Novel MT1-MMP Small-Molecule Inhibitors Based on Insights into Hemopexin Domain Function in Tumor Growth

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Abstract
Membrane type-1 matrix metalloprotease (MT1-MMP) is a promising drug target in malignancy. The structure of MT1-MMP includes the hemopexin domain (PEX) that is distinct from and additional to the catalytic domain. Current MMP inhibitors target the conserved active site in the catalytic domain and, as a result, repress the proteolytic activity of multiple MMPs instead of MT1-MMP alone. In our search for noncatalytic inhibitors of MT1-MMP, we compared the protumorigenic activity of wild-type MT1-MMP with an MT1-MMP mutant lacking PEX (ΔPEX). In contrast to MT1-MMP, ΔPEX did not support tumor growth in vivo, and its expression resulted in small fibrotic tumors that contained increased levels of collagen. Because these findings suggested an important role for PEX in tumor growth, we carried out an inhibitor screen to identify small molecules targeting the PEX domain of MT1-MMP. Using the Developmental Therapeutics Program (National Cancer Institute/NIH), virtual ligand screening compound library as a source and the X-ray crystal structure of PEX as a target, we identified and validated a novel PEX inhibitor. Low dosage, intratumoral injections of PEX inhibitor repressed tumor growth and caused a fibrotic, ΔPEX-like tumor phenotype in vivo. Together, our findings provide a preclinical proof of principle rationale for the development of novel and selective MT1-MMP inhibitors that specifically target the PEX domain. Cancer Res; 72(9); 2339–49. ©2012 AACR.

Introduction
Membrane type-1 matrix metalloprotease (MT1-MMP) is an archetype of membrane-type MMPs (1). MT1-MMP expression is associated with multiple pathophysiological conditions, including tumor cell invasion (2–6). In migrating cells, MT1-MMP accumulates at the leading and trailing edges to contribute efficiently to matrix cleavage and cell locomotion (7, 8). MT1-MMP is regulated both as a protease and as a membrane protein by coordinated mechanisms including activation of the MT1-MMP proenzyme, inhibition by tissue inhibitor of metalloproteinase (TIMP), self-proteolytic inactivation, homodimerization, trafficking throughout the cell to the plasma membrane, the internalization into the transient compartment inside the cells and recycling back to the plasma membrane [reviewed in (9, 10)]. These events stimulate intracellular cytoskeleton rearrangements and set in motion cellular invasion machinery.

There is a consensus among researchers that invasion-promoting MT1-MMP plays the pivotal and multiple roles in tumor progression and metastasis, especially in cell invasion and directional cell motility (6). The MT1-MMP multidomain structure includes the N-terminal prodomain, the catalytic domain (CAT), the hinge linker, the hemopexin domain (PEX), the transmembrane, and the C-terminal cytoplasmic domains (11). MT1-MMP is synthesized as a latent zymogen that requires proteolytic processing of the N-terminal inhibitory prodomain to generate the mature enzyme (12, 13). CAT is the most important domain for the protumorigenic MT1-MMP function (14–16). PEX, however, is also crucial for many specific MMP functions (17–19). In MMPs, including MT1-MMP, the PEX structure is a 4-bladed β-propeller (20). PEX is essential for the association, the lateral diffusion, and the proteolysis of the underlying collagen substratum by MT1-MMP (19, 21). PEX is directly involved in MT1-MMP homodimerization and interactions with CD44, TIMP-2, and MMP-2 (22–24). According to the results by others, blade IV of PEX is necessary for MT1-MMP homodimerization while blade I is required for heterodimerization with CD44 (20, 25). In the symmetrical MT1-MMP homodimer, blades II and III of the first monomer seems to interact with blades III and II of the second monomer while in the asymmetrical homodimer the contacts involve blades I/II and I/III/IV of the monomers (20). The requirement of PEX in the MMP-2 activation process also remains insufficiently understood. In a few studies, MT1-MMP lacking PEX was capable of accomplishing MMP-2 activation (26, 27). Other studies suggested that homodimerization and MMP-2 activation were interconnected (20, 28, 29).

