Proteolytic activity of cell surface-associated MT1-matrix metalloproteinase (MMP) (MMP-14) is directly related to cell migration, invasion, and metastasis. MT1-MMP is regulated as a proteinase by activation and conversion of the latent proenzyme into the active enzyme, and also via inhibition by tissue inhibitors of MMPs (TIMPs) and self-proteolysis. MT1-MMP is also regulated as a membrane protein through its internalization and recycling. Routine immunohistochemistry, flow cytometry, reverse transcription-PCR, and immunoblotting methodologies do not allow quantitative imaging and assessment of the cell-surface levels of the active, TIMP-free MT1-MMP enzyme. Here, we developed a fluorescent reporter prototype that targets the cellular active MT1-MMP enzyme alone. The reporter (MP-3653) represents a liposome tagged with a fluorochrome and functionalized with a PEG chain spacer linked to an inhibitory hydroxamate warhead. Our studies using the MP-3653 reporter and its inactive derivative demonstrated that MP-3653 can be efficiently used not only to visualize the trafficking of MT1-MMP through the cell compartment, but also to quantify the femtomolar range amounts of the cell surface-associated active MT1-MMP enzyme in multiple cancer cell types, including breast carcinoma, fibrosarcoma, and melanoma. Thus, the levels of the naturally expressed, fully functional, active cellular MT1-MMP enzyme are roughly equal to 1 × 10^5 molecules/cell, whereas these levels are in a 1 × 10^6 range in the cells with the enforced MT1-MMP expression. We suggest that the reporter we developed will contribute to the laboratory studies of MT1-MMP and then, ultimately, to the design of novel, more efficient prognostic approaches and personalized cancer therapies.

To establish metastasis from a primary tumor to distal organs, cancer cells down-regulate cell adhesion molecules to acquire motility and produce matrix metalloproteinases (MMPs) to degrade the extracellular matrix and basement membrane barriers (1–4). The MMPs family is represented by 18 soluble and 6 membrane-type (MT-MMP) individual zinc-dependent endopeptidases in humans. In contrast with soluble MMPs, MT-MMPs exhibit either a transmembrane domain followed by a short cytoplasmic tail (MT1-, MT2-, MT3-, and MT5-MMP) or a glycosylphosphatidylinositol moiety (MT4- and MT6-MMP) that tethers MT-MMPs to the plasma membrane (3, 5).

Because MT-MMPs can be controlled and concentrated at subcellular locations, these proteinases seem to play more significant roles than soluble MMPs during cancer invasion (6). There is a consensus among researchers that pro-invasive MT1-MMP is a key element in tumor cell migration and pericellular proteolysis (7–11). To become catalytically active, the latent MT1-MMP proenzyme requires proteolytic removal of its inhibitory prodomain (12–16). The active MT1-MMP enzyme cleaves extracellular matrix components, cell adhesion, and signaling receptors, and also initiates the activation pathway of soluble MMP-2 and MMP-13 (17–24). As a result, pro-invasive, pro-tumorigenic MT1-MMP usurps tumor growth control and stimulates cancer cell invasion and metastasis (25–30). MT1-MMP also plays a critical role in normal development: in contrast with other MMP knockouts with minor developmental defects, MT1-MMP null mice are dwarfs and die at adulthood (31).

Once activated, MMPs, including MT1-MMP, can be inhibited by tissue inhibitors of MMPs (TIMPs) (32, 33). The balance
between active MMPs and TIMPs is critical for the net proteolytic activity of MMPs. There are four individual TIMPs (TIMP-1, -2, -3, and -4) in humans (34). TIMP-2, -3, and -4 are potent, low nanomolar range inhibitors of MT1-MMP, whereas TIMP-1 is an inefficient MT1-MMP inhibitor (35, 36).

Because MMPs are promising drug targets in malignancy, multiple small-molecule inhibitors have been developed and tested in vitro and in vivo. Among them, hydroxamates (e.g. Ilomastat/GM6001) have been most extensively studied as small molecule drug leads characterized by an effective zinc-binding group and an additional side chain responsible for the selectivity (37).

MT1-MMP is regulated both as a proteasine and as a membrane-tethered protein by coordinated mechanisms including activation of the MT1-MMP proenzyme, inhibition by TIMPs, self-proteolytic inactivation, homodimerization, trafficking throughout the cell to the plasma membrane, internalization into the transient endosomal compartments inside the cell and recycling back to the plasma membrane (10, 38–42). To support directional cell locomotion, the de novo synthesized MT1-MMP is specifically trafficked to the leading front and the trailing edge in migrating cancer cells (6, 25–30, 43–45).

Because of its migration-promoting capabilities, MT1-MMP can be detected in a wide range of human cancers in clinical samples and its expression is elevated in the most aggressive cancer types, including triple-negative breast cancer (46, 47). It is likely that MT1-MMP activity in breast tumors is also essential for blood vessel invasion (48). Thus, the highest expression of MT1-MMP is present in the specimens showing lymph node metastasis (49).

A ligand that specifically binds to MT1-MMP may facilitate the labeling of this molecule, allow the imaging at the cellular and organism levels, and provide a means for targeted drug delivery specific to MT1-MMP (50–52). However, in addition to the TIMP-free active MT1-MMP enzyme, there is an excess of the latent proenzyme and the enzyme to the TIMP-free active MT1-MMP enzyme, there is an excess delivery specific to MT1-MMP (50–52). However, in addition and organism levels, and provide a means for targeted drug labeling of this molecule, allow the imaging at the cellular and additional side chain responsible for the selectivity (37).

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**General Reagents and Antibodies**—All reagents were purchased from Sigma unless indicated otherwise. A murine monoclonal antibody (clone 3G4), a rabbit polyclonal antibody (AB8345), and a broad spectrum hydroxamate inhibitor (GM6001) were purchased from EMD Millipore. A murine monoclonal antibody to α-tubulin was obtained from Molecular Probes. The SuperSignal West Dura Extended Duration Substrate kit was from Pierce. The secondary species-specific antibodies conjugated with horseradish peroxidase and Alexa Fluor 594 were purchased from Jackson ImmunoResearch and Molecular Probes, respectively. (7-Methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-l-2,3-diaminopropionyl)-Ala-Arg-NH$_2$ (MCA-PLGL-Dpa-AR-NH$_2$) was obtained from R&D Systems. Human TIMP-1 was obtained from Invitrogen. Hydrogenated soybean L-α-phosphatidylcholine (PC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-PEG(5000) were from Lipoid. Cholesterol was purchased from Fisher. Diethylenetriamine pentaaetic acid and DSPE-PEG(2000)-carboxyfluorescein were obtained from Acros and Avanti, respectively.

**Cells**—Madin-Darby canine kidney (MDCK), human breast carcinoma MCF-7, MDA-MB-231, and fibrosarcoma HT1080 cells were obtained from ATCC (Manassas, VA). Murine melanoma B16F1 cells were kindly provided by Dr. Ralph A. Reisfeld (The Scripps Research Institute, La Jolla, CA). All cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and gentamicin (10 μg/ml). MCF-7 cells stably transfected with the empty pcDNA3-zeo vector (MCF7-mock cells) or the pcDNA3-zeo plasmid encoding the full-length MT1-MMP (MCF7-MT1 cells) or the C-end truncated mutant (ΔCT) lacking the amino acids 563–582 of the cytoplasmic tail (MCF7-ΔCT cells) were obtained earlier (55, 56). MCF-7 cells were also stably transfected with the full-length human FLAG-tagged MT3-MMP and MT6-MMP to generate the MCF7-MT3 and MCF7-MT6 cells, respectively (57). HT1080 cells with shRNA transcriptional silencing of MT1-MMP (HT-shMT1 cells) were isolated and characterized earlier (58). B16F1 cells stably transfected with the empty pcDNA3-zeo vector (B16F1-mock cells) or the pcDNA3-zeo plasmid encoding the murine full-length MT1-MMP (B16F1-mMT1) were also generated and described earlier (59).

