

# Design and Characterization of a Metalloproteinase Inhibitor-Tethered Resin for the Detection of Active MMPs in Biological Samples

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## Summary

Matrix metalloproteinases (MMPs), zinc-dependent endopeptidases, are implicated in tumor progression. We describe herein the development of a resin-immobilized, broad-spectrum synthetic MMP inhibitor for selective binding of the active forms of MMPs from different experimental samples. We confirmed the activity-based binding of MMPs to the inhibitor-tethered resin with purified human recombinant MMP-2, -9, and -14, samples of cultured cells, and tissue extracts. Our results show that only the free active MMPs, and not the zymogens or MMP/TIMP (enzyme-protein inhibitor) complexes, bound specifically to the resin. In our comparison of benign and carcinoma tissue extracts, we detected active MMP-2 and MMP-14 forms only in the cancerous tissue samples, indicating that a pool of the tumor MMPs is free of endogenous inhibitors (TIMPs), and is thus likely to contribute to proteolytic events that precipitate tumor metastasis.

## Introduction

Metastasis is the single most important clinical feature associated with poor prognosis and survival in cancer patients, resulting in mortality in over 90% of patients [1]. Unfortunately, in spite of the significant advances in the past two decades in our understanding of the genetic and molecular processes that regulate cancer development and progression, treatment options for the prevention of cancer metastasis do not exist.

Tumor metastasis requires the activity of proteolytic enzymes to hydrolyze extracellular matrix (ECM) components and a variety of bioactive molecules, which together regulate tumor cell migration, invasion, and growth. Matrix metalloproteinases (MMPs), a group of at least 26 known enzymes, constitute a family of extracellular zinc-dependent endopeptidases that are involved in degradation of ECM components and other bioactive non-ECM molecules, and thus are involved in

many normal and pathological processes, including tumor cell invasion and metastasis [2–5]. It is known that the expression of the genes of many MMPs is upregulated in human cancers, implicating their activity in cancer progression [6–9]. A plethora of studies in animal models of experimental metastasis have shown that inhibition of activity or expression of members of the MMP family significantly inhibits tumor growth, decreases metastatic burden, and improves survival [2, 10–18]. However, recent evidence demonstrates that, in fact, MMPs play complex roles in cancer progression. Thus, while certain MMPs promote cancer progression, others can act in an inhibitory way. This new evidence highlights the importance of identifying and characterizing the expression and specific roles of active MMPs in human tumors [19–21].

Virtually all of the inhibition studies of MMPs have been carried out with broad-spectrum inhibitors. These inhibitors were developed mostly as zinc-ion chelators, and, as such, they inhibit a broad range of MMPs and often other related enzymes as well [12]. The initial success of various broad-spectrum synthetic MMP inhibitors in animal models of cancer metastasis and angiogenesis [22, 23] prompted the implementation of human clinical trials. Although some studies showed promise, others have been disappointing [12, 16, 24]. Several reasons were postulated to explain the poor performance of broad-spectrum MMP inhibitors in clinical trials, including advance stage of disease of the patient population, side effects due to inhibition of closely related enzymes such as the ADAMs (a disintegrin and metalloproteinases), lack of specificity, toxicity, and inability to assess inhibitory efficacy [12, 16, 24, 25]. An important aspect that has been lacking in all these studies is the identification of specific MMPs (out of the 26 known) that are important for the disease progression. Thus, determination of the protease profile for each cancer type, in particular the active form, will help to target specific MMPs on an individual basis with specific/selective inhibitors, avoiding unwanted side effects [26].

Profiling of MMP expression in cancer patients has been limited to detection of gene or protein levels in tissues and/or biological fluids. Although these methods can provide important information on the relative levels of MMPs during tumor progression they are not designed to identify which MMP(s) are active and thus responsible for the proteolytic activity in cancerous tissues. MMPs are produced as inactive zymogenic (also known as “latent”) forms. The zymogens have N-terminal propeptides of approximately 80–90 amino acids that block the active sites of the MMPs within the catalytic domains. Removal of the propeptide by proteolytic or nonproteolytic mechanisms results in zymogen activation and acquisition of catalytic competence [27–30]. Once active, the MMPs are specifically inhibited by endogenous protease inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs) of which four, TIMP-1 to TIMP-4, have been identified [31]. Binding of TIMPs to the catalytic domain results in efficient inhibition of enzymatic activity [32]. It has been postulated