To shed more light on PEX's function, we compared the proinvasive, protumorigenic capacity of the wild-type
MT1-MMP and MT1-MMP mutant lacking PEX. Because our results pointed to an important, albeit underestimated, role of PEX in the MT1-MMP–dependent tumorigenesis, we also identified a small molecule, PEX-targeting inhibitor. This inhibitor selectively targeted PEX and, as a result, repressed the protumorigenic function of MT1-MMP.

Materials and Methods

General reagents and antibodies
Reagents were purchased from Sigma-Aldrich unless indicated otherwise. Murine monoclonal and rabbit polyclonal MT1-MMP antibodies (3G4 and Abb8345, respectively) and a hydroxamate inhibitor (GM6001) were from Chemicon. Rat tail type 1 collagen (COL-I) was from BD Biosciences. Rabbit polyclonal antibodies to Ki-67 and COL-I were from Thermo Scientific and Novus Biologicals, respectively. A murine monoclonal V5 antibody was from Invitrogen.

Cells
All of the original cell lines were obtained from American Type Culture Collection in 2011. Human breast carcinoma MCF-7 and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)-10% FBS. Human mammary epithelial 184B5 cells were maintained in mammary epithelial cell growth medium (MEGM; Invitrogen). 184B5 cells stably transfected with the original pLent6/V5-D-TOPO lentiviral vector (184B5-MT cells) or the lentiviral vector encoding the wild-type MT1-MMP (MCF7-MT cells), MT1-MMP C-terminally tagged with a V5 tag (184B5-MT-V5 cells) or the latter with MT1-MMP (MCF7-MT-V5 cells) or the MT1-MMP mutant lacking the 319–328 PEX sequence (MCF7-β3/ΔPEX cells) were obtained earlier (12, 27, 30).

Cleavage of fluorescent peptide
CAT was expressed in E. coli, purified from the inclusion bodies, and refolded to restore its native conformation (33). The activity assay was conducted at 37°C in triplicate in wells of a 96-well plate in 0.2 mL, 50 mmol/L HEPES, pH 7.5 containing 1 mmol/L CaCl2, 50 mmol/L ZnCl2, 20% glycerol, the (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH2 fluorescent substrate (10 μmol/L; Bachem) and CAT (10 mmol/L; ref. 34). Reaction velocity was monitored at λex = 320 nm and λem = 400 nm. The increasing concentrations of the compounds were coincubated for 30 minutes with CAT at ambient temperature. The residual activity of CAT was measured. IC50 values were calculated by determining the compound concentrations that inhibited the cleavage activity by 50%.

Cell viability assays
Assays were conducted in wells of a 96-well flat bottom, white wall plates. 184B5-MT and MCF7-β3/ΔMT cells (5 × 104) were grown for 16 hours in MEGM-10% FBS and DMEM-10% FBS, respectively. 184B5-MT cells were replenished with fresh MEGM (0.1 mL per well) and incubated for an additional 24 hours in the presence of the compounds (100 μmol/L) or vehicle (1% dimethyl sulfoxide; DMSO). MCF7-β3/ΔMT cells were replenished with fresh DMEM-10% FBS (0.1 mL per well) and incubated for an additional 6 hours in the presence of the compounds (400 μmol/L) or vehicle (2% DMSO). The viable cells were counted using a luminiscent ATP-Lite assay (PerkinElmer). Each datum point represented the results of at least 3 independent experiments carried out in triplicate.