**Recombinant Proteins**—The individual catalytic domains of MT1-, MT3-, MT5-, and MT6-MMP were expressed in *Escherichia coli*, purified from the inclusion bodies using metal-chelating chromatography and refolded to restore their native conformation (60, 61). The MMP-2 and MMP-9 proenzymes were purified from the serum-free medium conditioned by stably transfected HEK293 cells and then activated using 4-aminophenylmercuric acetate (62). The purity of the isolated MMPs was confirmed using SDS-gel electrophoresis. Only the MMP samples with the purity over 95% were used in our further stud-
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Recombinant Human TIMP-2—The full-length secretory TIMP-2 construct was cloned from the Human Lung QUICK-Clone™ cDNA library (Clontech) using 5′-CACCATTGGCGCCTGGGCGGGCCG-3′ and 5′-TGGTCCGTAGTGCGAGAAACTCG-3′ oligonucleotides as the forward and reverse primers, respectively. The TIMP-2 construct was then C-terminally tagged with the V5 and His6 tags and inserted into the pcDNA3.1 vector (Invitrogen) (Fig. 1). MDCK cells were stably transfected with the pcDNA3.1-TIMP-2 construct. After selecting the most efficient MDCK cell clones, the latter were stably transfected with the pcDNA3.1-TIMP-2 construct. MDCK cells. The medium was collected, centrifuged at 3,000 × g, filtered through a 0.22-μm filter, and concentrated 100-fold using a 30-kDa cut-off filter to reach a 4-ml volume. As determined by measuring the total lipid content of the samples and the particle size.

Warhead and Carboxyfluorescein Insertion into the Liposomes—The inhibitory hydroxamate warhead (MP-3601) N-hydroxy-4-((4-phenoxyphenyl)sulfonyl)piperidine-4-carboxamide-PEG(5000)-DSPE conjugate was synthesized as described (64). To accomplish the incorporation of the warhead and the carboxyfluorescein moieties into the lipid bilayer, the liposomes (4 ml) were mixed with 2 ml of MP-3601 (1 mg/ml) and 1 ml of DSPE-PEG(2000)-carboxyfluorescein (1 mg/ml). The mixture was incubated with stirring at 37 °C for 12 h in the dark. The resulting sample was purified using a Sepharose CL-4B column (GE Healthcare; column size 1.9 × 23 cm) equilibrated in PBS. The purified material was concentrated using a centrifugal filter unit to reach a 4-ml volume. As determined by using a Malvern particle size analyzer, a mean particle size of the final sample was 61.65 nm. The amounts of the incorporated warhead (70 warheads per particle) and DSPE-PEG(2000)-carboxyfluorescein (440 fluorochromes per particle) were determined by HPLC using the respective calibration curves. This final liposomal formulation was further referred to as MP-3653.

A similar procedure was used to prepare the inactive liposome derivative (called MP-3655; a mean particle size of 61.89 nm). The latter was coupled with the inactive methyl ester warhead (MP-3616) rather than with the hydroxamate inhibitory warhead MP-3601 (Fig. 2A). The final MP-3655 sample contained 50 warheads and 380 fluorochromes per particle.

In Vitro Inhibitory Assays—MMP activity was measured in triplicate in wells of a 96-well plate in 0.2 ml of buffer, containing 50 mM HEPES, pH 7.5, 1 mM CaCl2, 50 μM ZnCl2, 0.5 mM methanol (3:1 v/v) in a 500-ml round-bottom flask. After mixing, the solvent was evaporated at 40 °C under reduced pressure and constant rotation to form a thin lipid film. Residual solvent was additionally removed for 18 h under reduced pressure at ambient temperature. Lipid film was hydrated with 50 ml of diethylenetriamine pentaacetic acid solution (100 mM in PBS, pH 7.7) at 65 °C for 2 h. The resulting sample was then sized by repeated extrusion through polycarbonate membranes of decreasing pore size (0.1 and 0.03 μm; 5 times under nitrogen at 400 p.s.i. and 10 times at 800 p.s.i., respectively; 65 °C) to prepare small unilamellar liposomes. The dialfiltration using a 30-kDa cut-off filter was further used to obtain the final liposome sample (50 ml; pH 6.98). Liposome concentration was determined by measuring the total lipid content of the samples and the particle size.

Preparation of Liposomes—Liposomes were composed of hydrogenated soybean PC, cholesterol, and DSPE-PEG(2000). Hydrogenated soybean PC, cholesterol, and DSPE-PEG(2000) (11:8:1 molar ratio) were dissolved in 40 ml of chloroform:
MgCl₂, MCA-PLGL-Dpa-AR-NH₂ (10 μM) was used as a substrate. To measure the potency of MP-3653 and MP-3655, their increasing concentrations (1 nM to 5 μM) were co-incubated with the individual MMPs (10 nM) for 30 min at ambient temperature. After adding the substrate, the reaction velocity of the samples was monitored continuously at 320 nm and 400 nm using a fluorescence spectrophotometer. Residual activity of MMPs was then determined. The IC₅₀ values were calculated by determining the inhibitor concentrations that inhibited the MMP activity by 50%. GraphPad Prism was used as fitting software. The results were highly reproducible without any significant day to day variations.

**Cell-based Assays**—Cells (2–10 × 10⁴) in 0.1 ml of DMEM containing 10% FBS were seeded for 24 h in triplicate in wells of a 96-well plate. After washing and blocking with 0.2% BSA in DMEM, the indicated concentrations of MP-3653 (10–100 nM) alone or jointly with TIMP-2 (1 μM) or GM6001 (1 μM) were added to the wells for 45–180 min at 37 °C. After extensive washing to remove the unbound MP-3653, 0.05 ml of 100 mM HEPES, pH 7.5, supplemented with 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1% Triton X-100 were added to the wells. The plate was incubated for an additional 15 min in the dark and the levels of the cell-bound MP-3653 were then measured at λₑₓ = 492 nm and λₑₘ = 520 nm using a fluorescence plate reader. The levels of the cell-associated MP-3653 were calculated using a calibration curve. For this purpose, MP-3653 (15–150 fmol in 0.05 ml of 100 mM HEPES, pH 7.5, supplemented with 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1% Triton X-100) was added to the adherent cells (2–10⁴) plated in wells of a 96-well plate and then the level of fluorescence was measured at λₑₓ = 492 nm and λₑₘ = 520 nm using a fluorescence plate reader. Where indicated, cells were co-incubated for 30 min at 37 °C with TIMP-2 (1 μM) or GM6001 (1 μM) or stimulated for 18 h with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) prior to adding MP-3653.

**Fluorescence Uptake Assays**—Cells (at a 25–70% confluence range) grown on a coverslip were washed using ice-cold DMEM containing 25 mM HEPES, pH 7.0, and 0.2% BSA, and then incubated on ice for 15 min in the same medium. Increasing concentrations of MP-3653 (8–1,000 nM) were added as a control. RFU, relative fluorescence units.