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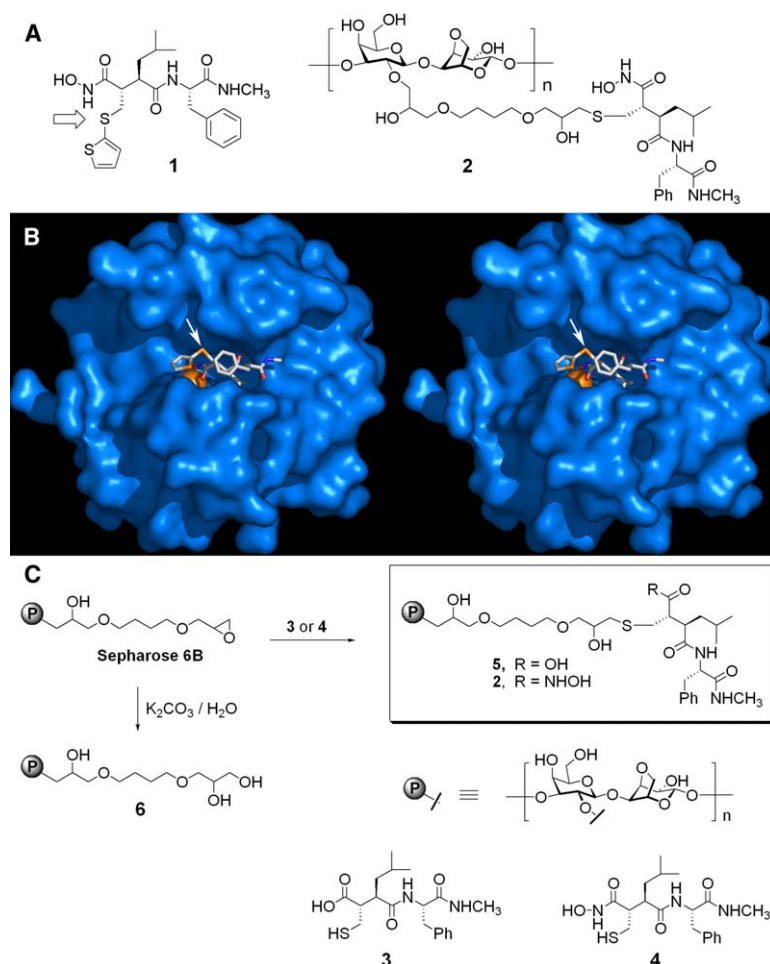


Figure 1. Inhibitor-Tethered Resins for Use with MMPs

(A) The structure of the Batimastat (1) used in the design of the inhibitor-tethered resin; the site chosen for attachment to the resin is indicated by the arrow. The inhibitor-tethered resin (2) based on the structure of inhibitor 1. The Sepharose 6B resin is shown as its monomeric building block in brackets.

(B) The stereoview of the water-accessible Connolly surface constructed on the X-ray coordinates of the catalytic domain of MMP-12. The bound inhibitor, Batimastat (1), is shown in capped-sticks representation (C, N, O, S in white, blue, red and orange, respectively). The catalytic zinc ion is shown as an orange sphere deep in the active site, a portion of which is visible from this perspective. The white arrow points to the sulfur that will be the point of attachment to resin 2. (C) Synthetic scheme for the preparation of the three targeted resins 2, 5, and 6.

that in cancer there exists an imbalance between active MMPs and TIMPs, which may lead to excessive enzymatic activity and eventually promote metastasis [16, 24]. Thus, the presence of active MMPs depends on both the rate of zymogen activation and the levels of endogenous TIMPs. At present, there are no established approaches to determine what MMP(s), among all the members of the MMP family, is catalytically active and free of TIMPs in cancer tissues. Knowing this information would help the development of approaches aimed at targeting the relevant MMPs for selective inhibition.

Here we report the development of an inhibitor-tethered resin with a broad-spectrum inhibitor for MMPs. The inhibitor that we have selected (the criteria for choice are described below) for attachment to this resin is Batimastat [10] (referred to here as inhibitor 1), which is a potent and broad-spectrum MMP inhibitor. The targeted resin is given as structure 2 (based on a variation of 1) (Figure 1). This tool should be useful for monitoring biological samples, including tumor tissues, for the presence of active and uninhibited MMP.

## Results and Discussion

### Design of the Inhibitor-Tethered Resin

As the first prerequisite for designing the inhibitor-tethered resin, it was important to select a broad-spectrum

MMP inhibitor that would allow binding of multiple MMPs. The second prerequisite was the availability of crystal structures for the inhibitor bound to the active sites of these MMPs for judicious selection of the point of attachment of the resin to the inhibitor.

An extensive survey of all the existing X-ray structures of MMP-inhibitor complexes from the RCSB database (<http://www.rcsb.org>) was carried out by systematically visualizing the complexes with the Sybyl 6.9.1 software package (Tripos, Inc.). At the time of this effort, there were 25 three-dimensional structures available for catalytic domains of MMPs bound to various inhibitors. The goal of this exercise was to find inhibitor-MMP complexes in which the bound inhibitor contained a structural moiety that was solvent exposed. Furthermore, we expected that the solvent-exposed site would be synthetically accessible for facile structure alteration, such that a linkage to a chromatographic resin through this site could be made. The structure of the broad-spectrum inhibitor 1 (Batimastat) bound to the catalytic domain of MMP-12 [33, 34] met the above criteria. As shown in Figure 1, the P1' moiety of the broad-spectrum MMP inhibitor 1 was exposed to solvent in the complex (shown by arrows in the structure of 1 in Figures 1A and 1B). This was chosen as the site of attachment to Sepharose 6B. A synthetically feasible modification of this side chain was designed to allow chemoselective

