, tissue preparation, regular and reverse zymography procedures, and zymogram analysis are presented. Additional helpful tips to troubleshoot problems in the zymography technique and to enhance the quality of the zymograms should make it more feasible to determine the changes in MMPs and assess the efficacy of MMPIs in modulating MMP activity in various biological systems and pathological conditions.

Keywords

blood vessels; uterus; extracellular matrix; matrix metalloproteinase; TIMP

1 Introduction

Matrix metalloproteinases (MMPs) are structurally and functionally related Ca^{2+} -dependent and Zn^{2+} -containing endopeptidases that degrade the extracellular matrix (ECM) and connective tissue proteins [1–3]. Since its discovery, the MMP family has grown to 28 members in vertebrates, at least 23 in humans, and 14 in blood vessels [2]. Based on their activity toward specific substrates and the organization of their different domains, MMPs are classified into collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs, and other MMPs [4–6]. MMPs are secreted as inactive or latent pro-MMPs which

undergo proteolytic activation by other MMPs or other proteases before they can degrade ECM proteins [7] (Fig. 1). Activated MMPs play a role in vascular remodeling and angiogenesis [1], the uterine and vascular changes associated with pregnancy and preeclampsia [8], and many pathological conditions such as neoplasm, connective tissue disorders, and vascular disease.

Because there are no specific activators of MMPs, MMP inhibitors (MMPIs) are often used to test the role of MMPs in different processes. Also, in many tissues, MMP activity is modulated by endogenous tissue inhibitors of metalloproteinases (TIMPs) [9,10] (Fig. 1). TIMPs include four homologous members; TIMP-1, -2, -3 and -4 [11–13]. While all TIMPs can inhibit all MMPs, the efficacy of MMP inhibition varies with each TIMP. For example, TIMP-1 is a poor inhibitor of membrane type 1-MMP (MT1-MMP), MT3-MMP, MT5-MMP, and MMP-19 [12]. Also, TIMP-1 and -3 interact with pro-MMP-9 while TIMP-2, -3 and -4 interact with pro-MMP-2 [12]. Although TIMPs could restrict ECM deposition, their ultimate effect on ECM turnover depends on the TIMP/MMP ratio in the tissue. Other endogenous MMPIs include α 2-macroglobulin, a glycoprotein consisting of four identical subunits that is found in blood and tissue fluids and acts as a general proteinase inhibitor. Most endopeptidases are inhibited by being entrapped within the macroglobulin and the complex is then cleared by endocytosis via a low density lipoprotein receptor-related protein-1 [14].

In addition to endogenous MMPIs, several classes of synthetic MMPIs have been developed and evaluated as diagnostic and therapeutic tools in cancer, autoimmune and vascular disease [15] (Table). Early investigations on MMPIs focused on developing compounds that contain a group that chelates the catalytic Zn^{2+} ion and a backbone that mimics the natural peptide substrate of MMPs [16]. The first-generation hydroxamate-based MMPIs have the Zn^{2+} -binding group hydroxamate and the basic backbone of collagen [17]. Batimastat, a low-molecular-mass hydroxamate derivative, was the first MMPI to enter clinical trials [18], but the results were disappointing due to the metabolically labile nature of the hydroxamate Zn^{2+} -binding group, the metabolic inactivation and chelation of metal ions of other metalloproteins, and the serious side effects such as musculoskeletal pain experienced by patients [19].

Non-hydroxamate-based Zn^{2+} -binding MMPIs such as carboxylates, hydrocarboxylates, sulphhydryls, phosphoric acid derivatives and hydantoins are more stable and do not have the limitations associated with hydroxamate-based MMPIs. Rebimastat is a broad-spectrum MMPI that has a thiol Zn^{2+} -binding group [20,21]. Tanomastat has a thioether Zn^{2+} -binding group and a biphenyl deep-pocket-binding segment and is well-tolerated, but may show variable efficacies and outcomes depending on the timing of administration [22].

Mechanism-based MMPIs bind to the MMP active site and cause covalent enzyme modification. SB-3CT, a thiol-based inhibitor that contains a diphenylether deep-pocket-binding scaffold, is a mechanism-based selective inhibitor of MMP-2 and -9 through a process involving slow-binding inhibition similar to that of TIMP-1 and -2 [23,16,24]. SB-3CT directly binds the catalytic Zn^{2+} ion of MMP-2 and changes the conformation around the Zn^{2+} active site to that of the proenzyme [25].

Pyrimidine-based MMPIs include Ro 28-2653, an orally bioavailable MMPI that inhibits MT1-MMP, MT3-MMP, and MMP-2, -8 and -9 [26,27]. Phosphorous-based MMPIs have phosphinate as Zn^{2+} -binding group and include 582311-81-7 and the MMP11-selective inhibitor RXP03 [28,29]. Tetracyclines, such as minocycline and doxycycline, have innate MMPI capacity. Doxycycline is the only MMPI approved by the United States Food and Drug Administration, and is indicated for periodontal disease [30]. Metastat (COL-3) is a chemically modified tetracycline that has been tested in a Phase I clinical trial in patients with refractory metastatic cancer [31].

Because of the high structural homology of the MMP catalytic site, most of the early MMPIs show broad-spectrum effects on different MMPs. In order to reduce off-target effects of MMPIs, investigations have shifted from targeting the catalytic site to alternative sites in the MMP molecule. MMPs have unprimed subsites S1, S2 and S3 on the left side of the Zn^{2+} ion and primed S1', S2' and S3' on the right side of Zn^{2+} ion [32,33]. The S1' pocket is the main substrate recognition subsite and is the most variable among different MMPs in terms of amino acid sequence and pocket depth (shallow, intermediate and deep) [34,35,16,36]. These variabilities in MMP structure have been utilized to design more specific MMPIs. For example, extending the P1' substituent (the group in MMPI or substrate that binds to the S1' pocket of MMP) was used to enhance selectivity of MMP-13 over the highly homologous MMP-2 by taking advantage of the steric limitations of the shorter S1' loop of MMP-2 [37]. However, identifying alternative MMP-specific sites could be challenging as they are scattered in different locations on the surface and even hidden inside the MMP molecule. Combining structural spectroscopic analyses, nuclear magnetic resonance and protein crystallography with computational prediction of binding sites have helped to reveal these hidden sites and made it possible to design novel molecular effectors and therapeutic agents [38,39]. For example, peptide-based MMPIs interact with secondary binding sites on MMPs and thereby have greater selectivity [40]. Also, phage display peptide libraries have been used to identify selective MMP-2, MMP-9 and MT1-MMP inhibitors that are effective *in vivo* [41,42].

Antibody-based MMPIs have been designed to achieve high selectivity and potency [43]. For example, combining a human antibody phage display library with automated selection and screening strategies resulted in the identification of the highly selective antibody-based MMP14 inhibitor DX-2400 [44,45]. Also, the neutralizing monoclonal antibody REGA-3G12 is a selective inhibitor of MMP-9 directed against the catalytic domain but not the fibronectin or Zn^{2+} -binding domains [46,47]. Another strategy for generating inhibitory antibodies that effectively target the *in vivo* activity of dysregulated MMPs is mimicking the mechanism used by TIMPs [48].