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**FIGURE 2.** MP-3653/MP-3655 liposomal formulation structure and inhibitory activity. **A**, the structure and composition of MP-3653 and MP-3655. MP-3653 contains the inhibitory hydroxamate warhead (MP-3601) inserted into the lipid bilayer via a PEG(5000) linker. MP-3655 contains the inactive methyl ester warhead derivative (MP-3616) inserted into the lipid bilayer via a PEG(5000) linker. The DSPE-carboxyfluorescein moiety is inserted into the lipid bilayer via a PEG(2000) linker. Diethylene triamine pentaacetic acid (DTPA) is used for hydration of the liposomal samples. **B**, IC₅₀ values for MP-3653 against multiple MMPs. **Left**, the MMP activity was measured in the presence of increasing concentrations of MP-3653 (1 nM to 2 μM) using MCA-PLGL-Dpa-AR-NH₂ as a substrate. The MMP concentrations in the reactions were 10 nM. **Right**, a representative dose-response curve of MP-3653 against the catalytic domain of MT1-MMP. The inactive MP-3655 liposomal formulation (1 nM to 5 μM) was used as a control. RFU, relative fluorescence units.
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absence of the MT1-MMP 3G4 antibody (50 nM) were then added to the cells in DMEM containing 0.2% BSA and 25 mM HEPES, pH 7.0, and incubation was continued for an additional 1 h at 4 °C. Cells were next extensively washed using the above medium to remove the unbound reagents. Cells were then either immediately fixed with 4% paraformaldehyde (PFA) or shifted for 20–60 min at 37 °C in the same medium to allow the internalization of both the cell-bound MP-3653 and the MT1-MMP 3G4 antibody and then fixed with PFA. To visualize the MT1-MMP 3G4 antibody, the cells were additionally permeabilized for 4 min with 0.1% Triton X-100 in 4% PFA, blocked for 1 h in 0% BSA, and then stained using DAPI and analyzed using a fluorescence microscope equipped with a digital camera.

Immunofluorescence Microscopy—For routine observations, cells grown on a coverslip were washed with PBS, fixed for 16 min with 4% PFA, permeabilized for 4 min using 0.1% Triton X-100 in 4% PFA, blocked for 1 h in 10% BSA, and then stained for 16–18 h at 4 °C using the MT1-MMP 3G4 antibody (dilution 1:800), the monoclonal anti-FLAG M2 antibody (dilution 1:800), or stimulated for 18 h with PMA (50 ng/ml) prior to adding MP-3653. Cells were then fixed with 4% PFA in PBS for 10 min, mounted in the VectaShield mounting medium containing DAPI for the nuclear staining (Vector Lab), and examined on a fluorescence microscope equipped with a digital camera.

Gelatin Zymography and Western Blotting—Gelatin zymography of the conditioned medium aliquots and Western blotting of the cell lysate samples were performed as described earlier (65, 66). For gelatin zymography, where indicated, the purified MMP-2 proenzyme (0.5 nM) was added to the cells. In Western blotting, the use of either the mouse monoclonal or the rabbit polyclonal MT1-MMP antibodies was followed by the secondary horseradish peroxidase-conjugated species-specific antibody and a SuperSignal West Dura Extended Duration Substrate kit. Where indicated, cells were co-incubated for 18 h with GM6001 (25 μM) or PMA (50 ng/ml).

RESULTS

In Vitro Inhibition of MT1-MMP by MP-3653—The MP-3653 liposome formulation includes a potent hydroxamate inhibitory warhead conjugated with the DSPE-PEG(5000) moiety. The coupling conditions we used to obtain MP-3653 preserved the inhibitory potency of the warhead (Fig. 2A). A structurally matched inactive liposome formulation (MP-3655) containing the inactive methyl ester warhead derivative (MP-3616) was used as a control in our experiments (Fig. 2A). Both liposomal formulations included the DSPE-PEG(2000)-carboxyfluorescein moiety. The latter facilitated the identification of the MP-3653 and MP-3655 liposomes via fluorescence.

In *in vitro* cleavage tests, MP-3653 performed as a potent, low nanomolar, inhibitor of MT1-MMP (Fig. 2B). In turn, the MP-3655 inactive derivative did not show any inhibition of MT1-MMP. MP-3653, however, exhibited an ability to inhibit the catalytic activity of soluble MMP-2 and MMP-9, albeit less efficiently as compared with MT1-MMP. There was also a noticeable inhibitory efficacy of MP-3653 against MT3-MMP and MT5-MMP, both of which are close structural analogs of MT1-MMP, whereas the distantly related MT6-MMP was not efficiently inhibited by MP-3653 control.

Imaging of Cellular MT1-MMP—To test the ability of MP-3653 to bind the cellular MT1-MMP enzyme, we used breast carcinoma MCF7-MT1 and MCF7-ΔCT cells with the enforced expression of the full-length MT1-MMP and the C-end-truncated MT1-MMP mutant, respectively. In contrast with the full-length protease, the ΔCT mutant is not efficiently internalized (40, 67). As a result, the continuing presence of this mutant at the cell surface facilitates its binding to MP-3653. MCF7-mock cells, which do not express any significant level of MT1-MMP, served as a control. As expected, immunostaining using the MT1-MMP 3G4 antibody confirmed high levels of MT1-MMP in both the MCF7-MT1 and MCF7-ΔCT cells, whereas the MCF7-mock were clearly deficient in MT1-MMP (Fig. 3).

Following a 1-h co-incubation with MP-3653, the cells were washed to remove the unbound reagent, fixed, and observed using a fluorescence microscope. The inactive MP-3655 derivative was inefficient in both the interaction with cellular MT1-MMP and the imaging of the MCF7-MT1 and MCF7-ΔCT cells. In contrast, MP-3653 binding and the resulting cell fluorescence was readily observed in MCF7-MT1 and MCF7-ΔCT cells but not in MT1-MMP-deficient MCF7-mock cells (Fig. 3). GM6001 (1 μM), a potent inhibitor of MMPs, including MT1-MMP (k_i = 0.5 nM against MT1-MMP), when added jointly with MP-3653, fully abolished the binding of the latter to cellular MT1-MMP and, therefore, cell fluorescence was not observed in MCF7-MT1 and MCF7-ΔCT cells.

Uptake of MP-3653 by the Cells—To determine whether the MP-3653-MT1-MMP complex was actively internalized by the cells, MCF7-MT1 cells were co-incubated with increasing concentrations of MP-3653 for 1 h at 4 °C to allow the reagent to associate with cellular MT1-MMP. After washing the unbound MP-3653, the cells were transferred to 37 °C for 20–60 min to stimulate the uptake of the pre-formed MP-3653-MT1-MMP complex. Microscopic evaluation revealed that, after its predominant association with the cell surface at t = 0 min, MP-3653 fluorescence was rapidly internalized in a dose-dependent manner and then detected at both t = 20 and 60 min as the multiple vesicles in the cell cytoplasm, especially in the perinuclear region at t = 60 (Fig. 4, A and B). To corroborate these results, MCF7-MT1 cells were treated with nocodazole prior to MP-3653 uptake. As expected, nocodazole disrupted the microtubular cytoskeleton in the cells as visualized by immunostaining of the cells with the α-tubulin antibody (Fig. 4B). As a result, the internalized vesicular MP-3653-MT1-MMP complex was largely accumulated at the cell periphery rather than
being distributed throughout the cell compartment alongside the microtubule network. Overall, our results suggest that the cell-surface MP-3653/H18528 MT1-MMP complex is normally internalized by the cells and accumulated in the endocytic pathway as vesicles in the cytoplasm. These results agree well with the observation by us and others (39, 40).