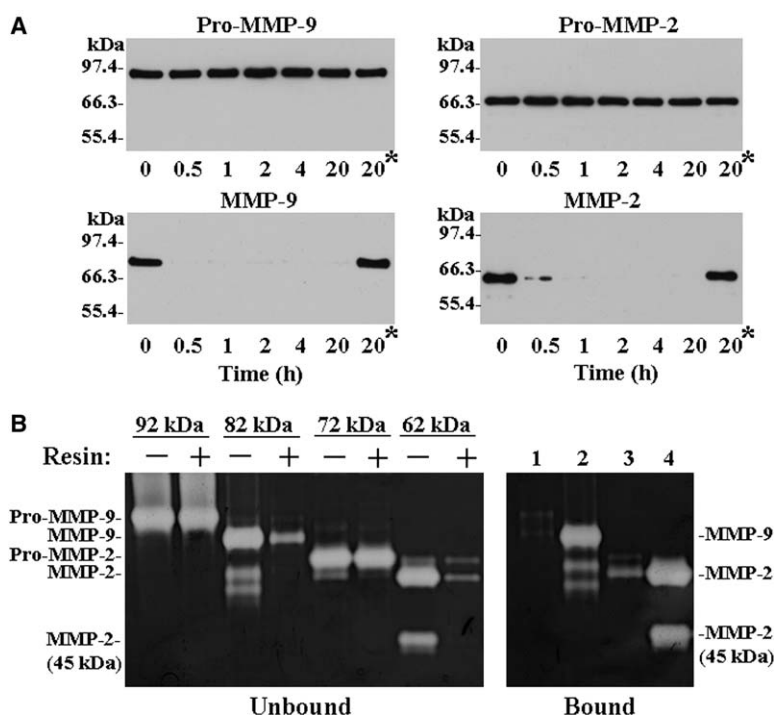


Figure 2. Binding of Recombinant Gelatinases to the Inhibitor-Tethered Resin

(A) Time course of gelatinase binding. Latent (pro-MMP-9 and pro-MMP-2) and active (MMP-9 and MMP-2) recombinant gelatinases (10 nM) were incubated with or without 20  $\mu$ l resin 2 in 0.5 ml CB buffer. At various times (0–20 hr), aliquots (20 ng protein) of the unbound fractions were collected and subjected to reducing 10% SDS-PAGE, followed by immunoblot analysis using anti-gelatinase antibodies. Asterisks indicate the same enzymes incubated for 20 hr in CB buffer without resin.

(B) Gelatin zymography of bound and unbound fractions. Aliquots of the unbound fractions of latent (92 and 72 kDa) and active (82 and 62 kDa) gelatinases incubated (20 hr) with (+) or without (-) resin 2, and aliquots of the bound fractions (lane 1, pro-MMP-9; lane 2, MMP-9; lane 3, pro-MMP-2; lane 4, MMP-2) eluted from the resin 2 were analyzed by gelatin zymography.

immobilization to a suitable solid support (see resin 2 in Figure 1A). Out of several immobilization strategies, we selected a nucleophilic opening of a Sepharose-tethered oxirane ring. The choice of sulfur as a nucleophilic species on the modified inhibitor represented a synthetically viable structure alteration.

A model of a Sepharose chain was connected in silico to the modified structural variant of the Batimastat molecule, at which point and an energy minimization was carried. The minimum length of the linker in Sepharose for optimum binding to MMP in solution was chosen by systematically lengthening the chain in silico, such that binding of MMP to the resin bound inhibitor would not create a contact between the resin and the enzyme. This exercise led to the structure of the desired inhibitor-tethered resin (structure 2).

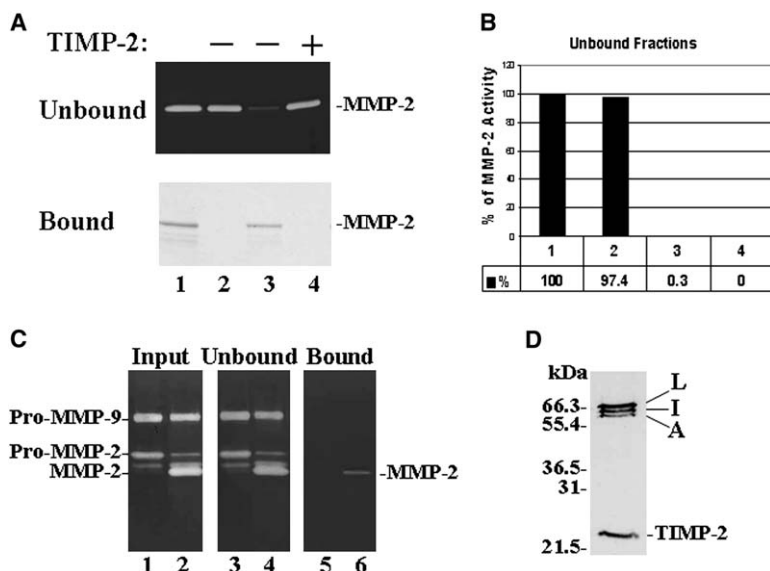
#### Synthetic Route to the Inhibitor-Tethered Resin

The synthetic routes for the preparation of resins 2, 5, and 6 are given in Figure 1C. Resin 2 is hydroxamate-based (as is Batimastat). The hydroxamate moiety is the zinc chelating function. We have also prepared the carboxylate-based resin 5. In this resin, we expect that the carboxylate would coordinate to the active site zinc ion of the MMP analogously to the case of the hydroxamate group, except with somewhat lower affinity. We also set out to prepare the control resin 6, which essentially contained the Sepharose resin and the linker, after the opening of the epoxide function to eliminate the electrophilic group.