Migration assays
Assays were conducted in wells of a 24-well, 8 μm pore size Transwell plate (Corning Costar). A 6.5-mm insert membrane was coated with 0.1 mL COL-I (300 μg/mL in MEGM) and then air dried for 16 hours. The collagen coating was rehydrated for 1 hour in 0.2 mL MEGM. The inner chamber contained MEGM-10% FBS as a chemoattractant. The compounds (10–100 μmol/L) or DMSO (0.1%–1%) were added to both inner and outer chambers. Before plating in the outer chamber, cells (5 × 104) were coincubated for 20 minutes with the compounds or DMSO in MEGM. Cells were allowed to migrate for 16 to 18 hours. The cells remaining on the surface of the membrane were removed with a cotton swab. The cells on the bottom surface of the membrane were fixed and stained (0.2% crystal violet). The incorporated dye was extracted with 1% SDS and the A570 was measured. Data are means ± SE from 3 individual experiments conducted in triplicate.

Adhesion assays
Assays were conducted in wells of a 96-well clear bottom well plate. Wells were coated for 16 to 18 hours at 4°C using 150 μL COL-I, Matrigel, and bovine serum albumin (BSA) solutions (50 μg/mL, 100 μg/mL, and 1%, respectively, in PBS). Wells were blocked for 1 hour with 1% BSA. Cells were coincubated for 15 minutes at 37°C with the compounds (25–100 μmol/L). Cells (1 × 105/0.1 mL) were then allowed to adhere to the wells for 1 hour at 37°C. Unattached cells were removed by washings. Attached cells were fixed and stained (0.2% crystal violet). The incorporated dye was extracted using 1% SDS and the A570 was measured. Data are means ± SE from 3 individual experiments carried out in triplicate.

Collagen degradation assays
Assays were conducted in wells of a 24-well culture plate. Wells were coated for 1 hour at 37°C with neutralized, chilled rat tail COL-I (0.2 mg/mL, 0.25 mL in DMEM). Cells were coincubated for 20 minutes at 37°C with the compounds (100 μmol/L) or 1% DMSO. Cells (2.5 × 104 per well) were then seeded on COL-I and cultured for 3 days. Cells were detached with 2 mmol/L EDTA. Collagen was fixed with 4% paraformaldehyde, stained with Coomassie Blue, and the images were captured using a Nikon TE-2000 microscope with a ×20 objective and a CCD camera.

Pull down and Western blotting
Cells grown in wells of a 12-well plate were lysed with 50 mmol/L N-octyl-β-D-glucopyranoside in TBS supplemented with 1 mmol/L phenylmethylsulphonyl fluoride. 10 mmol/L
MT1-MMP Hemopexin Domain as a Drug Target

EDTA, and a protease inhibitor cocktail set III (buffer A). Insoluble material was removed by centrifugation (14,000 × g; 15 minutes). The supernatant aliquots (4 μg total protein) were analyzed by Western blotting with the MT1-MMP, FLAG, V5, and actin antibodies followed by the secondary horseradish peroxidase–conjugated antibody (Jackson ImmunoResearch) and a SuperSignal West Dura Extended Duration Substrate kit (Pierce). Where indicated, GM6001 (50 μmol/L) was added to the cells 24 hours before cell lysis.

For pull-down experiments, MCF7-MT and MCF7-MT-V5 cells were transiently transfected with the pcDNA3.1-zeo plasmid encoding the FLAG-tagged MT1-MMP construct (32). Cells were cocentrifuged for 24 hours with the compounds (100 μmol/L) or DMSO (1%). In 48 hours posttransfection, the cells were lysed with buffer A. Insoluble material was removed by centrifugation (14,000 × g; 15 minutes). The supernatant aliquots (200 μg total protein) were cocentrifuged for 16 hours with the FLAG M2 antibody beads (10 μL; 50% slurry). The beads were collected by centrifugation and washed in buffer A. The FLAG construct was eluted from the beads using the FLAG peptide (20 μL; 150 μg/mL; 1 hour). The beads were removed by centrifugation. The supernatant samples were then analyzed by Western blotting with the V5 antibody.

Gelatin zymography

Cells (2 × 10⁶) were grown for 24 hours in DMEM-10% FBS in wells of a 24-well plate. The medium was replaced with fresh DMEM (0.2 mL per well), and cells were incubated for 16 to 18 hours. Where indicated, the medium was supplemented with the MMP-2 proenzyme (0.5 mmol/L). In 24 hours, medium aliquots (20 μL) were analyzed by gelatin zymography.