While several MMPIs have been developed, only one MMPI is approved by the Food and Drug Administration [1]. This could be due to the numerous side effects of MMPIs and their lack of specificity in various MMP assays. In order to determine the role of MMPs in pathological conditions and the efficacy of various MMPIs, it is critical to have reliable methods for measuring MMP activity. Zymography is a simple technique first described in 1980 [49], and is now widely used to measure MMP activity in various systems. The zymography technique is based on separation of proteins by nonreducing sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Special polyacrylamide gel is made by inducing acrylamide polymerization in the presence of the specific substrate of the MMP(s) of interest. For instance, using gelatin as a substrate, zymography has been used to assess the activity of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [50]. During electrophoresis, MMPs are activated in a nonproteolytic manner by SDS [51]. Following electrophoretic separation and a “renaturation” step, the gel is incubated at 37°C in a Ca^{2+} and Zn^{2+} -containing buffer optimized for measuring MMP activity toward the specific substrate (Fig. 2).

If performed carefully and analyzed accurately, zymography can be a very valuable technique to measure MMP activity in various biological systems including plasma, cells, culture media, and tissue extracts [52,53]. Zymography has also been used to measure the changes in MMP activity in vascular remodeling and vascular disorders [3]. Also, reverse zymography is a modified technique to study the activity of endogenous TIMPs. However, problems encountered during the zymography procedure may lead to inaccurate assessment of MMP activity in control specimen, and thus make it difficult to determine the pathological changes in MMP activity and their responsiveness to MMPIs.

In this chapter, we will discuss the materials needed for a good zymography experiment, the preparation of experimental buffers, sample preparation, running and developing the gels during the regular and reverse zymography procedures, and zymogram analysis and interpretation. We will also provide helpful notes to troubleshoot problems and some tips to enhance the quality of the zymograms and assess the efficacy of MMPIs.

2 Materials

2.1 Materials for Zymography Experiment

The following materials should be prepared before starting a zymography experiment. All materials should be clean and stock solutions should be clear (see Note 1).

1. Glass plates with 0.75-mm-thick spacers and 10 well combs.
2. 30% w/v Acrylamide/bis-acrylamide (A/C) (Bio-Rad).
3. Separating gel buffer stock: 1.5 M Tris/HCl (Sigma), pH 8.8. Use 27.23 g Tris-base (Sigma) in 150 ml dH_2O , adjust pH to 8.8 with 38% HCl (Sigma).
4. Stacking gel buffer stock: 0.5 M Tris/HCl, pH 6.8. Use 6 g Tris-base in 100 ml dH_2O , adjust pH to 6.8 with 38% HCl.
5. 1% w/v gelatin (Sigma). Use 100 mg gelatin in 10 ml dH_2O . Warm the solution to 60°C in a water bath and vortex repeatedly until it becomes almost translucent (~20 min). Cool down the gelatin solution to room temperature before use.
6. 10% w/v SDS (Bio-Rad). Use 1 g SDS in 10 ml dH_2O .
7. 10% w/v ammonium persulfate (APS) (Sigma). Use 1 g APS in 10 ml dH_2O .
8. N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma).

9. Running buffer stock: For 1× running buffer use 0.024 M or 2.9 g Tris-base (Sigma), 0.192 M or 14.4 g glycine (Sigma), and 0.1% w/v or 1 g SDS in 1 liter dH₂O, pH 8.3. For 10× running buffer use 0.24 M or 29 g Tris-base, 1.92 M or 144 g glycine and 1% w/v or 10 g SDS in 1 liter dH₂O, pH 8.3. Remember to dilute the 10× running buffer 1:10 in dH₂O before use.
10. Sample buffer (2×): Use 2.5 ml of 0.5 M Tris/HCl pH 6.8, 2 ml glycerol, 4 ml of 10% w/v SDS (1 g SDS in 10 ml dH₂O), and 0.5 ml of 0.1% w/v bromophenol blue (Sigma) (0.001 g in 1 ml dH₂O). Add dH₂O to 10 ml. When ready to load the samples in the gel and depending on the protein concentration in the sample tissue homogenate, add one part of the sample to one part sample buffer.
11. Renaturing buffer. For 1× renaturing buffer (Triton X-100 2.5% v/v) add 5 ml Triton X-100 (Sigma) in 195 ml deionized dH₂O. For 10× solution stock (Triton X-100 25% v/v) add 25 ml Triton X-100 in 75 ml deionized dH₂O.
12. Developing buffer (1×): 50 mM or 6.06 g Tris-base, 0.2 M or 11.688 g NaCl, 5 mM or 0.555 g CaCl₂, 0.02% w/v or 0.2 g Brij35 (Fisher Scientific), and 1 μM or 0.136 mg ZnCl₂ (Sigma) in 1 liter dH₂O, pH 7.6.
13. Staining solution: 0.5% w/v or 2 g Coomassie blue R-250 (Sigma), 25% v/v or 100 ml isopropanol (Sigma), and 10% v/v or 40 ml acetic acid (Fisher Scientific) to 260 ml dH₂O. Filter through filter paper before use.
14. Destaining solution: Add methanol : acetic acid : dH₂O in the following proportion, 50 : 10 : 40. Final concentration 50% v/v methanol (Sigma) and 10% v/v acetic acid in dH₂O.
15. Homogenization buffer: 0.02 M or 0.418 g 3-[N-Morpholino]propanesulfonic acid (MOPS) (Sigma), 4% w/v or 4 g SDS, 10% v/v or 10 ml glycerol, and 90 ml dH₂O. Immediately before use, add the following solutions: 1.5 ml homogenization buffer, 35 μl of 50 mM ethylenediaminetetraacetic acid (EDTA) (Sigma), and 75 μl of 20× anti-protease cocktail.
16. Anti-protease cocktail (20×): To make 1.5 ml stock, use 0.4% w/v bovine serum albumin (BSA) (take 0.3 ml of 2% w/v or 2 g BSA in 100 ml dH₂O), 0.11 mM or 165 μl of 1 mM leupeptin, 0.11 mM or 165 μl of 1 mM pepstatin, 0.15 TIU or 60 μl of 7.6 TIU/ml aprotinin, 0.4 mM or 60 μl of 10 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 0.1 M or 750 μl of 200 mM ethylene glycol tetraacetic acid (EGTA) (Sigma).

2.2 Materials for Studying MMPIs using Zymography

1. Prepare all materials as for regular zymography.
2. Prepare stock solution for the MMPI of interest. For example, stock solution (10⁻² M) of SB-3CT (MMP-2/MMP-9 inhibitor IV) (EMD Millipore), Ro-28-2653 (5-biphenyl-4-yl-5-[4-(4-nitro-phenyl)-piperazin-1-yl]-

pyrimidine-2,4,6-trione, Roche Diagnostics) and Batimastat (BB-94, Tocris Bioscience) is prepared in dimethylsulfoxide (DMSO) (Sigma) [54].

2.3 Materials for Reverse Zymography

1. Prepare all materials as for regular zymography.
2. Prepare human recombinant MMP-2 or MMP-9 (0.13 µg/ml) (MyBiosource).