The desired immobilized broad-spectrum inhibitor-tethered resins 2 and 5 were prepared by shaking of Sepharose 6B in the presence of an excess of thiol reagents 3 or 4 under an atmosphere of argon. The control resin 6 was prepared by shaking of epoxy-activated Sepharose 6B resin in a carbonate solution in the absence of inhibitor.

#### The Inhibitor-Tethered Resin Binds TIMP-free Active Gelatinases

We first examined the ability of the inhibitor-tethered resin to bind latent and active purified human recombinant gelatinases. To this end, the enzymes were incubated with resin 2 for various times (0–20 hr) and aliquots of the unbound fractions were analyzed by immunoblot analyses with specific anti-gelatinase antibodies. As expected, neither pro-MMP-9 nor pro-MMP-2 bound to the resin, even after a 20 hr incubation period, and were recovered in the unbound fraction (Figure 2A, upper panels). In contrast, both active MMP-9 and MMP-2, obtained as described under Experimental Procedures, bound to the resin within 30 min of the addition of the enzymes to the resin and, consequently, were not seen in the unbound fraction (Figure 2A, lower panels). Gelatin zymography also demonstrated that pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa) were completely recovered in the unbound fraction after incubation with resin 2, while only marginal amounts of active forms (82 and 67–60 kDa, MMP-9; 62 and 45 kDa, MMP-2) were detectable in these fractions (Figure 2B, left panel). The marginal active proteins that were seen in the unbound fraction by zymography were undetectable by immunoblotting, as the quantities were very small (Figure 2A, at 20 hr). In contrast, virtually all of the active forms, but conspicuously not the zymogens, were recovered in the bound fraction (Figure 2B, right panel). Whereas most of the experiments described herein have been performed with resin 2, it should be noted that both resins 2 and 5 could be used interchangeably. In biological samples, the existence of a large, diverse, and complex protein mixture in relatively high concentration might potentially limit the effectiveness of active MMP binding to the resin. After all, the solid support is a chromatographic resin that is functionalized with the inhibitor. Furthermore, other



(D) Serum-free conditioned media collected from TPA-treated HT1080 cells were subjected to gelatin affinity chromatography. Aliquots of the eluted proteins were subjected to reducing 15% SDS-PAGE, and MMP-2 and TIMP-2 were detected by immunoblot analysis. L = latent; I = intermediate; A = active MMP-2.

Figure 3. The MMP-2:TIMP-2 Complex Does Not Bind to the Inhibitor-Tethered Resin

(A and B) Active MMP-2 (200 nM) was incubated without (lane 1) or with control resin 6 (lane 2) or with inhibitor-tethered resin 2 (lanes 3 and 4). MMP-2 was preincubated 1 hr with TIMP-2 (lane 4). Aliquots of the unbound fractions were analyzed by gelatin zymography (A, upper), and the relative enzyme activity was measured (B) as described in *Experimental Procedures*. Total (100%) of MMP-2 activity refers to the activity of MMP-2 (200 nM), which was incubated under the same conditions but without resins. Aliquots of the bound fractions were subjected to reducing 12% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue (A, lower).

(C) Serum-free conditioned media collected from untreated (lane 1) or ConA-treated (lane 2) HT1080 cells were incubated with resin 2. Aliquots of the unbound and bound fractions of untreated (lanes 3 and 5) and ConA-treated (lanes 4 and 6) samples were analyzed by gelatin zymography.

metalloenzymes might interact with the ligand and, as such, diminish capacity for binding to the MMPs. To overcome any such potential problem, we use an excess of the resin and allow copious washing of the resin after binding has taken place to make certain that the eluted active MMPs are obtained in high purity. As will be described subsequently here, this protocol was successful. We predicted that the amounts of active MMPs in our samples (media, extract, or homogenates) are much lower than the estimated binding capacity for the resins used in the quantities that have been specified in *Experimental Procedures*.