To additionally confirm that the selective MP-3653 uptake took place through cellular MT1-MMP, we performed the MT1-MMP 3G4 antibody uptake using MCF7-MT1 cells. This antibody recognizes the catalytic domain of MT1-MMP. However, in contrast with the active site-targeting GM6001, the antibody did not compete with MP-3653 binding to the active site of MT1-MMP. Thus, we next co-incubated the cells jointly with the 3G4 antibody and MP-3653. As a result, a 40:1 antibody MP-3653 molar excess did not affect the binding of MP-3653 to cell-surface MT1-MMP in MCF7-MT1 cells (Fig. 5A).

We then subjected the MCF7-MT1 cells to a double uptake of the MT1-MMP antibody and MP-3653. Thus, the cells were co-incubated at 4 °C with the MT1-MMP antibody and MP-3653 (50 nM each). After washing the unbound reactants, the incubation was continued for 1 h at 37 °C followed by immunostaining of MT1-MMP and the detection of MP-3653 fluorescence. These results demonstrated a predominant direct co-localization of the vesicular MT1-MMP immunoreactivity (red) with MP-3653 fluorescence (green) in the cells, suggesting that cellular MT1-MMP functioned as a vehicle in MP-3653 uptake (Fig. 5B).

**Effects of TIMPs**—To corroborate our results further, we tested if TIMP-1 and TIMP-2 (an inefficient and a very potent inhibitor of MT1-MMP, respectively) affected the binding of
MP-3653 to cellular MT1-MMP (35, 36, 68). For these purposes, MCF-7 MT1 cells were co-incubated with TIMP-1 or TIMP-2 (at a 40:1 TIMP-MP-3653 molar ratio) prior to adding MP-3653. GM6001 was used as a control. As expected, both GM6001 and TIMP-2 totally abolished the binding of MP-3653 to cellular MT1-MMP, whereas TIMP-1 was without any significant effect (Fig. 6A).

We next determined if the inhibition of MP-3653 binding by TIMP-2 occurred in a dose-dependent manner in MCF-7 MT1 cells. For this purpose, the cells were co-incubated with the indicated concentrations of TIMP-2 prior to adding MP-3653 (25 nM). Fluorescence microscopy revealed that as low as 10 nM levels of TIMP-2 were sufficient to partially suppress MP-3653 binding to cellular MT1-MMP, whereas a 33 nM TIMP-2 concentration blocked almost quantitatively MP-3653 binding, suggesting the dose-dependent inhibition of MP-3653 binding by TIMP-2 (Fig. 6B). Because TIMP-2 is a subnanomolar inhibitor of MT1-MMP, it is not surprisingly that a near equimolar amount of TIMP-2 relative to that of MP-3653 almost quantitatively abolished the binding of the latter to cellular MT1-MMP. Conversely, these results suggest that MP-3653 can bind only the TIMP-2-free MT1-MMP but not the pre-formed TIMP-2-MT1-MMP stoichiometric complex.

**MP-3653 Binds Cellular MT3-MMP but Not MT6-MMP**—Because there is a significant level of structure homology among MT-MMPs (69), especially in the active site region, we tested if MP-3653 interacted with cellular MT3-MMP and MT6-MMP. Structurally, MT3-MMP is closely related to MT1-MMP, whereas MT6-MMP is the most distant relative of MT1-MMP. For these purposes, we used MCF-7 cells stably transfected with the FLAG-tagged MT3-MMP and MT6-MMP constructs (MCF7-MT3 and MCF7-MT6 cells, respectively). Immunostaining using the M2-FLAG antibody clearly demonstrated high expression levels of the tagged MT3-MMP and MT6-MMP constructs in MCF7-MT3 and MCF7-MT6 cells, respectively (Fig. 7A). These cells were then incubated for 1 h with MP-3653, fixed, and observed using a fluorescence microscope. Our results demonstrated that, similar to cellular MT1-MMP, MP-3653 was capable of interacting efficiently with cell-surface MT3-MMP in MCF7-MT3 cells but not with MT6-MMP in MCF7-MT6 cells (Fig. 7A). These results are consistent with the IC$_{50}$ values of MP-3653 we recorded in our *in vitro* assays (Fig. 2B). MP-3653 was also able to associate with cell-surface MT2-MMP, which is the closest relative of MT1-MMP (data not shown). As expected, GM6001 and TIMP-2 readily repressed the binding of MP-3653 with cellular MT3-MMP in MCF7-MT3 cells (TIMP-2 data not shown), suggesting MT3-MMP as a primary target of MP-3653 in these cells.

**Both GM6001 and MP-3653, but Not TIMP-2, Bind the Catalytically Inert MT1-MMP Mutant**—To substantiate our data even further, we next evaluated the capability of MP-3653 to interact with the catalytically inert E240A MT1-MMP mutant. In this mutant Ala substituted for the essential Glu-240 of the proteinase active site. Immunostaining using the MT1-MMP 3G4 antibody confirmed a high expression level of the mutant in MCF7-E240A cells (Fig. 7B). Earlier data by us and others suggested that because of the structural re-arrangement of the active site caused by the E240A mutation, the resulting mutant construct does not efficiently bind TIMP-2 (34). For these purposes, MCF7-E240A cells were co-incubated with TIMP-2 or GM6001 prior to adding MP-3653. In agreement, according to our current data the E240A mutant lost the ability to interact efficiently with TIMP-2. As a result, TIMP-2 (at a 20-fold molar excess relative to MP-3653) did not significantly interfere with MP-3653 binding to MCF7-E240A cells (Fig. 7B). In turn, because both MP-3653 and GM6001 bind directly the zinc atom of the proteinase active site, GM6001 (at a 20-fold molar excess relative to MP-3653) efficiently abrogated the association of MP-3653 with MCF7-E240A cells.

**MP-3653 Binds Murine MT1-MMP**—Mice are the most frequently used for modeling human diseases, including malignancies. There is a 4-amino acid residue difference in the protein sequence of the catalytic domain of murine MT1-MMP as compared with the human proteinase. We next determined if, in addition to efficiently interacting with human MT1-MMP, MP-3653 was also capable of interacting with murine MT1-MMP. For this purpose, we used murine melanoma B16F1 cells with the enforced expression of murine MT1-MMP (B16F1-mMT1 cells). Mock-transfected B16F1 cells (B16F1-mock cells) were employed as a control. As demonstrated earlier by us and others (59), the MT1-MMP 3G4 antibody efficiently cross-reacted with the murine proteinase. Immunostaining using the MT1-MMP 3G4 antibody confirmed high levels of MT1-MMP in B16F1-mMT1 cells. In turn, the levels of endogenous MT1-MMP immunoreactivity were low in the B16F1-mock cell control (Fig. 8A). To determine whether MP-3653 was capable of interacting with murine MT1-MMP, B16F1-mock and B16F1-
mMT1 cells were incubated with MP-3653. Fluorescence microscopy demonstrated the ability of MP-3653 to bind to cell-surface murine MT1-MMP in B16F1-mMT1 cells but not in B16F1-mock cells (Fig. 8A). GM6001, if jointly added with MP-3653 to B16F1-mMT1 cells, abrogated MP-3653 binding. These results confirm murine MT1-MMP as a target of MP-3653.