3 Methods

3.1 Gel Preparation

1. Prepare 8% A/C separating gel (for two 0.75mm gels) (*see Note 2*): 4.6 ml dH₂O, 1 ml 1% w/v gelatin (100 mg/10 ml) (*see Note 3*), 3.0 ml 1.5 M Tris/HCl (pH 8.8), 3.2 ml 30% A/C stock, 120 µl 10% w/v SDS (1 g/10 ml), 120 µl 10% w/v APS (1 g/10 ml).
2. Prepare 5% A/C stacking gel: 3.6 ml dH₂O, 1.56 ml 0.5 M Tris/HCl (pH 6.8), 0.6 ml 30% A/C stock, 20 µl 10% w/v SDS (1 g/10 ml), 140 µl 10% w/v APS (1 g/10 ml).
3. TEMED will polymerize the gel and should be added last to the freshly prepared separating and stacking gel, immediately before pouring into the cassette. Add 5 µl of TEMED to the separating gel solution to initiate the polymerization process. Swirl the solution rapidly and avoid bubble formation. Pipette 3 ml of the separating gel solution into each cassette, avoiding the formation of bubbles. Carefully, overlay the separating gel with dH₂O, filling up to the top of the cassette. Do not disturb the surface of the separating gel solution. Allow the gel to polymerize for at least 30 min at room temperature. The warmer the condition the gel is in, the less polymerization time. Do not touch the gels until polymerization is complete as indicated by a clear straight line of separation between the gel phase and the water phase.
4. Decant the water from the top of the separating gel and keep the gel upside down on bench top for a few minutes to drain away all the water. Use filter paper to absorb residual water.
5. Add 10 µl of TEMED to the stacking gel solution, swirl rapidly, and immediately pipette the solution on top of the already polymerized separating gel until it reaches the top of the front glass plate. Rapidly insert the appropriate comb (10 wells or lanes) into the liquid stacking gel and ensure that no bubbles are trapped under the comb. Allow the stacking gel to polymerize at room temperature (about 30 min). Place the gels (comb on top) in a sealed plastic bag or a container containing 1× running buffer to keep them moist. The gels can be stored at 4°C for up to 2–3 weeks before running.

3.2 Sample Preparation

1. Preparation of the biological sample is critical for a successful zymography. It is relatively easy to detect MMP activity in culture media and cell lysates.

However, analysis of MMPs in tissues such as the aorta or uterus could be difficult (*see* Note 4).

2. Weigh approximately 50 mg of the tissue of interest. Cut the tissue into small pieces in a small weighing boat on ice. Transfer the tissue to a tissue grinder or mortar (Kimble) on ice. Add cold homogenization buffer with anti-protease cocktail (for 50 mg tissue add 300 μ l homogenization buffer). The volume of homogenization buffer may vary depending on the tissue used. Homogenize the tissue completely using the tissue grinder or mortar and pestle (Kimble) on ice.
3. Transfer homogenate to labeled microcentrifuge tubes. Centrifuge the homogenate at 10000 rpm for 2 min at 4°C. If the tissue homogenate contains floating lipids, repeat centrifugation at least two times to obtain a clear supernatant. Save the supernatant, discard the pellet, and measure the protein concentration in the supernatant using Bradford protein assay [55]. Before loading, adjust the protein concentration in the samples using sample buffer. Ideally, mix one part sample with one part sample buffer and let stand at room temperature for at least 30 min to allow coating of the protein with SDS. DO NOT HEAT.

3.3 Running the Gel and Electrophoresis

1. Gently pull the comb out of the stacking gel and peel off the rubber band at the bottom of the cassette.
2. Gently place the cassette in the gel protean II apparatus (Bio-Rad). Fill the buffer chambers with 1 $\times$ running buffer. Reducing agents such as dithiothreitol (DDT) are omitted because of possible interference with subsequent refolding of gelatinases. Load the samples in the gel lanes. Typically 10 to 25 μ l of each sample which contains 0.1–10 μ g of the enzyme is loaded to each of the gel lanes (*see* Note 5). Molecular weight markers are included on each gel preferably in lanes 1 and 6.
3. Run the gel in the protean II apparatus (Bio-Rad) using gel electrophoresis at the standard running conditions (125 V, constant voltage) and until the bromophenol blue tracking dye reaches the bottom of the gel (*see* Notes 6 and 7). Running time could range between 60–120 min depending on the gel A/C percentage, running buffer concentration and pH. The proteins will be separated according to their molecular weight; whereby the low molecular weight proteins will run faster and farther than the high molecular weight proteins which will be lagging behind (Fig. 2).

3.4 Renaturing, Developing, Staining and Destaining the Gel

1. Dilute the renaturing buffer (10 $\times$) 1:10 with deionized dH₂O to obtain 1 $\times$ renaturing buffer solution. Carefully remove the gel from the cassette and place it in a plastic tray containing 1 $\times$ renaturing solution (50 ml for one mini-gel). Incubate the gel for 30 min at room temperature with gentle shaking in order to

remove SDS which causes MMPs to denature and become inactive (*see* Notes 8 and 9).

2. Decant the zymogram 1× renaturing buffer and replace with fresh 1× developing buffer (50 ml for one mini-gel). Equilibrate the gel at room temperature for an additional 30 min in the developing buffer with gentle shaking. Decant the developing buffer and replace with 50 ml of fresh 1× developing buffer and incubate the gel at 37°C overnight for ~18 h for maximum sensitivity (*see* Note 10). Optimal results are determined empirically by varying the sample load and incubation time (Fig. 3).
3. Decant the developing buffer and stain the gel with commassie blue R-250 staining solution for at least 30 min until the gel is uniformly dark blue. Destain the gel with destaining solution until areas of gelatinolytic activity appear as clear sharp bands against dark blue background.

3.5 Zymogram Analysis

The goal of gelatin zymography is to obtain clear and sharp bands of the digested substrate against a blue background of the undegraded substrate (Fig. 3). Comparison of the location of the gelatinolytic with molecular weight standards run simultaneously on the same gel should help identify the specific MMP involved (*see* Note 11). The bands in the gel are quantified using ImageJ 1.38X (NIH). The gel image is scaled using a grey scale such that the intensity of each pixel would range from 0 to 255. The integrated intensity of the band of interest is calculated by first outlining and measuring the band area in pixels then transferring it into mm² using a calibration bar. The total pixel intensity is measured by summing the pixel values within the band area, and the average pixel intensity is measured by dividing the total pixel intensity by the number of pixels. The average pixel density of the background is then subtracted from the average band intensity. The integrated intensity of the selected band is then measured as average pixel intensity × band area in mm² [56,54]. The integrated intensity of the band can also be normalized to the housekeeping protein actin to correct for loading. Comparison of the integrated intensity of the bands would help determine the changes in MMP activity in specimens in different physiological and pathological conditions (*see* Notes 12 and 13).

3.6 Study of MMPI

1. Prepare all the materials and follow all the methods as described above for gelatin zymography.
2. For sample preparation, incubate the sample overnight in physiological buffer solution in the presence of appropriate concentration of MMPI. For example, SB-3CT (MMP-2/MMP-9 inhibitor IV, 10⁻⁶ M), BB-94 (10⁻⁶ M) or Ro-28-2653 (10⁻⁶ M) (*see* Note 14).
3. Compare the integrated intensity of the gelatinolytic bands in control samples in the absence of MMPI with those in the presence of MMPI. The integrated intensity of the gelatinolytic bands should be less in the presence of MMPI compared to the control samples in the absence of MMPI.

3.7 Reverse Zymography

1. Prepare all the materials and follow the methods as described above for regular gelatin zymography.
2. When preparing the separating gel, make the 8% separating gel as in regular gelatin zymography but add human recombinant MMP-2 or MMP-9 (0.13 µg/ml) (*see* Note 15).
3. For the staining step, decant the developing buffer and stain the gel with Coomassie Blue R-250 staining solution for at least 30 min until the areas representing the undigested gelatin due to the presence of endogenous TIMPs appear as clear dark blue bands against faint blue background of digested gelatin caused by the added MMP-2 or MMP-9.