Because the physiological inhibitors of MMPs, the TIMPs, bind to the catalytic sites of the active enzymes, they are expected to preclude resin binding. Indeed, preincubation of active MMP-2 (62 kDa species) with a 1.5-fold excess TIMP-2 prior to treatment with the inhibitor-tethered resin prevented binding of the 62 kDa species to the resin. Consistently, the enzyme was recovered in the unbound fraction (Figure 3A, lane 4). Without preincubation with TIMP-2, the active MMP-2 appeared in the bound fraction (Figure 3A, lane 3, lower panel), as expected. Relative enzyme activity was measured in the unbound fractions by following peptide substrate hydrolysis with a fluorescence spectrophotometer. MMP-2 activity disappeared from the unbound fraction as the active enzymes bound to the inhibitor-tethered resin (Figure 3B, lane 3), but it remained in the supernatant when the control resin 6 (without bound inhibitor) was used (Figure 3B, lane 2). These results further demonstrated the specific interaction between active MMP-2 and the immobilized inhibitor. Taken together, these results show that resin 2 was capable of discriminating between the latent and active gelatinases and between the free and TIMP-complexed enzymes, as was expected by the principle behind the design of the resin. It is important to note that the existing methodology in investigations of MMP, such as immu-

noblots or zymography, cannot reveal any information on the important issue of enzymatic activity. Furthermore, even when the MMP exists in the active forms, discernable by both immunoblotting and by zymography, we cannot draw any conclusion as to whether the active form is free-standing or in complex with TIMPs. The methodology disclosed herein differentiates among these forms, opening new avenues of investigation in activity-based functional proteomics.

Next, we examined the ability of resin 2 to bind natural gelatinases present in cultured cells or in human tissue extracts (carcinoma or benign). First, we used conditioned media of human HT1080 fibrosarcoma cells, which are known to secrete progelatinases and TIMPs [35]. Upon treatment with concanavalin A (ConA), HT1080 cells activate pro-MMP-2 in a process that is mediated by the membrane-anchored MT1-MMP (MMP-14) and requires TIMP-2 [36, 37]. Active MMP-2 can then be detected in the supernatant, as shown in Figure 3C, lane 2. We used resin 2 to analyze the distribution of free versus TIMP-complex gelatinases in the media of ConA-treated HT1080 cells. These studies showed that pro-MMP-9, pro-MMP-2, and the intermediate form of MMP-2 (~68 kDa) were all recovered in the unbound fraction, as expected (Figure 3C, lanes 3 and 4). Interestingly, relatively high levels of active MMP-2 were also recovered in the flowthrough (Figure 3C, lane 4), while the bound fraction contained only trace amounts of active MMP-2 (Figure 3A, lane 6), suggesting that the presence of TIMP-2 in the culture supernatant inhibits binding of active MMP-2 to resin 2. To test this hypothesis, the conditioned media of HT1080 cells were subjected to gelatin-affinity purification to pull out all forms of gelatinases that were present. As shown in the immunoblot in Figure 3D, the gelatin bound fraction contained all three forms of MMP-2 (latent, intermediated, and active). In addition, TIMP-2 coeluted with the bound MMP-2 forms, which is

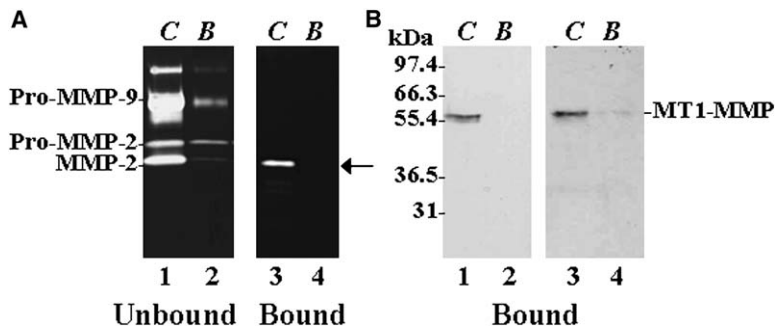


Figure 4. Binding of Active MMPs from Human Tumors to the Inhibitor Resin

(A) Gelatin zymography of carcinoma (lanes 1 and 3) and benign (lanes 2 and 4) breast tissue extracts after incubation with resin 2. Aliquots of unbound and bound fractions were analyzed. The arrow indicates active MMP-2.

(B) Immunoblot of laryngeal (lanes 1 and 2) and breast (lanes 3 and 4) tissue extracts (carcinoma, lanes 1 and 3; benign, lanes 2 and 4) after incubation with resin 2. Aliquots of bound fractions were analyzed with an antibody to MT1-MMP. C = carcinoma; B = benign.

consistent with its ability to form a complex with latent and active MMP-2 forms. TIMP-2 would not have been retained by the chromatographic resin if it were not already in complex with the gelatinases. These data lend further support to the notion that a major fraction of active MMP-2 present in the supernatant of ConA-treated HT1080 cells is in complex with TIMP-2. These results further show that resin 2 can differentiate between inhibitor-free and TIMP-complexed MMPs, and thus it is a useful tool in identification of TIMP-free MMPs in experimental biological systems.