Western blotting of the cell lysate aliquots confirmed high levels of MT1-MMP in B16F1-mMT1 cells, especially when compared with B16F1-mock cells (Fig. 8B). Co-incubation of cells with GM6001 increased the detected levels of the mature MT1-MMP enzyme in B16F1-mMT1 cells (Fig. 8B, short exposure). In turn, the inhibitor did not significantly increase the levels of endogenous MT1-MMP in the control cells (Fig. 8B, long exposure). Based on our previous results (59), these data can be easily explained by the presence of the TIMP-2-free proteinase in B16F1-mMT1 cells. In these MT1-MMP-overexpressing cells, the levels of the proteinase exceed the available amounts of TIMP-2. Normally, this TIMP-2-free MT1-MMP enzyme is prone to self-proteolysis. In the presence of GM6001, however, this TIMP-2-free portion is inactivated and stabilized and, as a result, a high level of the MT1-MMP enzyme is observed in Western blots. However, in B16F1-mock cells the low levels of the endogenous MT1-MMP enzyme are roughly equal to those of TIMP-2. As a result, the endogenous, inactivated and stabilized proteinase already exists as a TIMP-2-MT1-MMP complex. The addition of GM6001 to B16F1-mock cells, therefore, did not result in any additional stabilization of MT1-MMP enzyme and did not increase the levels of the latter observed in Western blots. In addition, these data are consistent with our gelatin zymography results in B16F1-mock and B16F1-mMT1 cells. Thus, B16F1-mMT1 cells readily activated the 68-kDa MMP-2 proenzyme (a well-known activation target of cellular MT1-MMP) and generated the 62–64-kDa MMP-2 enzyme, whereas the B16F1-mock control was incapable of MMP-2 activation (Fig. 8C).

**MP-3653 Interacts with MT1-MMP Produced by Cancer Cells Naturally**—To determine whether MP-3653 interacts with MT1-MMP naturally produced in cancer, we used human breast carcinoma MDA-MB-231 and fibrosarcoma HT1080 cells. We also used HT1080 cells with the transcriptionally silenced expression of MT1-MMP (HT-shMT1 cells; a negative control). Where indicated, cells were stimulated with PMA (50 ng/ml) to up-regulate the levels of the natural MT1-MMP activity.

**FIGURE 6. TIMP-2 competes MP-3653 binding.** A, MCF7-MT1 cells were co-incubated at 37 °C for 30 min with TIMP-1, TIMP-2, or GM6001 (1 μM each). The incubation was then continued for an additional 2 h in the presence of the respective inhibitor and MP-3653 (25 nM), Intact, cells were co-incubated with MP-3653 alone. Scale bars, 30 μm. B, MCF7-MT1 cells were co-incubated at 37 °C for 30 min with the indicated concentrations of TIMP-2. The incubation was then continued for an additional 2 h in the presence of the respective TIMP-2 concentrations and MP-3653 (25 nM). MP-3653 (green), DAPI (blue). Scale bars, 20 μm.
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![Figure 7](image1.png)  
**Figure 7.** MP-3653 binds MT3-MMP but not MT6-MMP. A, left panels, MCF7-MT3 and MCF7-MT6 cells were stained using the M2-FLAG antibody followed by secondary anti-mouse antibody conjugated with Alexa Fluor 594. Right two panels, cells were co-incubated at 37°C for 30 min with GM6001 (1 µM) or left untreated. The incubation was then continued for an additional 2 h in the presence or absence of GM6001 and MP-3653 (50 nM). MT3-MMP and MT6-MMP (red), MP-3653 (green), DAPI (blue). Scale bars, 20 µm. B, left panel, MCF7-E240A cells were stained using the MT1-MMP 3G4 antibody followed by secondary anti-mouse Alexa Fluor 594-conjugated antibody. Right three panels, MCF7-E240A cells were co-incubated at 37°C for 30 min with GM6001 or TIMP-2 (1 µM each). The incubation was then continued for an additional 2 h in the presence of the respective inhibitor and MP-3653 (25 nM). Intact cells were co-incubated with MP-3653 alone. MT1-MMP (red), MP-3653 (green), DAPI (blue). Scale bars, 25 µm.

Gelatin zymography of the conditioned medium aliquots demonstrated low levels of conversion of the 68-kDa MMP-2 proenzyme into the activated, 62–64-kDa MMP-2 enzyme in the unstimulated HT1080, HT-shMT1, and MDA-MB-231 cells (Fig. 9A). Following PMA stimulation, the 92-kDa MMP-2 proenzyme was up-regulated in the cells, indicating the expected malignant transformation of the cells. The activated MMP-2 enzyme emerged in the stimulated HT1080 cells alone. MMP-2 activation was not observed in the stimulated HT-shMT1 and MDA-MB-231 cells. These data indicated that PMA treatment caused generation of the proteolytically active MT1-MMP enzyme in HT1080 cells alone. In agreement with these results, there was a significant level of MP-3653 association with the cellular endogenous MT1-MMP in PMA-stimulated HT1080 cells but not in the HT-shMT1 and MDA-MB-231 cells (Fig. 9B).

**Assessment of the Proteolytically Active Cellular MT1-MMP Enzyme Using MP-3653 Binding**—To assess quantitatively the levels of the net proteolytic activity of the MT1-MMP enzyme in cancer cells, we established several important parameters including the optimal concentrations of MP-3653, the optimal cell number, and incubation time. To determine the optimal concentrations of MP-3653 for the cell-binding experiments, we co-incubated MP-3653 (10–100 nM) with MCF7-MT1 cells (100,000 cells/well of a 96-well plate) for 45 and 90 min. The unbound MP-3653 was washed out and the levels of the internalized/cell-bound MP-3653 were measured using a fluorescence plate reader. Fig. 10A demonstrates that the binding of MP-3653 to the cells occurred in a time- and concentration-dependent manner. Based on these results, we then used a 100 nM MP-3653 concentration and a long, 3-h incubation to increase the level of the cell-bound MP-3653 in our further cell experiments.

We next determined an optimal number of cells for MP-3653 binding experiments. For these purposes, we co-incubated increasing numbers of MCF7-MT1 cells (30 s and 5 min, respectively) was used to visualize MT1-MMP in the B16F1-mock cells. C, conditioned medium aliquots of B16F1-mock and B16F1-mMT1 cells were subjected to gelatin zymography. Where indicated, purified MMP-2 (0.5 nM) was added to the cells.

We next determined an optimal number of cells for MP-3653 binding experiments. For these purposes, we co-incubated increasing numbers of MCF7-MT1 cells (100,000 cells/well of a 96-well plate) for 45 and 90 min. The unbound MP-3653 was washed out and the levels of the internalized/cell-bound MP-3653 were measured using a fluorescence plate reader. Fig. 10A demonstrates that the binding of MP-3653 to the cells occurred in a time- and concentration-dependent manner. Based on these results, we then used a 100 nM MP-3653 concentration and a long, 3-h incubation to increase the level of the cell-bound MP-3653 in our further cell experiments.
MT1 cells (=100%). In both MCF7-mock and B16F1-mock cells, the levels of active MT1-MMP were ~20-fold lower relative to that in MCF7-MT1 cells. GM6001, if added jointly with MP-3653 to the MCF7-MT1 and B16F1-mMT1 cells, reduced MP-3653 binding to comparable levels we recorded in the respective control mock cells. TIMP-2 demonstrated the effects, which were highly similar to those of GM6001. Similarly, no cell fluorescence was recorded when MCF7-MT1 cells were incubated with the inactive MP-3655 formulation (data not shown).