4 Notes

Although zymography could be a sensitive and quantifiable assay to analyze MMP activity, problems related to the nature, source and preparation of samples, the substrate in the gel, and the distinction between inactive and active forms of MMPs could compromise the validity of the technique and complicate interpretation of the results. In order to obtain consistent and reliable zymograms it is important to pay attention to all the different steps including the gel preparation, sample preparation, running and developing the gels, and analysis of the zymograms.

1. For gel electrophoresis, all stock solutions should be prepared using electrophoresis-grade reagents and kept fresh. If any precipitations are observed, the quality of the solution and the concentration of the different ingredients are likely altered and should not be used. Also, avoid bacterial contamination of the buffers and solutions as bacterial proteases may result in the appearance of nonspecific bands. This can be minimized by filter sterilization of the buffers and stock solutions and storage at 4°C.
2. An 8% polyacrylamide gel is generally used for separating gelatinases. However, the percentage of acrylamide and the thickness of the separating gel may vary depending on the MMP type, form and molecular mass. For instance, to better visualize the dimeric form of MMP-9 (~200 kDa) or to obtain a better resolution of bands with close molecular weights (latent and active forms), a lower 4–6% polyacrylamide solution should be used. However, by taking this approach the gelatinolytic bands may become less sharp. On the other hand, to better visualize the lower molecular weight MMPs such as MMP-1 and -7, a higher 10–12% polyacrylamide solution can be used. The gels can be prepared in advance and stored at 4°C for 2 or 3 weeks without significant effects on the resolution.
3. Gelatin is commonly used as the protein substrate because it is inexpensive, easily hydrolyzed by several peptidases, and does not tend to migrate out of the resolving gel in electrophoretic tests performed at 4°C [57]. Gelatin is the substrate of choice to detect the gelatinases MMP-2 and MMP-9. Other MMPs such as MMP-1, MMP-8 and MMP-13 can degrade gelatin; however, the

gelatinolytic bands will be weak because gelatin is not their preferred substrate [58,59]. For improved detection of these MMPs, modified zymography has been developed by incorporating a more suitable substrate such as casein or collagen into the gel, or by enhancing the gelatinolytic signal with the addition of heparin to the samples [60–62].

4. Fresh tissues should be used for measuring MMP activity, and the whole procedure of tissue preparation and homogenization should be performed on ice at 4°C. Because of inherent differences in tissue structure, protein extractability may vary between and within specimens [63]. For example, tissues such as the liver and placenta are easy to cut and homogenize due to their fragile nature, while the aorta and uterus are more difficult to cut into small pieces and to homogenize because of their large content of collagen and elastin fibers. The homogenized samples should not be boiled as high temperature causes protein precipitation of the enzymes. Also, reducing agents such as DTT or 2-mercaptoethanol should not be added as reducing agents break the disulfide bond and thereby prevent some forms of tertiary protein folding, inhibit MMP refolding after electrophoresis [53], and break up quaternary protein structure in oligomeric subunits. The tissue extraction procedure itself may activate MMPs or cause inhibition of active enzymes by interacting with some of the components of the homogenization buffer. Some studies suggest that EDTA, other Zn^{2+} chelators and protease inhibitors should not be added to the tissue extract [64]. While EDTA may prevent MMP activation by binding with the Ca^{2+} and Mg^{2+} ion in the homogenization buffer, MMPs are reactivated after incubation in the developing buffer which contains Ca^{2+} and Zn^{2+} ions.
5. The amount of sample loaded in the gel is critical for successful zymography as large amounts of tissue extracts may produce saturated and distorted bands. The sensitivity of zymography is much greater than that of Western blots, which depends on the antibody affinity for MMPs. Because zymography is a sensitive technique, gelatinolytic bands can be detected with MMP levels as low as 10 pg [56,65]. However, these low MMP levels are not often detected because the ratio of MMPs to total protein in crude samples is extremely low. This may make it necessary to load larger amounts of the tissue extract. Overloading of total protein extracts into the wells or lanes may lead to saturated gelatinolytic bands in the zymogram. For instance, in pregnant rat uterus extracts, gelatin zymography using different protein amounts showed dose-dependent increases in MMP-2 and MMP-9 proteolytic activity at 0.1, 0.2 and 0.5 μg , clearly discernible bands at 1–2 μg , but saturated bands at 5, 10 and 20 μg (Fig. 3).
6. For the proteins to move from the cathode to the anode through the gel, the gel system and running buffer should have the proper pH as it may affect the mobility of the different components of the gel system relative to the proteins. For example, depending on the pH, glycine can exist in three different charge states; positive, neutral or negative. Control of the charge of glycine in the different buffers is key to the mobility of proteins in the gel. All of the proteins in the gel have an electrophoretic mobility that is intermediate between the mobility

of glycine and Cl^- so that as the glycine and Cl^- fronts sweep through the sample well the proteins are concentrated in the narrow zone between the two fronts. This process continues through the stacking gel until the proteins hit the separating gel, where the pH switches to 8.8. At this pH the glycine becomes mostly negatively charged and migrates much faster than the proteins. As the glycine front accelerates past the proteins, the proteins become concentrated in a very narrow region at the interface of the stacking and separating gels. Because the separating gel has a greater acrylamide concentration it slows the mobility of the proteins according to their molecular weight and the protein separation begins [66]. If any of the buffers or gel system pH is altered, the protein mobility will be affected in areas of the gel with “improper” pH. This explains the odd protein migration behavior and the distorted shape of the protein bands in some gels.

7. Careful attention to the temperature could provide the best conditions for running the gel. It is important to keep the gels cold while running, otherwise the lower part of the gels may become distorted and show wavy bands. Putting the gel apparatus on ice during running could minimize overheating of the apparatus and distortion of the gel.
8. SDS is a strongly denaturing anionic detergent which unfolds and fully denatures all proteins including MMPs, essentially disregarding secondary structures or hydrophobic domains, and generates SDS-protein complexes that are mostly characterized by a uniform charge-to-mass ratio. This makes SDS-PAGE in general a very simple and reliable technique for protein separation and molecular mass determination. The ratio of 1.4 gram of SDS bound per gram of protein is often quoted as a typical stoichiometric value [67]. When the proteins are saturated with micelles of SDS (SDS-protein), this amount of highly charged surfactant molecules is sufficient to overwhelm the intrinsic charges on the protein chains so that the net charge per unit of mass becomes almost constant, thus allowing the protein chains to separate through SDS-PAGE, mostly according to their molecular weight [68].
9. Pro-MMPs are secreted as inactive zymogens with an inhibitory propeptide domain. The pro-MMP architecture in which Cys^{73} is located in the vicinity of the Zn^{2+} ion makes the $\text{Cys}^{73}\text{-Zn}^{2+}$ complex vulnerable to disruption by multiple stimuli. Dissociation of the Cys^{73} residue from the Zn^{2+} ion “switches” it from a non-catalytic to catalytic Zn^{2+} . Because the sequences surrounding Cys^{73} in the propeptide and the Zn^{2+} binding site in the catalytic domain of the MMPs are highly homologous, the “cysteine switch” mechanism applies to all MMPs. For example, during the electrophoresis step of the gelatin zymography experiment the propeptide is unfolded in the denaturing conditions induced by SDS. After electrophoresis, other non-ionic detergents such as Triton X-100 are used in the renaturing buffer to replace SDS and remove it from the SDS-complex. This allows the pro-MMPs in the sample to renature, become partially refolded, and autoactivate, resulting in the appearance of a partially catalytically active pro-MMP portion of the originally inactive pro-MMPs. Only about 35% of the MMP

catalytic activity is recovered during the protein refolding [63], which may not represent the true biological activity of the pro-MMP when activated *in vivo*. Because these propeptides are covalently anchored to the enzyme proforms, the pro-MMPs are detected at higher molecular weights than the activated MMPs from which the propeptides are cleaved off. Furthermore, non-covalently bound complexes, such as TIMP-MMP complexes, are dissociated by SDS during the electrophoresis step [56,64,69]. Hence, the gelatinolytic bands of the zymography may not be a measure of the net MMPs activity present in the sample, but should rather be seen as a measure of potential activity of MMPs [56,69,70].