#### Identification of Gelatinases and MT1-MMP in Human Tumor Extracts

To test the ability of resin 2 to capture active MMPs from human tumor samples, we prepared tissue extracts from breast and laryngeal carcinomas, as described in [Experimental Procedures](#). Equal protein amounts from each extract were incubated with resin 2, and the bound MMPs (gelatinases and MT1-MMP) were detected by immunoblot analysis and gelatin zymography. As shown in [Figure 4A](#), the bound fraction of tissue extracts derived from breast carcinoma showed the presence of active MMP-2 ([Figure 4A](#), lane 3) and active MT1-MMP ([Figure 4B](#), lane 3). Active MT1-MMP (~57 kDa) was also detected in the bound fraction of extracts of laryngeal cancer tissue ([Figure 4B](#), lane 1). In contrast, neither gelatinases nor MT1-MMP was detected in the bound fraction of extracts derived from benign breast ([Figures 4A and 4B](#), lane 4) and laryngeal tissues ([Figure 4B](#), lane 2). Interestingly, the unbound fractions of carcinoma ([Figure 4A](#), lane 1) and, to a lesser extent, that of benign ([Figure 4A](#), lane 2) breast tissue extracts contained significant amounts of latent and active gelatinases, suggesting that the active forms are in complex with TIMPs, and thus they were recovered in the flowthrough. Although here we have focused on detection of gelatinases and MT1-MMP, it is expected that other active MMPs would also bind to the inhibitor-tethered resin, and thus the levels of these enzymes detected in the tumor extracts may be underestimated. Because tumors are likely to express multiple MMPs, the ability to detect TIMP-free gelatinases and MT1-MMP under those conditions is consistent with the abundant expression of these enzymes in the tumor microenvironment.

We add here that inhibitor-based affinity purification of MMPs with simpler MMP inhibitors have been reported previously, but the scope of those studies did not envision the kinds of application undertaken in this

study [38–42]. Here we show for the first time that an inhibitor-tethered resin (resin 2) has the ability to capture two important proteases in cancer, namely, MMP-2 and MT1-MMP, in extracts of human cancer tissues. Most importantly, because binding of MMPs to the resin is inhibited by TIMPs, the enzymes detected represent active species that are free of TIMPs, and thus likely to contribute to tumor proteolysis. It should be mentioned, however, that differences in amounts and activation of MMPs in tissue extracts detected by the method described here may be affected by a variety of factors, including the structure of the tissue, the extraction procedure, the presence of detergents, and the storage of the samples before processing, just to mention a few. Therefore, careful attention should be given to these issues before reaching conclusions on the pattern of active MMPs present in tumor extracts.

The affinity approach described here is also useful for isolating active MMPs from tissues and cultured cells. Indeed, this resin has been instrumental in detection of the soluble ectodomain of MT1-MMP in detergent-free extracts of human breast carcinoma tissues, as we have recently reported [43]. Recent studies have shown that MT1-MMP is a key enzyme involved in the migration and invasion of tumor and endothelial cells through interstitial collagen matrix, and is thus essential for tumor metastasis and angiogenesis [44–46]. Therefore, the ability to detect active TIMP-free MT1-MMP in tumor samples may be of clinical value. Future investigations of this methodology with additional samples from pathologically characterized tumor samples are needed. Only through such investigations can we gain a deeper knowledge of active MMP profiles in cancer for identification of active MMPs worthy of targeting for selective inhibition. These efforts are especially important in light of the fact that analyses of gene expression have been instrumental in describing what MMP genes are upregulated or downregulated [24, 44, 47]. However, there is a clear information gap in what happens posttranscriptionally. There are the issues of longevity of RNA, the facility of translocation of the MMPs to the surface of the cell and to the milieu, the process of proteolytic activation of these enzyme, or even compartmentalization and turnover at the protein level, not to mention inhibition by TIMPs. All these are profoundly complicating matters that have obscured our understanding of the critically important contributions of MMPs to progression of cancer. Our studies and the general approach have the potential to have an impact at the understanding of these

enzymes at the active protein level, which is the true instigator of manifestation of pathological outcome in cancer. The results presented here are in line with recent developments aimed at profiling active proteases in biological samples [48–51], which may aid in the development of more rational approaches to target-specific enzymes in pathological conditions.

## Significance

The integrity and normal remodeling of extracellular matrix (ECM) is critical for the well-being of the organism. A number of pathological conditions, including metastasis of tumors, involve remodeling of ECM, for which matrix metalloproteinases (MMPs) have been implicated. The functions of MMPs are regulated at multiple levels, including zymogen activation, cellular localization, and enzyme inhibition by tissue inhibitors of metalloproteinases (TIMPs). MMPs play complex roles in tumor progression. Whereas there is compelling information, at the transcriptional level on what MMPs are upregulated in cancer, the detection of activities of MMPs is a challenge and cannot distinguish the functional status of these enzymes (whether they exist as zymogens, active forms, or complexed with TIMPs). It is the TIMP-free active species of MMPs that are responsible for uncontrolled catalytic activity in cancer tissues, which precipitate tumor metastasis. Development of a method for selective binding of active MMPs from biological samples using a resin-immobilized potent broad-spectrum synthetic MMP inhibitor is described. The synthesis of resin and its characterization with recombinant gelatinases (MMP-2 and MMP-9), samples of cultured cell lines, and tissue extracts are reported herein. We document for the first time that only active MMPs and not zymogen forms or MMP/TIMP complexes bound specifically to the inhibitor-tethered resin. Benign breast tissue extracts showed no detectable bound active MMPs, while breast carcinoma tissue extracts contained both active MMP-2 and MMP-14, indicating that a pool of the tumor MMPs is active, free of endogenous protein inhibitors, and thus likely to contribute to uncontrolled proteolytic events. Monitoring the changes in the levels of active MMPs using approaches such as the one described in this report may serve as a valuable tool in both detection and determination of the stage of the cancer and of potential outcome of cancer treatments.