We next determined if MP-3653 could be employed to quantify the natural net activity of the cellular MT1-MMP enzyme. For this purpose, we used untreated and PMA-stimulated HT1080 cells. Our results suggested that 40,000–60,000 cells were optimal for our experiments, whereas as low as 20,000 cells were sufficient to the quantitative assessment of the cell-associated fluorescence of MP-3653 (Fig. 10D).

To estimate the levels of the cell-associated MP-3653, we used a dose-dependent curve of MP-3653 fluorescence. The fluorescence levels of MP-3653 were linear in a 15–150 fmol range (Fig. 10D). We then used this dose-dependent curve to measure the levels of cell-associated MP-3653 and, therefore, to estimate the levels of the net catalytic activity of the surface-associated MT1-MMP enzyme in the cells. Thus, according to the calibration curve, we estimated that there were 40 fmol of the cell-bound MP-3653 and, consequently, 40 fmol of active MT1-MMP were associated with 60,000 PMA-stimulated HT1080 cells (4 \times 10^5 active MT1-MMP molecules/cell), whereas only to 10 fmol were detected in the intact HT1080 cells, suggesting a 4-fold increase in the TIMP-2-free MT1-MMP activity following PMA stimulation of the cells. This 10
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fmol level was largely associated with minor amounts of the active TIMP-2-free MT1-MMP enzyme in the intact HT1080 cells or the insignificant off-target effects of MP-3653 or both.

Using the same approach, we determined that in MCF7-MT1 cells the levels of the true active MT1-MMP were roughly 45 fmol/25,000 cells (11 \times 10^3 active MT1-MMP molecules/cell), whereas in MCF7-mock cells were as low as 2 fmol/25,000 cells. Similarly, over 36 fmol of active MT1-MMP were recorded per 25,000 B16F1-mMT cells (9 \times 10^3 active MT1-MMP molecules/cell) versus 2 fmol (roughly, a 20-fold difference) in B16F1-mock cells.

To support these measurements, we then determined by Western blotting analysis with MT1-MMP antibodies the levels of MT1-MMP in MCF7-mock and MCF7-MT1 cells, and also in intact and PMA-stimulated HT1080 cells (Fig. 10E). The 3G4 antibody recognizes the 55-kDa mature full-length MT1-MMP enzyme alone, whereas the AB8345 antibody recognizes the 55-kDa MT1-MMP enzyme and, in addition, the 45-kDa degraded species that lack the catalytic domain. The naturally synthesized MT1-MMP was represented by the 55-kDa mature full-length MT1-MMP alone in HT1080 cells. PMA stimulation of HT1080 cells did not significantly affect the 55-kDa enzyme levels, however, the 45-kDa degraded MT1-MMP emerged in the cells. These results in HT1080 cells are consistent with our zymography data (Fig. 9A) and with the decreased net levels of cellular TIMP-2 in PMA-treated cells (70). As a result, the net MT1-MMP activity increased without significantly affecting the levels of the 55-kDa enzyme as observed in Western blotting.

In MCF7-MT1 cells, however, overexpressed MT1-MMP was mainly represented by its 45-kDa degraded species. The levels of the 55-kDa active MT1-MMP were roughly equal in PMA-stimulated HT1080 cells and MCF7-MT1 cells that overexpressed MT1-MMP.

**DISCUSSION**

Non-invasive molecular imaging is an indispensable diagnostic tool in both clinical and research laboratories. Because molecular imaging visualizes the molecular, biochemical, and cellular processes in living biological targets, it holds potential for detection of diseases. Discovery of efficient and selective ligands that specifically bind to molecular targets is a critical step for further advances. MMPs are a 24-member family of zinc-dependent proteinases. Because MMPs are responsible for multiple pathological disorders, they are important drug targets. MMPs are classified as soluble extracellular and membrane-type MMPs. Regardless of the important biological functions and roles assigned to all of the individual MMPs, there are volumes of pre-existing studies by us and others indicating that pro-invasive, pro-tumorigenic MT1-MMP is the most critical cell surface-associated enzyme in cell migration, invasion, and metastasis (25–30).

A listing of non-invasive imaging techniques able to show clear visualization of cellular MT-MMP activity is exceedingly limited. Cellular MT1-MMP exists as the proenzyme, as the enzyme–TIMP-2 proteolytically inactive complex, as the partially proteolyzed, inactivated species, lacking the catalytic domain, and also as the proteolytically functional, mature enzyme. However, only the measurements of the mature enzyme activity, rather than the combined levels of MT1-MMP, are sufficient for reliable cancer diagnostic and prognostic as well as for laboratory researchers.

Here, we designed and extensively characterized a molecular reporter that specifically and efficiently binds cell surface-associated MT1-MMP. This reporter represents a hydroxamate warhead linked, via a PEG linker, to a fluorescent reagent-loaded liposome. Among the MMPs we tested, the reporter most efficiently recognizes MT1-MMP rather than other members of the MMP family. We appreciate, however, that the reporter we used may bind other MMPs depending on the levels of their expression.

The hydroxamate warhead binds the essential zinc atom of the MT1-MMP active site. In both the latent MT1-MMP proenzyme and the MT1-MMP enzyme–TIMP-2 proteolytically inactive complex, the active site is inaccessible and the zinc atom is shielded by the prodomain and the inhibitory loop of the inhibitor, respectively. As a result, the fluorescent reporter we designed interacts with the active enzyme of MT1-MMP alone and, following the binding, the level of fluorescence of the reporter is directly proportional to the active MT1-MMP levels present at the cell surface. The *in vitro* and cell-based studies we performed directly support our above conclusions.

Our extensive, well controlled studies directly demonstrated that the imaging reporter can be efficiently used not only to visualize the trafficking of MT1-MMP through the cell compartment but also to quantify, with a significant precision, the amounts of cell surface-associated active MT1-MMP in several cancer cell types, including breast cancer and melanoma. According to our measurements based on the dose dependence studies in several cancer cell models, the levels of the naturally expressed, fully functional, active cellular MT1-MMP enzyme are roughly equal to 1 \times 10^5 molecules/cell.

This ability to quantify the true active cell-surface proteinase is a significant advantage of our approach. This advantage greatly and favorably discriminates the reporter and the methodology we developed from other laboratory techniques such as immunohistochemistry, Western blotting, and RT-PCR, none of which are capable of quantitatively reporting active, cellular MT1-MMP. Furthermore, because MT1-MMP is regulated both as a proteinase by activation, self-proteolysis and inhibition and as a membrane protein through internalization and re-cycling, the results of immunohistochemistry, Western blotting, and RT-PCR could be not only inconclusive but also misleading. We, however, appreciate that the imaging agent is based on the use of an inhibitory hydroxamate warhead, whose interaction with MT1-MMP will inhibit surface activity and accordingly may affect cell behavior in live imaging applications.

We are now confident that our approach and the reporter prototype we developed will contribute to the laboratory studies of MT1-MMP. Furthermore, our studies ultimately will lead to a high-throughput, non-invasive, convenient and low-cost assay to perform a highly accurate prognosis of cancers and for the monitoring of personalized cancer therapy. In addition, many anti-cancer drugs show improved therapeutic properties if administered in a liposome-encapsulated form. As modifying
liposome surface with PEG normally leads to further improvements in drug pharmacokinetics, the liposomal formulation we developed and characterized here may be readily re-designed for interacting with MT1-MMP and using the latter as a vehicle for drug delivery.