10. The incubation time of the gel in the developing buffer is critical for proper renaturation and proteolysis. Since the appearance of the gelatinolytic bands depends on enzymatic activity, changing the incubation time will affect the intensity of the bands. Incubating the gel in the developing buffer at 37°C for 4 h in a closed tray may be sufficient to detect the gelatinolytic bands. However, in most cases, overnight incubation (16 to 18 h) may be needed to obtain better resolution and reproducible results. If the bands remain barely visible, it may be necessary to develop the gels for a longer period of time, even up to 72 h.
11. The identity of the MMP type in the gelatinolytic band is usually determined by comparing the band location with known molecular weight standards run simultaneously in the same gel. This could also help in discerning the latent inactive from the active forms of MMPs [51]. Also, pro-MMPs are usually activated in a process involving the generation of an inactive intermediate forms which are then processed to generate the fully mature active forms [71,72]. Some commercially available molecular weight standards contain a reducing agent, and when they are used under nonreducing conditions, they may indicate different molecular weights [51]. Also, detection of small differences in molecular weight between intermediate and fully active MMP species would require further optimization of the conditions to enhance the sensitivity of the zymography assay. Hence, for an exact identification of MMPs, Western blot analysis using specific antibodies should be performed. While Western blot analysis is more specific than zymography, antibodies may not be sensitive enough to detect low levels of MMPs. On the other hand, while zymography is more sensitive than Western blots in detecting small amounts of MMPs, some of the limitations regarding the resolution of gelatin zymography may make it difficult to analyze the data. Both gelatin zymography and Western blots techniques could complement each other in studying MMPs.
12. Gelatin zymography can be used to detect the changes in specific MMP activity in vascular remodeling and angiogenesis [1], and in the uterine and vascular changes associated with pregnancy [8]. We have recently used gelatin zymography to measure the changes in MMP activity during uterine wall stretch and in response to sex hormones during pregnancy in rats [54]. We found that oxytocin-induced contraction of uterine strips was reduced in pregnant compared with virgin rats. Gelatin zymography showed increased activity of MMP-2 and -9

in uterus of pregnant versus virgin rats. Prolonged stretch of uterine strips of virgin rats was associated with reduced contraction and enhanced activity of MMP-2 and -9. Treatment of stretched uterus of virgin rats with 17β -estradiol (E2) or progesterone (P4) or E2+P4 caused further reduction in contraction and increases in MMP activity. MMP-2 and -9 decreased oxytocin-induced contraction in uterus of virgin rat. These data suggested that during pregnancy uterine stretch and increased sex hormone levels cause increases in activity of MMP-2 and -9, which in turn reduce uterine contraction and enhance uterine relaxation [54].

13. Gelatin zymography can be used to measure the changes in MMP activity in vascular disease. We have used gelatin zymography to measure the changes in uteroplacental and vascular MMPs in an animal model of hypertension in pregnancy produced by reduction in uterine perfusion pressure (RUPP) [73]. We observed a decrease in gelatinase activity of MMP-2 and -9 in uterus, placenta and aorta of RUPP compared with normal pregnant rats. Also, collagen was more abundant in uterus, placenta and aorta of RUPP than Norm-Preg rats. The anti-angiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1) decreased MMP activity in uterus, placenta and aorta of normal pregnant rats, and vascular endothelial growth factor (VEGF) reversed the decreases in MMPs in tissues of RUPP rats. These observations suggested that placental ischemia and anti-angiogenic sFlt-1 decrease uterine, placental and vascular MMP-2 and -9, leading to increased uteroplacental and vascular collagen, and growth restriction in hypertensive pregnancy, and that angiogenic factors may reverse the decrease in MMP activity and enhance uteroplacental and vascular growth in preeclampsia [73].
14. Gelatin zymography can be used to test the specificity and efficacy of MMPIs. Selective MMPIs can be added to a tissue sample *ex vivo* to determine if they reduce MMP activity and alter tissue function. MMPIs can also be added to the gel (or part of a gel cut in half) during the incubation in the developing buffer to determine if they decrease the activity of the MMP of interest. The optimal dose and reaction time of MMPIs with MMPs can be determined by performing a concentration-response curve and time course studies. We have recently tested the effects of MMPIs on uterine function and MMP activity. We found that pretreatment of uterine strips with the MMPIs SB-3CT, BB-94 or Ro-28-2653 (10^{-6} M) did not change oxytocin contraction in virgin uterus but enhanced contraction in uterine strips of pregnant rats. Also, gelatin zymography revealed that the intensity of the pro-MMP-2, MMP-2 and MMP-9 digested gelatin bands was reduced in uterine strips treated with the MMP-2/MMP-9 inhibitor IV (SB-3CT, 10^{-6} M) compared with control non-treated uterine strips of virgin or pregnant rats [54]. These observations support the contention that MMPIs could be used to test the role of changes in MMP activity in modulating the function of different systems.
15. There is mounting evidence that MMPs regulate tissue remodeling under physiological and pathological conditions, and reliable detection and quantitative

methods such as zymography are needed to determine the role of MMPs in various processes. This classical technique for measuring MMP activity is a highly sensitive, cost-effective and relatively simple to perform. Modification of the substrate in the zymography assay could enhance the detection spectrum to include MMPs with different substrate preferences. Improvement in the sensitivity and accuracy of gelatin zymography will further enhance its value in assessing the changes in MMP activity in biological samples and in disease conditions. Tissue remodeling is controlled by a balance between endogenous TIMPs and MMPs such that an increase in TIMP/MMP activity ratio would decrease ECM protein degradation and vice versa. Reverse zymography could be utilized to test the activity of endogenous TIMPs (Fig. 4). Also, synthetic MMPIs have been evaluated as diagnostic and therapeutic tools in cancer, autoimmune and vascular disease [15]. Zymography could be a valuable technique to test the specificity and efficacy of MMPIs, and could help in the development of more potent and selective inhibitors for specific MMPs.

Acknowledgments

This work was supported by grants from National Heart, Lung, and Blood Institute (HL-65998, HL-98724, HL-111775). Dr. Zongli Ren was a visiting scholar from the Department of Cardiovascular Surgery, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, P. R. China, and a recipient of scholarship from the China Scholarship Council. Dr. Juanjuan. Chen was a visiting scholar from the Department of Obstetrics & Gynecology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China 510150.