## Experimental Procedures

### General Preparation of the Inhibitor-Tethered Resin

Epoxy-activated Sepharose 6B (Sigma, St. Louis, MO) (0.56 g) was washed thoroughly with distilled water (500 ml) before use. A solution of sodium bicarbonate (0.81 g) and potassium carbonate (6.91 g) in water (100 ml, pH 10.8), was prepared and degassed by sonication for 10 min under an atmosphere of argon (to remove oxygen). The swollen resin was placed in a flask, which was then purged with argon. The carbonate solution (5 ml) was added to the resin under argon. Compound 3 (174 mg, 0.4 mmol), with  $K_2CO_3$  (56 mg, 0.4 mmol) in water (1 ml), was sonicated for 10 min and then transferred via cannula to the swollen Sepharose 6B resin. The resultant suspension was swirled using a gyratory water bath shaker (model G76; New Brunswick Scientific) under argon at 42°C for 1 day. The resin was filtered, washed with water (200 ml), and then washed

with methanol (200 ml). The resin was transferred to a solution of 100 mM sodium phosphate buffer (pH 8.0) and the suspension was swirled under argon at 42°C for 2 additional hr. The resin was filtered and washed with methanol (5 × 40 ml), followed by five cycles (50 ml each) of alternating washes with 100 mM sodium acetate buffer (pH 4.0, 0.5 M NaCl) and 100 mM Tris/HCl buffer (pH 8.0, 0.5 M NaCl). Finally, the resin was washed with 0.5 M NaCl (100 ml) containing 1% sodium azide, and was stored at 4°C in the same solution. Sodium azide was removed before use of the resin by washing with water. Elemental analyses of the resin for both nitrogen and for sulfur indicated incorporation of the ligand at 0.4  $\mu\text{mol}/1\text{ g}$  of resin dry weight.

Synthesis of the inhibitor-tethered resin 2 was achieved in the same manner, following the general procedure described for the synthesis of 5 above using intermediate 4 under nitrogen. Elemental analyses of the resin for both nitrogen and sulfur indicated incorporation of the ligand at 0.4–0.5  $\mu\text{mol}/1\text{ g}$  of resin dry weight. The product of the control reaction (resin 6) was prepared in the carbonate solution by agitating the epoxy-activated Sepharose 6B resin in a shaker for 24 hr at room temperature under an atmosphere of argon.

### Cell Culture

Nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) and human fibrosarcoma HT-1080 (CCL-121) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Human HeLa S3 (CCL-2.2) cells were purchased from ATCC and cultured in MEM Spinner medium (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 5% horse serum and antibiotics.

### Recombinant Proteins and Antibodies

Purified human recombinant pro-MMP-2, pro-MMP-9, and TIMP-2 were expressed in HeLa S3 cells using the vaccinia expression system, and purified to homogeneity as previously described [52]. The monoclonal antibodies (mAb) to human gelatinases and to TIMP-2 were previously described [53, 54]. The mAb to the catalytic domain of MT1-MMP (LEM-2/15) [55] was a generous gift from Dr. A. Arroyo (Hospital de la Princesa, Madrid, Spain).

### Binding of Recombinant Gelatinases to the Inhibitor-Tethered Resin

To obtain active MMP-9, purified pro-MMP-9 was incubated (37°C, 2 hr) with a recombinant catalytic domain of MMP-3 (Calbiochem, San Diego, CA) at 1:12 (enzyme:substrate) molar ratio in CB buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 5 mM  $CaCl_2$ , and 0.02% Brij 35). Under these conditions, all the pro-MMP-9 is converted to the active 82 kDa species and a minor 67 and 60 kDa species. After incubation, the active MMP-9 was isolated by gelatin-Agarose chromatography, as described previously [52]. Active MMP-2 (62 kDa) was obtained by incubation (16 hr, 37°C) of pro-MMP-2 with a recombinant catalytic domain of MT1-MMP (EMD Biosciences, Inc., La Jolla, CA) in CB buffer at a 2:1 molar ratio, as previously described [54]. Pro-MMP-2 was also activated by incubation (2 hr, 37°C) with 1 mM *p*-aminophenylmercuric acetate (APMA), followed by dialysis against CB buffer to remove APMA, as previously described [56]. The inhibitor-tethered resin 2 was used in all experiments; however, resin 5 works equally well.