REFERENCES

1. Berrier, A. L., and Yamada, K. M. (2007) Cell-matrix adhesion. J. Cell Physiol. 213, 565–573
2. Egeblad, M., and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer 2, 161–174
3. Kessenbrock, K., Plaks, V., and Werb, Z. (2010) Matrix metalloproteinases. Regulators of the tumor microenvironment. Cell 141, 52–67
4. Stetler-Stevenson, W. G., Aznavorian, S., and Liotta, L. A. (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu. Rev. Cell Biol. 9, 541–573
5. Nagase, H., and Woessner, J. F., Jr. (1999) Matrix metalloproteinases. J. Biol. Chem. 274, 21491–21494
6. Sabeh, F., Li, X. Y., Saunders, T. L., Rowe, R. G., and Weiss, S. J. (2009) Secreted versus membrane-anchored collagenases: relative roles in fibroblast-dependent collagenolysis and invasion. J. Biol. Chem. 284, 23001–23011
7. Barbolina, M. V., and Stack, M. S. (2008) Membrane type 1-matrix metalloproteinase. Substrate diversity in pericellular proteolysis. Semin. Cell Dev. Biol. 19, 24–33
8. Gingras, D., and Béliveau, R. (2010) Emerging concepts in the regulation of membrane-type 1 matrix metalloproteinase activity. Biochim. Biophys. Acta 1803, 142–150
9. Gonzalo, P., Moreno, V., Gálvez, B. G., and Arroyo, A. G. (2010) MT1-MMP and integrins. Hand-to-hand in cell communication. Biofactors 36, 248–254
10. Strongin, A. Y. (2010) Proteolytic and non-proteolytic roles of membrane type 1-matrix metalloproteinase in malignancy. Biochim. Biophys. Acta 1803, 133–141
11. Wolf, K., and Friedl, P. (2009) Mapping proteolytic cancer cell-extracellular matrix interfaces. Clin. Exp. Metastasis 26, 289–298
12. Golubkov, V. S., Chekanov, A. V., Shiryev, S. A., Aleshin, A. E., Ratnikov, B. I., Gawlik, K., Radichev, I., Motamedchaboki, K., Smith, J. W., and Strongin, A. Y. (2007) Proteolysis of the membrane-type 1 matrix metalloproteinase prodomain. Implications for a two-step proteolytic processing and activation. J. Biol. Chem. 282, 36283–36291
13. Golubkov, V. S., Chernov, A. V., and Strongin, A. Y. (2011) Intradomain cleavage of inhibitory prodomain is essential to protumorigenic function of membrane type 1 matrix metalloproteinase (MT1-MMP) in vivo. J. Biol. Chem. 286, 34215–34223
14. Golubkov, V. S., Cieplak, P., Chekanov, A. V., Ratnikov, B. I., Aleshin, A. E., Golubkova, N. V., Postnova, T. I., Radichev, I. A., Rozanov, D. V., Zhu, W., Motamedchaboki, K., and Strongin, A. Y. (2010) Internal cleavages of the autoinhibitory prodomain are required for membrane type 1 matrix metalloproteinase activation, although furin cleavage alone generates inactive proenzyme. J. Biol. Chem. 285, 27726–27736
15. Pei, D., and Weiss, S. J. (1995) Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature 375, 244–247
16. Pei, D., and Weiss, S. J. (1996) Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 proprotein cleavage site. J. Biol. Chem. 271, 9135–9140
17. Belkin, A. M., Akimov, S. S., Zaritskaya, L. S., Ratnikov, B. I., Deryugina, E. I., and Strongin, A. Y. (2001) Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. J. Biol. Chem. 276, 18415–18422
18. Deryugina, E. I., Bourdon, M. A., Jungwirth, K., Smith, J. W., and Strongin, A. Y. (2000) Functional activation of integrin αβ3 in tumor cells expressing membrane type 1 matrix metalloproteinase. Int. J. Cancer 86, 15–23
19. Deryugina, E. I., Ratnikov, B., Monosov, E., Postnova, T. I., DiScipio, R., Smith, J. W., and Strongin, A. Y. (2001) MT1-MMP initiates activation of pro-MMP-2 and integrin αβ3 promotes maturation of MMP-2 in breast carcinoma cells. Exp. Cell Res. 263, 209–223
20. Endo, K., Takino, T., Miyamori, H., Kinsen, H., Yoshizaki, T., Furukawa, M., and Sato, H. (2003) Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration. J. Biol. Chem. 278, 40764–40770
21. Golubkov, V. S., Chekanov, A. V., Cieplak, P., Aleshin, A. E., Chernov, A. V., Zhu, W., Radichev, I. A., Zhang, D., Dong, P. D., and Strongin, A. Y. (2010) The Wnt/planar cell polarity protein-tyrosine kinase-7 (PTK7) is a highly efficient proteolytic target of membrane type-1 matrix metalloproteinase. Implications in cancer and embryogenesis. J. Biol. Chem. 285, 35740–35749
22. Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kino, H., and Seiki, M. (2001) Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J. Cell Biol. 153, 893–904
23. Knauper, V., Will, H., López-Otin, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M., and Murphy, G. (1996) Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase (MMP-2) are able to generate active enzyme. J. Biol. Chem. 271, 17124–17131
24. Rozanov, D. V., Hahn-Dantona, E., Strickland, D. K., and Strongin, A. Y. (2004) The low density lipoprotein receptor-related protein LRP is regulated by membrane type-1 matrix metalloproteinase (MT1-MMP) proteolysis in malignant cells. J. Biol. Chem. 279, 4260–4268
25. Hidalgo, M., and Eckhardt, S. G. (2001) Development of matrix metalloproteinases (MMPs) regulate fibrin-invasive activity via MT1-MMP-dependent and -independent processes. J. Exp. Med. 195, 295–308
26. Itoh, Y., and Seiki, M. (2000) MT1-MMP: A potent modulator of pericellular microenvironment. J. Cell Physiol. 206, 1–8
27. Zhao, Y., Hori, K. B., Nan, B., Bosch, F. X., Muñoz, N., Weiss, S. J., and Cho, K. R. (2005) Expression of membrane type 1 matrix metalloproteinase is associated with cervical carcinoma progression and invasion. Cancer Res. 65, 6543–6550
28. Clark, I. M., Swingler, T. E., Sampieri, C. L., and Edwards, D. R. (2008) The regulation of matrix metalloproteinases and their inhibitors. Int. J. Biochem. Cell Biol. 40, 1362–1378
29. Edwards, D. R., Beaudry, P. P., Laing, T. D., Kowal, V., Leco, K. J., Leco, K. J., Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Uhnert, A. S., Mankani, M., Robey, P. G., Poole, R. A., Pitoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell 99, 81–92
30. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) The soluble catalytic domain of membrane type 1 matrix metalloproteinase-1 stimulates cell migration. J. Biol. Chem. 271, 17119–17123
31. Hidalgo, M., and Eckhardt, S. G. (2001) Development of matrix metallo-
Imaging of the Active MT1-MMP Enzyme in Cancer