List of Abbreviations

A/C	acrylamide/bis-acrylamide
ECM	extracellular matrix
MMP	matrix metalloproteinase
MMPI	MMP inhibitor
MT-MMP	membrane type-MMP
RUPP	reduction in uterine perfusion pressure
TIMP	tissue inhibitor of MMP
Zn²⁺	zinc ion

References

1. Benjamin MM, Khalil RA. Matrix metalloproteinase inhibitors as investigative tools in the pathogenesis and management of vascular disease. *EXS (Experientia Supplementum)*. 2012; 103:209–279.
2. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res*. 2003; 92(8):827–839. [PubMed: 12730128]
3. Galis ZS, Khatir JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res*. 2002; 90(3):251–262. [PubMed: 11861412]

4. English WR, Holtz B, Vogt G, Knauper V, Murphy G. Characterization of the role of the “MT-loop”: an eight-amino acid insertion specific to progelatinase A (MMP2) activating membrane-type matrix metalloproteinases. *J Biol Chem.* 2001; 276(45):42018–42026. [PubMed: 11555661]
5. Kucukguven A, Khalil RA. Matrix metalloproteinases as potential targets in the venous dilation associated with varicose veins. *Curr Drug Targets.* 2013; 14(3):287–324. [PubMed: 23316963]
6. Pei D, Kang T, Qi H. Cysteine array matrix metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. *J Biol Chem.* 2000; 275(43):33988–33997. [PubMed: 10945999]
7. Ellerbroek SM, Wu YI, Overall CM, Stack MS. Functional interplay between type I collagen and cell surface matrix metalloproteinase activity. *J Biol Chem.* 2001; 276(27):24833–24842. [PubMed: 11331272]
8. Palei AC, Granger JP, Tanus-Santos JE. Matrix metalloproteinases as drug targets in preeclampsia. *Curr Drug Targets.* 2013; 14(3):325–334. [PubMed: 23316964]
9. Bode W, Fernandez-Catalan C, Grams F, Gomis-Ruth FX, Nagase H, Tschesche H, Maskos K. Insights into MMP-TIMP interactions. *Ann N Y Acad Sci.* 1999; 878:73–91. [PubMed: 10415721]
10. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res.* 2006; 69(3):562–573. [PubMed: 16405877]
11. Brew K, Dinakarandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta.* 2000; 1477(1–2):267–283. [PubMed: 10708863]
12. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci.* 2002; 115(Pt 19):3719–3727. [PubMed: 12235282]
13. Murphy G. Tissue inhibitors of metalloproteinases. *Genome Biol.* 2011; 12(11):233. [PubMed: 22078297]
14. Strickland DK, Ashcom JD, Williams S, Burgess WH, Migliorini M, Argraves WS. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J Biol Chem.* 1990; 265(29):17401–17404. [PubMed: 1698775]
15. Bode W, Maskos K. Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinases. *Biol Chem.* 2003; 384(6):863–872. [PubMed: 12887053]
16. Jacobsen JA, Major Jourden JL, Miller MT, Cohen SM. To bind zinc or not to bind zinc: an examination of innovative approaches to improved metalloproteinase inhibition. *Biochim Biophys Acta.* 2010; 1803(1):72–94. [PubMed: 19712708]
17. Whittaker M, Floyd CD, Brown P, Gearing AJ. Design and therapeutic application of matrix metalloproteinase inhibitors. *Chem Rev.* 1999; 99(9):2735–2776. [PubMed: 11749499]
18. Macaulay VM, O’Byrne KJ, Saunders MP, Braybrooke JP, Long L, Gleeson F, Mason CS, Harris AL, Brown P, Talbot DC. Phase I study of intrapleural batimastat (BB-94), a matrix metalloproteinase inhibitor, in the treatment of malignant pleural effusions. *Clin Cancer Res.* 1999; 5(3):513–520. [PubMed: 10100701]
19. Fingleton B. Matrix metalloproteinases: roles in cancer and metastasis. *Front Biosci.* 2006; 11:479–491. [PubMed: 16146745]
20. Miller KD, Saphner TJ, Waterhouse DM, Chen TT, Rush-Taylor A, Sparano JA, Wolff AC, Cobleigh MA, Galbraith S, Sledge GW. A randomized phase II feasibility trial of BMS-275291 in patients with early stage breast cancer. *Clin Cancer Res.* 2004; 10(6):1971–1975. [PubMed: 15041714]
21. Leigh NB, Paz-Ares L, Douillard JY, Peschel C, Arnold A, Depierre A, Santoro A, Betticher DC, Gatzemeier U, Jassem J, Crawford J, Tu D, Bezjak A, Humphrey JS, Voi M, Galbraith S, Hann K, Seymour L, Shepherd FA. Randomized phase III study of matrix metalloproteinase inhibitor BMS-275291 in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: National Cancer Institute of Canada-Clinical Trials Group Study BR.18. *J Clin Oncol.* 2005; 23(12):2831–2839. [PubMed: 15837997]
22. Lutz J, Yao Y, Song E, Antus B, Hamar P, Liu S, Heemann U. Inhibition of matrix metalloproteinases during chronic allograft nephropathy in rats. *Transplantation.* 2005; 79(6):655–661. [PubMed: 15785371]

23. Lee M, Bernardo MM, Meroueh SO, Brown S, Fridman R, Mobashery S. Synthesis of chiral 2-(4-phenoxyphenylsulfonylmethyl)thiiranes as selective gelatinase inhibitors. *Org Lett*. 2005; 7(20): 4463–4465. [PubMed: 16178559]
24. Olson MW, Gervasi DC, Mobashery S, Fridman R. Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. *J Biol Chem*. 1997; 272(47):29975–29983. [PubMed: 9368077]
25. Kleifeld O, Kotra LP, Gervasi DC, Brown S, Bernardo MM, Fridman R, Mobashery S, Sagi I. X-ray absorption studies of human matrix metalloproteinase-2 (MMP-2) bound to a highly selective mechanism-based inhibitor. comparison with the latent and active forms of the enzyme. *J Biol Chem*. 2001; 276(20):17125–17131. [PubMed: 11278946]
26. Grams F, Brandstetter H, D'Alo S, Geppert D, Krell HW, Leinert H, Livi V, Menta E, Oliva A, Zimmermann G, Gram F, Livi VE. Pyrimidine-2,4,6-Triones: a new effective and selective class of matrix metalloproteinase inhibitors. *Biol Chem*. 2001; 382(8):1277–1285. [PubMed: 11592410]
27. Fisher JF, Mobashery S. Recent advances in MMP inhibitor design. *Cancer Metastasis Rev*. 2006; 25(1):115–136. [PubMed: 16680577]
28. Reiter LA, Mitchell PG, Martinelli GJ, Lopresti-Morrow LL, Yocum SA, Eskra JD. Phosphinic acid-based MMP-13 inhibitors that spare MMP-1 and MMP-3. *Bioorg Med Chem Lett*. 2003; 13(14):2331–2336. [PubMed: 12824028]
29. Dive V, Georgiadis D, Matziari M, Makaritis A, Beau F, Cuniasse P, Yiotakis A. Phosphinic peptides as zinc metalloproteinase inhibitors. *Cell Mol Life Sci*. 2004; 61(16):2010–2019. [PubMed: 15316651]
30. Sorsa T, Tjaderhane L, Kontinen YT, Lauhio A, Salo T, Lee HM, Golub LM, Brown DL, Mantyla P. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med*. 2006; 38(5):306–321. [PubMed: 16938801]
31. Rudek MA, Figg WD, Dyer V, Dahut W, Turner ML, Steinberg SM, Liewehr DJ, Kohler DR, Pluda JM, Reed E. Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer. *J Clin Oncol*. 2001; 19(2):584–592. [PubMed: 11208854]
32. Schechter I, Berger A. On the active site of proteases. 3. Mapping the active site of papain; specific peptide inhibitors of papain. *Biochem Biophys Res Commun*. 1968; 32(5):898–902. [PubMed: 5682314]
33. MacColl E, Khalil RA. Matrix Metalloproteinases as Regulators of Vein Structure and Function: Implications in Chronic Venous Disease. *J Pharmacol Exp Ther*. 2015; 355(3):410–428. [PubMed: 26319699]
34. Park HI, Jin Y, Hurst DR, Monroe CA, Lee S, Schwartz MA, Sang QX. The intermediate S1' pocket of the endometase/matrixlysin-2 active site revealed by enzyme inhibition kinetic studies, protein sequence analyses, and homology modeling. *J Biol Chem*. 2003; 278(51):51646–51653. [PubMed: 14532275]
35. Aureli L, Gioia M, Cerbara I, Monaco S, Fasciglione GF, Marini S, Ascenzi P, Topai A, Coletta M. Structural bases for substrate and inhibitor recognition by matrix metalloproteinases. *Curr Med Chem*. 2008; 15(22):2192–2222. [PubMed: 18781944]
36. Fabre B, Ramos A, de Pascual-Teresa B. Targeting matrix metalloproteinases: exploring the dynamics of the s1' pocket in the design of selective, small molecule inhibitors. *J Med Chem*. 2014; 57(24):10205–10219. [PubMed: 25265401]
37. Hu Y, Xiang JS, DiGrandi MJ, Du X, Ipek M, Laakso LM, Li J, Li W, Rush TS, Schmid J, Skotnicki JS, Tam S, Thomason JR, Wang Q, Levin JI. Potent, selective, and orally bioavailable matrix metalloproteinase-13 inhibitors for the treatment of osteoarthritis. *Bioorg Med Chem*. 2005; 13(24):6629–6644. [PubMed: 16216515]
38. Udi Y, Fragai M, Grossman M, Mitternacht S, Arad-Yellin R, Calderone V, Melikian M, Toccafondi M, Berezovsky IN, Luchinat C, Sagi I. Unraveling hidden regulatory sites in structurally homologous metalloproteases. *J Mol Biol*. 2013; 425(13):2330–2346. [PubMed: 23583775]
39. Overall CM, Kleifeld O. Towards third generation matrix metalloproteinase inhibitors for cancer therapy. *Br J Cancer*. 2006; 94(7):941–946. [PubMed: 16538215]