Active MMP-2 or MMP-9 (10 nM each) were incubated (22°C) for various time periods with or without 20  $\mu\text{l}$  of resin 2 in 0.5 ml (final volume) of CB buffer. At various times (0, 0.5, 1, 2, 4, and 20 hr), the samples were centrifuged (15,000 × g, 1 min) and aliquots of the supernatants (unbound fractions) were collected, mixed with reducing Laemmli sample buffer [57], and resolved by SDS-PAGE, followed by immunoblot analysis [37] with mAbs to human gelatinases (mAb801 to MMP-2 and mAb209 to MMP-9). After 20 hr incubation, the resin was washed several times with CB buffer, and the bound proteins were eluted with Laemmli sample buffer without reducing agents. The bound fractions were analyzed by gelatin zymography, as previously described [58]. In some experiments, active MMP-2 was incubated (22°C, 1 hr) with or without TIMP-2 at 1:1.5 molar ratio (MMP-2:TIMP-2). Then, the free and TIMP-2-complexed MMP-2 (200 nM each) were each incubated (18 hr, 4°C) with 10  $\mu\text{l}$  resin 6

(control) or resin 2 in 0.6 ml CB buffer (final volume). The samples were centrifuged ( $15,000 \times g$ , 1 min), and the supernatants (unbound fractions) were analyzed by gelatin zymography and for enzyme activity with the peptide substrate MOCAC-Pro-Leu-Gly-Leu-A<sub>2</sub>pr(Dnp)-Ala-Arg-NH<sub>2</sub> (Peptides International, Louisville, KY) [53]. Substrate hydrolysis was monitored for 15 min in a Varian Cary Eclipse fluorescence spectrophotometer at excitation and emission wavelengths of 328 and 393 nm, respectively. After consecutive washes of the resin with CB buffer and CB buffer supplemented with 1 M NaCl, the bound MMP-2 was eluted with Laemmli sample buffer. Aliquots of the samples of bound and unbound fractions were resolved by reducing 12% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue.

Resins 2 and 5 (in the range of 0.25–1.00  $\mu$ l) were incubated (18 hr, 4°C) with 200 nM active MMP-2 in 0.6 ml CB buffer (final volume). The samples were centrifuged ( $15,000 \times g$ , 1 min), and the supernatants were collected and analyzed for MMP-2 activity with the fluorescence-quenching peptide substrate as described above.

#### HT1080 Cell Treatment and Analysis of Conditioned Media

HT1080 cells were treated (12 hr) with 10  $\mu$ g/ml concanavalin A (ConA; Sigma) or 100 nM phorbol ester (referred to as TPA; Sigma) in serum-free media. The serum-free media (15 ml) were collected and, after a brief centrifugation, were incubated with 100  $\mu$ l of either resin 2 for 18 hr at 4°C or with gelatin-Agarose beads for 2 hr at 4°C. The gelatin beads were washed with CB buffer, and the bound proteins were eluted with Laemmli sample buffer and subjected to immunoblot analysis with antibodies to MMP-2 (mAb 801) and to TIMP-2 (mAb 101). The supernatants (unbound fractions) of resin 2-containing samples were collected after a brief centrifugation. The resins were then thoroughly washed with CB buffer, and the bound proteins were eluted with 25  $\mu$ M Marimastat (150  $\mu$ l). Aliquots of the unbound and bound fractions were analyzed by gelatin zymography.

#### Identification of Gelatinases and MT1-MMP in Human Tumor Extracts

Samples of benign and carcinoma breast and laryngeal tissues were collected from excess tissues sent for histopathological diagnosis to the Department of Pathology at Harper Hospital (Detroit, MI). The collection and use of human tissue samples was approved by the Wayne State University Institutional Review Board according to federal regulations (HIC no. 022803MP2E). The tissue samples were homogenized in cold homogenization buffer (25 mM Tris/HCl [pH 7.5], 8.5% sucrose, 50 mM NaCl, and protease inhibitors, with the exception of metalloproteinase inhibitors), the extracts were clarified, and the inhibitor-tethered resin was applied to the equal total protein amounts of tissue homogenate, as described previously [43]. The protein concentration was measured in carcinoma and benign homogenates (either by OD<sub>280</sub> reading or by BCA protein assay kit) and adjusted to equal protein amounts with homogenization buffer. Aliquots of the eluates and the unbound fractions were then subjected to gelatin zymography to detect the secreted active gelatinases. Individual pairs of malignant and benign tissue samples (derived from laryngeal or breast tissues) were homogenized in 10 vol lysis buffer (25 mM Tris/HCl [pH 7.5], 100 mM NaCl, 1% NP-40, and protease inhibitors, with the exception of metalloproteinase inhibitors) to solubilize membrane-type MMPs. The lysates were centrifuged, and the floating lipid fraction was removed. The clarified tissue extracts were diluted with CB buffer, and 100  $\mu$ l inhibitor-tethered resin was applied at 4°C overnight. The resin was washed several times with CB buffer. The bound proteins were eluted with the same volume of sample buffer. Equal aliquots of the bound fractions from benign and carcinoma tissue samples were resolved by reducing SDS-PAGE, followed by immunoblot analysis using the mAb LEM-2/15 antibody against the catalytic domain of MT1-MMP.

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