proteinase inhibitors in cancer therapy. *J. Natl. Cancer Inst.* 93, 178–193
38. Itoh, Y., Ito, N., Nagase, H., Evans, R. D., Bird, S. A., and Seiki, M. (2006) Cell surface collagenolysis requires homodimerization of the membrane-bound collagenase MT1-MMP. *Mol. Biol. Cell* 17, 5390–5399
39. Remacle, A., Murphy, G., and Roghi, C. (2003) Membrane type 1-matrix metalloproteinase (MT1-MMP) is internalized by two different pathways and is recycled to the cell surface. *J. Cell Sci.* 116, 3905–3916
40. Remacle, A. G., Rozanov, D. V., Baciu, P. C., Chekanov, A. V., Golubkov, V. S., and Strongin, A. Y. (2005) The transmembrane domain is essential for the microtubular trafficking of membrane type-1 matrix metalloproteinase (MT1-MMP). *J. Cell Sci.* 118, 4975–4984
41. Remacle, A. G., Rozanov, D. V., Fugere, M., Day, R., and Strongin, A. Y. (2006) Furin regulates the intracellular activation and the uptake rate of cell surface-associated MT1-MMP. *Oncogene* 25, 5648–5655
42. Strongin, A. Y. (2006) Mislocalization and unconventional functions of cellular MMPs in cancer. *Cancer Metastasis Rev.* 25, 87–98
43. Chun, T. H., Hotary, K. B., Sabeh, F., Saltiel, A. R., Allen, E. D., and Weiss, S. J. (2006) A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* 125, 577–591
44. Ota, I., Li, X. Y., Hu, Y., and Weiss, S. J. (2009) Induction of a MT1-MMP and MT2-MMP-dependent basement membrane transmigration program in cancer cells by Snail1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20318–20323
45. Sabeh, F., Ota, I., Holmbeck, K., Birkedal-Hansen, H., Soloway, P., Balbin, M., Lopez-Otin, C., Inada, M., Krane, S., Allen, E., Chung, D., and Weiss, S. J. (2004) Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. *J. Cell Biol.* 167, 769–781
46. Perentes, J. Y., Kirkpatrick, N. D., Nagano, S., Smith, E. Y., Shaver, C. M., Sgroi, D., Garkavtsev, I., Munn, L. L., Jain, R. K., and Boucher, Y. (2011) Cancer cell-associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors. *Cancer Res.* 71, 4527–4538
47. Takahashi, M., Tsunoda, T., Seiki, M., Nakamura, Y., and Furukawa, Y. (2002) Identification of membrane-type matrix metalloproteinase-1 as a target of the B-cat/en TC4 complex in human colorectal cancers. *Oncogene* 21, 5861–5867
48. Mimori, K., Fukagawa, T., Kosaka, Y., Ishikawa, K., Iwatsuki, M., Yokohori, T., Hirasaki, S., Takatsuno, Y., Sakashita, H., Ishii, H., Sasaki, M., and Mori, M. (2008) A large-scale study of MT1-MMP as a marker for isolated tumor cells in peripheral blood and bone marrow in gastric cancer cases. *Ann. Surg. Oncol.* 15, 2934–2942
49. Yonemura, Y., Endo, Y., Takino, T., Sakamoto, K., Bandou, E., Kinoshita, K., Fushida, S., Miwa, K., Sugiyama, K., and Sasaki, T. (2000) Membrane-type 1 matrix metalloproteinase enhances lymph node metastasis of gastric cancer. *Clin. Exp. Metastasis* 18, 321–327
50. Lowery, A., Onishko, H., Hallahan, D. E., and Han, Z. (2011) Tumor-targeted delivery of liposome-encapsulated doxorubicin by use of a peptide that selectively binds to irradiated tumors. *J. Control Release* 150, 117–124
51. Manjappa, A. S., Chaudhari, K. R., Venkataraju, M. P., Dantuluri, P., Nanda, B., Sidda, C., Sawant, K. K., and Murthy, R. S. (2011) Antibody derivatization and conjugation strategies. Application in preparation of therapeutic and/or diagnostic nanocarriers. United States Patent 92286–85688 (007210US)
52. Remacle, A. G., Golubkov, V. S., Shiryaev, A. Y., Dahl, R., Stebbins, J. L., Chern, A. Y., and Strongin, A. Y. (2009) Novel MT1-MMP small-molecule inhibitors based on insights into heparin domain function in tumor growth. *Cancer Res.* 72, 2339–2349
53. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Strongin, A. Y. (2006) Interference with the complement system by tumor cell membrane type-1 matrix metalloproteinase plays a significant role in promoting metastasis in mice. *Cancer Res.* 66, 6258–6263
54. Kridel, S. J., Sawai, H., Ratnikov, B. I., Chen, E. I., Li, W., Godzik, A., Strongin, A. Y., and Smith, J. W. (2002) A unique substrate binding mode discriminates membrane type-1 matrix metalloproteinase from other matrix metalloproteinases. *J. Biol. Chem.* 277, 23788–23793
55. Shiryaev, A. S., Remacle, A. G., Savinov, A. Y., Chernov, A. V., Cieplak, P., Radichev, I. A., Williams, R., Shiryaeva, T. N., Gawlik, W., Postnova, T. I., Ratnikov, B. I., Eroshkin, A. M., Matomeschaboki, K., Smith, J. W., and Strongin, A. Y. (2009) Inflammatory proprotein convertase-matrix metalloproteinase proteolytic pathway in antigen-presenting cells as a step to autoimmune multiple sclerosis. *J. Biol. Chem.* 284, 30615–30626
56. Chen, E. I., Li, W., Godzik, A., Howard, E. W., and Smith, J. W. (2003) A residue in the S2 subsite controls substrate selectivity of matrix metalloproteinase-2 and matrix metalloproteinase-9. *J. Biol. Chem.* 278, 17158–17163
57. Shiryaev, A. S., Savinov, A. Y., Cieplak, P., Ratnikov, B. I., Matomeschaboki, K., Smith, J. W., and Strongin, A. Y. (2009) Membrane metalloproteinase proteolytic pathway of the myelin basic protein isoforms is a source of immunogenic peptides in autoimmune multiple sclerosis. *PLoS One* 4, e9452
58. Rogers, T. E., and Freskos, J. N. (November 30, 2012) MMP-targeted therapeutic and/or diagnostic nanocarriers. United States Patent 92286–85688 (007210US)
59. Remacle, A. G., Golubkov, V. S., Shiryaev, A. S., Dahl, R., Stebbins, J. L., Chernov, A. Y., Cieplak, P. A., Pellecchia, M., and Strongin, A. Y. (2011) Novel MT1-MMP small-molecule inhibitors based on insights into heparin domain function in tumor growth. *Cancer Res.* 72, 2339–2349
60. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J. Biol. Chem.* 270, 5331–5338
61. Rozanov, D. V., Deryugina, E. I., Monosov, E. Z., Marchenko, N. D., and Strongin, A. Y. (2004) Aberrant, persistent inclusion into lipid rafts limits the tumorigenic function of membrane type-1 matrix metalloproteinase in malignant cells. *Exp. Cell Res.* 293, 81–95
62. Remacle, A. G., Shiryaev, S. A., Radichev, I. A., Rozanov, D. V., Stec, B., and Strongin, A. Y. (2011) Dynamic interdomain interactions contribute to the inhibition of matrix metalloproteinases by tissue inhibitors of metalloproteinases. *J. Biol. Chem.* 286, 21002–21012
63. Massova, I., Kotra, L. P., Fridman, R., and Mobashery, S. (1998) Matrix metalloproteinases. Structures, evolution, and diversification. *FASEB J.* 12, 1075–1095
64. Maquoi, E., Frankenne, F., Baramova, E., Munaut, C., Sounni, N. E., Remacle, A., Noël, A., Murphy, G., and Foidart, J. M. (2001) Matrix metalloproteinase type 1 matrix metalloproteinase-associated degradation of tissue inhibitor of metalloproteinase-2 in human tumor cell lines. *J. Biol. Chem.* 275, 11368–11378