To analyze the status of MMP-2 in tumor xenografts, snap-frozen tumors (~0.1 mg tissue) were extracted in 1 mL 20 mmol/L Tris-HCl, pH 7.4, supplemented with 150 mmol/L NaCl, 1% deoxycholate, 1% IGEPAL, a protease inhibitor cocktail set III, 1 mL/L phenylmethylsulfonyl fluoride, and 10 mmol/L EDTA. The pellet was removed by centrifugation (14,000 × g; 30 minutes). The protein concentration was then equalized among the samples (1 mg/mL). To determine the status of MMP-2, the samples (5 μg total protein) were analyzed by gelatin zymography.

Tumor xenografts

On day 1, MCF7-β3/WT and MCF7-β3/APEX cells (4 × 10⁵) were injected subcutaneously in 0.1 mL DMEM containing 3 mg/mL Matrigel into the mammary tissue of athymic BALB/c nu/nu 4-week-old female mice (Charles River). Because young, 4-week, female mice produce high levels of estrogen, no additional estrogen supplementation is required to support the growth of estrogen-dependent MCF7 cells. Tumors were measured weekly by caliper measurements of 2 perpendicular diameters of xenografts (D₁ and D₂). Tumor volume was calculated with the formula: \( V = \pi/6 \left(D_1 \times D_2 \right)^2 \). Starting on day 29, mice received intratumoral injections of compound 9 (0.5 mg/kg; 3 times per week). Control animals received vehicle injections (2% DMSO). On day 46, animals were sacrificed according to the NIH guidelines. Tumors were excised and either freeze molded in optimum cutting temperature (OCT) compound (Sakura Finetek) or embedded in paraffin. Tumor sections were stained with hematoxylin & eosin (H&E) and with the antibodies to MT1-MMP, Ki-67, and COL-1 followed by the secondary antibody conjugated with red Alexa Fluor 594 (Molecular Probes). Nuclear DNA was stained with DAPI. The fluorescence images were acquired using an Olympus BX51 fluorescence microscope equipped with a MagnaFire camera.

RT-PCR

To measure the expression levels of human MT1-MMP, Ki-67, and glyceraldehyde-3-phosphate dehydrogenase (loading control), total RNA was extracted from cultured cells and tumors using TRizol reagent and additionally purified using the RNeasy columns (QIAGEN). The RNA purity was estimated by measuring the A₂₆₀/₂₈₀ and the A₂₆₀/₂₃₀ ratios. The integrity of the RNA samples was validated with an Experion automated electrophoresis system (Bio-Rad). The RNA templates (50 ng) were used in the 25 μL reverse transcriptase (RT)-PCR reactions supplemented with the corresponding forward and reverse primers (0.6 μmol/L each) using the OneStep RT-PCR system (QIAGEN; ref. 12). After the completion of the first strand synthesis, RT-PCR was carried out for 35 cycles (denaturation at 95°C, annealing at 55°C, and elongation at 72°C; 30, 30, and 60 seconds, respectively). The amplified products were separated with agarose gel electrophoresis.

Protein-ligand docking

The protein-ligand docking simulations were carried out with the MT1-MMP crystal structure coordinates from PDB 3C7X as a target and an approximately 275,000 compound library of the Developmental Therapeutics Program (DTP) National Cancer Institute/NIH as a source of small-molecule ligands. The docking into the druggable pocket was carried out using Q-MOL modeling package (35). The PDB 3C7X molecule preparation included adding of hydrogen atoms and the assignment of the standard Optimized Potential for Liquid Simulations (OPLS) atom types (36). The ligand structures were prepared for docking using the Q-MOL small-molecule minimization protocol. Polyphenols and compounds with either a molecular mass below 220 Da or chlorine atoms attached to aliphatic carbons and compounds that failed minimization were discarded. The crystal structure was treated as a set of grid-based potentials. The minimized ligand structures were docked with a Monte Carlo simulation in the internal coordinate space of the crystal structure. The identified hits were ranked according to their binding energy. The resulting hits with the lowest binding energy were selected for our further studies.