40. Ndinguri MW, Bhowmick M, Tokmina-Roszyk D, Robichaud TK, Fields GB. Peptide-based selective inhibitors of matrix metalloproteinase-mediated activities. *Molecules*. 2012; 17(12): 14230–14248. [PubMed: 23201642]
41. Koivunen E, Arap W, Valtanen H, Rainisalo A, Medina OP, Heikkila P, Kantor C, Gahmberg CG, Salo T, Kontinen YT, Sorsa T, Ruoslahti E, Pasqualini R. Tumor targeting with a selective gelatinase inhibitor. *Nat Biotechnol*. 1999; 17(8):768–774. [PubMed: 10429241]
42. Suojanen J, Salo T, Koivunen E, Sorsa T, Pirila E. A novel and selective membrane type-1 matrix metalloproteinase (MT1-MMP) inhibitor reduces cancer cell motility and tumor growth. *Cancer Biol Ther*. 2009; 8(24):2362–2370. [PubMed: 19855192]
43. Devy L, Dransfield DT. New Strategies for the Next Generation of Matrix-Metalloproteinase Inhibitors: Selectively Targeting Membrane-Anchored MMPs with Therapeutic Antibodies. *Biochem Res Int*. 2011; 2011:191670. [PubMed: 21152183]
44. Devy L, Huang L, Naa L, Yanamandra N, Pieters H, Frans N, Chang E, Tao Q, Vanhove M, Lejeune A, van Gool R, Sexton DJ, Kuang G, Rank D, Hogan S, Pazmany C, Ma YL, Schoonbroodt S, Nixon AE, Ladner RC, Hoet R, Henderikx P, Tenhoo C, Rabbani SA, Valentino ML, Wood CR, Dransfield DT. Selective inhibition of matrix metalloproteinase-14 blocks tumor growth, invasion, and angiogenesis. *Cancer Res*. 2009; 69(4):1517–1526. [PubMed: 19208838]
45. Hoet RM, Cohen EH, Kent RB, Rookey K, Schoonbroodt S, Hogan S, Rem L, Frans N, Daukandt M, Pieters H, van Hegelsom R, Neer NC, Nastri HG, Rondon JJ, Leeds JA, Hufton SE, Huang L, Kashin I, Devlin M, Kuang G, Steukers M, Viswanathan M, Nixon AE, Sexton DJ, Hoogenboom HR, Ladner RC. Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. *Nat Biotechnol*. 2005; 23(3):344–348. [PubMed: 15723048]
46. Hu J, Van den Steen PE, Houde M, Ilenchuk TT, Opdenakker G. Inhibitors of gelatinase B/matrix metalloproteinase-9 activity comparison of a peptidomimetic and polyhistidine with single-chain derivatives of a neutralizing monoclonal antibody. *Biochem Pharmacol*. 2004; 67(5):1001–1009. [PubMed: 15104254]
47. Martens E, Leyssen A, Van Aelst I, Fiten P, Piccard H, Hu J, Descamps FJ, Van den Steen PE, Proost P, Van Damme J, Liuzzi GM, Riccio P, Polverini E, Opdenakker G. A monoclonal antibody inhibits gelatinase B/MMP-9 by selective binding to part of the catalytic domain and not to the fibronectin or zinc binding domains. *Biochim Biophys Acta*. 2007; 1770(2):178–186. [PubMed: 17137715]
48. Sela-Passwell N, Kikkeri R, Dym O, Rozenberg H, Margalit R, Arad-Yellin R, Eisenstein M, Brenner O, Shoham T, Danon T, Shanzer A, Sagi I. Antibodies targeting the catalytic zinc complex of activated matrix metalloproteinases show therapeutic potential. *Nat Med*. 2011; 18(1):143–147. [PubMed: 22198278]
49. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem*. 1980; 102(1): 196–202. [PubMed: 7188842]
50. Murphy G, Crabbe T. Gelatinases A and B. *Methods Enzymol*. 1995; 248:470–484. [PubMed: 7674939]
51. Hawkes SP, Li H, Taniguchi GT. Zymography and reverse zymography for detecting MMPs and TIMPs. *Methods Mol Biol*. 2010; 622:257–269. [PubMed: 20135288]
52. Lombard C, Saulnier J, Wallach J. Assays of matrix metalloproteinases (MMPs) activities: a review. *Biochimie*. 2005; 87(3–4):265–272. [PubMed: 15781313]
53. Snoek-van Beurden PA, Von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques*. 2005; 38(1):73–83. [PubMed: 15679089]
54. Yin Z, Sada AA, Reslan OM, Narula N, Khalil RA. Increased MMPs expression and decreased contraction in the rat myometrium during pregnancy and in response to prolonged stretch and sex hormones. *Am J Physiol Endocrinol Metab*. 2012; 303(1):E55–70. [PubMed: 22496348]
55. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]

56. Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal Biochem.* 1994; 218(2):325–329. [PubMed: 8074288]
57. Michaud D, Cantin L, Raworth DA, Vrain TC. Assessing the stability of cystatin/cysteine proteinase complexes using mildly-denaturing gelatin-polyacrylamide gel electrophoresis. *Electrophoresis.* 1996; 17(1):74–79. [PubMed: 8907521]
58. Knauper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. *J Biol Chem.* 1996; 271(3):1544–1550. [PubMed: 8576151]
59. Bjornland K, Winberg JO, Odegaard OT, Hovig E, Loennechen T, Aasen AO, Fodstad O, Maelandsmo GM. S100A4 involvement in metastasis: deregulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in osteosarcoma cells transfected with an anti-S100A4 ribozyme. *Cancer Res.* 1999; 59(18):4702–4708. [PubMed: 10493528]
60. Fernandez-Resa P, Mira E, Quesada AR. Enhanced detection of casein zymography of matrix metalloproteinases. *Anal Biochem.* 1995; 224(1):434–435. [PubMed: 7710105]
61. Gogly B, Groult N, Hornebeck W, Godeau G, Pellat B. Collagen zymography as a sensitive and specific technique for the determination of subpicogram levels of interstitial collagenase. *Anal Biochem.* 1998; 255(2):211–216. [PubMed: 9451506]
62. Yu WH, Woessner JF Jr. Heparin-enhanced zymographic detection of matrilysin and collagenases. *Anal Biochem.* 2001; 293(1):38–42. [PubMed: 11373076]
63. Woessner JF Jr. Quantification of matrix metalloproteinases in tissue samples. *Methods Enzymol.* 1995; 248:510–528. [PubMed: 7674942]
64. Kupai K, Szucs G, Cseh S, Hajdu I, Csonka C, Csont T, Ferdinandy P. Matrix metalloproteinase activity assays: Importance of zymography. *J Pharmacol Toxicol Methods.* 2010; 61(2):205–209. [PubMed: 20176119]
65. Masure S, Proost P, Van Damme J, Opdenakker G. Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *Eur J Biochem.* 1991; 198(2):391–398. [PubMed: 1645657]
66. Garfin DE. One-dimensional gel electrophoresis. *Methods Enzymol.* 2009; 463:497–513. [PubMed: 19892189]
67. Pitt-Rivers R, Impiombato FS. The binding of sodium dodecyl sulphate to various proteins. *Biochem J.* 1968; 109(5):825–830. [PubMed: 4177067]
68. Shapiro AL, Vinuela E, Maizel JV Jr. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem Biophys Res Commun.* 1967; 28(5):815–820. [PubMed: 4861258]
69. Ikeda M, Maekawa R, Tanaka H, Matsumoto M, Takeda Y, Tamura Y, Nemori R, Yoshioka T. Inhibition of gelatinolytic activity in tumor tissues by synthetic matrix metalloproteinase inhibitor: application of film in situ zymography. *Clin Cancer Res.* 2000; 6(8):3290–3296. [PubMed: 10955815]
70. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol.* 2002; 37(6):375–536. [PubMed: 12540195]
71. Springman EB, Angleton EL, Birkedal-Hansen H, Van Wart HE. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a “cysteine switch” mechanism for activation. *Proc Natl Acad Sci U S A.* 1990; 87(1):364–368. [PubMed: 2153297]
72. Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci U S A.* 1990; 87(14):5578–5582. [PubMed: 2164689]
73. Li W, Mata KM, Mazzuca MQ, Khalil RA. Altered matrix metalloproteinase-2 and -9 expression/activity links placental ischemia and anti-angiogenic sFlt-1 to uteroplacental and vascular remodeling and collagen deposition in hypertensive pregnancy. *Biochem Pharmacol.* 2014; 89(3):370–385. [PubMed: 24704473]

Fig. 1. Mechanisms of MMP activation, MMP-substrate interaction and MMP inhibition. Full-length pro-MMP can be activated in two ways. A: Proteolytic activation of MMPs by MT-MMP/TIMP or other proteases occurs by removal of the cysteine switch motif -SH autoinhibitory propeptide region resulting in a truncated active MMP. B: In the presence of oxidative stress, reactive O₂ species such as peroxynitrite (ONOO⁻) and cellular glutathione (GSH) the critical cysteine residue in the propeptide region undergoes S-glutathiolation, leading to the release of cysteine binding to the catalytic Zn²⁺ ion and active enzyme. C: Active MMP interacts with its substrate through a series of biochemical reactions. Using H⁺ from free H₂O, the substrate carbonyl binds to Zn²⁺, forming a Michaelis complex. The Zn²⁺-bound H₂O performs a nucleophilic attack on the substrate, resulting in the release of an H₂O molecule, breakdown of the substrate and the release of active MMP to be ready for interaction with another substrate. D: TIMP interacts with MMP in a manner similar to that of substrate substituent, further contributing to expelling Zn²⁺-bound H₂O and preventing substrate degradation. E: Zn²⁺-binding MMPis act as an anchor that is locked in the active site and direct the backbone of the inhibitor into the target substrate-binding pockets resulting in inactive MMP. Dashed lines indicate inhibition.

Gelatin Zymography Procedure

Fig. 2.
Flow chart of gelatin zymography procedure.

Fig. 3.

Concentration-dependent MMP-2 and MMP-9 gelatinase activity in uterus of pregnant rats. Uterine tissue strips from normal pregnant rats were homogenized and prepared for gelatin zymography analysis using different concentrations of loaded protein (0.1–20 µg). Pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 showed concentration-dependent gelatinolytic bands because of the presence of their preferred substrate gelatin. Other MMPs are only detected at higher protein concentration and are less clear because gelatin is not their preferred substrate.

Fig. 4. TIMP activity in uterus of pregnant rats. Uterine tissue strips from normal pregnant rats were homogenized and prepared for reverse zymography. Separating gel was prepared as in regular gelatin zymography experiment except that MMP-9 (0.13 $\mu\text{g/ml}$) was added. TIMP-1 appears as a darker blue band of the undigested substrate against a faint blue background of the degraded gelatin substrate.

Table

Representative synthetic MMPiIs and their IC₅₀ or K_i toward specific MMPs.

MMPi (other name)	MMP Specificity (IC ₅₀ or K _i) (nM)														
	MMP-1	2	3	7	8	9	10	11	13	14	16				
Hydroxamate-based Batimastat (BB-94)	3	4	20	6	10	1									
marimastat (BB-2516)	5	6	200	20	2	3				1.8					
Ilomastat (GM6001)	0.4	0.4	27		0.1	0.2				5.2					
CGS-27023-A (MMI-270)	33	20	43		8	8			6						
MMI-166		0.4			400	90				100					
ABT-770	4600	3.7	42	>10000		120									
PD-166793	6.1	47	12	7.2		7.9			8	240					
Prinomastat (AG3340)	8.3	0.05	0.3	54		0.26			0.03	0.33					
Cipemastat (Ro 32-3555)	3.0	154			4.4	59			3.4						
Non-hydroxamate Rebimastat (BMS-275291)	9	39	157	23		27				40					
Tanomastat (BAY 12-9566)		11	134			301			1470						
Mechanism-Based SB-3CT	206	14	15	96		600									
Pyrimidine-based Ro 28-2653	16000	7-246	1200		15	12-23				96	91				
Phosphorous-based RXP03	2000	55		20000	4	41	45	6.2	16	90					
Tetracyclines Doxycycline	>400000	56000	32000	28000	26000-50000				2000-50000						
Metastat (COL-3;CMT-3)	34 µg/ml				48 µg/ml				0.3 µg/ml						
Antibody-based DX-2400 (Ki)										0.8					

MMPI (other name)	MMP Specificity (IC ₅₀ or K _i) (nM)									
	MMPI-1	2	3	7	8	9	10	11	13	14 16
REGA-3G12						+++				

MMPI, MMP inhibitor; CMT, chemically modified tetracycline; IC₅₀ half-maximal inhibitory concentration; K_i, inhibition constant