Compound databases

The compound databases were obtained from "The DTP/NCI Open Chemical Repository." The compounds were more than 95% pure as certified by the DTP/NCI Services. The ligands were dissolved in 100% DMSO and stored at ~20°C.
Structure modeling

The predicted binding mode of compound 9 in a complex with PEX (PDB 3C7X) was built using the Q-MOL full atom flexible protein-ligand docking in the internal coordinates. The protein \( \psi, \phi, \) and \( \chi \) angles of all of the amino acid residues, which are within a 7.5Å distance from ligand atoms, were allowed to change while the positional and rotable torsion variables of a ligand molecule were unfixed.

Results

Role of PEX in tumor growth

To investigate the tumorigenic role of PEX, we selected MCF7-\( \beta3/\)MT and MCF7-\( \beta3/\)APEX cells. Normally, nonmigratory MCF-7 cells do not express MMP-2, MT1-MMP, and the \( \beta3 \) integrin. Expression of the \( \beta3 \) integrin subunit reconstitutes the \( \alpha v \beta3 \) integrin on the MCF-7 cell surface. Compared with the MT1-MMP expression alone, coexpression of MT1-MMP with the \( \beta3 \) integrin subunit confers an invasive phenotype on MCF-7 cells (31, 37, 38). MCF7-\( \beta3/\)MT and MCF7-\( \beta3/\)APEX cells expressed a similar level of the mature MT1-MMP enzyme (as detected by the 3G4 antibody to CAT). Because of MT1-MMP self-proteolysis, degradation species were present in both MCF7-\( \beta3/\)MT and MCF7-\( \beta3/\)APEX cells (as detected by the Ab1815 antibody to the hinge). According to the gelatin zymography analysis, both cell types were similarly efficient in activating the MMP-2 proenzyme. The expression level of MT1-MMP and the ability to activate MMP-2 by MCF7-\( \beta3/\)zeo cells were negligible (39; Fig. 1A).

In our in vivo studies, cells (4 \( \times \) 10^5) were embedded in Matrigel and then xenografted into young immunodeficient female mice (\( n = 5–6 \)). The size of the developing tumors was measured weekly for 46 days. Following an approximately 20-day lag period, MCF7-\( \beta3/\)MT xenografts acquired a rapid growth rate. In turn, the size of MCF7-\( \beta3/\)APEX tumors (\( \sim 25–30 \) mm^3) remained continually low. At day 46, the MCF7-\( \beta3/\)MT tumor volume was approximately 40-fold larger than that of MCF7-\( \beta3/\)APEX tumors (Fig. 1B).

Microscopic examination revealed extensive vascularization and infiltration of T cells in MCF7-\( \beta3/\)MT xenografts both at the periphery and in the central regions of the tumors. There was a limited development of blood vessels in the central regions of MCF7-\( \beta3/\)APEX tumors. The infiltration of T cells was also reduced in MCF7-\( \beta3/\)APEX tumors compared with MCF7-\( \beta3/\)MT xenografts. There were additional differences between the 2 tumor types. Thus, MCF7-\( \beta3/\)APEX tumors seemed fibrotic with an increased level of the stroma and connective tissues, but with less tumor cells (Fig. 1C).

Immunostaining confirmed the presence of high levels of COL-I in MCF7-\( \beta3/\)APEX tumors. In turn, the reduced levels of COL-I were evident in MCF7-\( \beta3/\)MT xenografts. MT1-MMP immunoreactivity was detected in the central and peripheral regions in MCF7-\( \beta3/\)MT tumors. MT1-MMP was mainly observed at the edges of MCF7-\( \beta3/\)APEX xenografts. Ki-67
immunostaining confirmed the presence of proliferating human carcinoma cells in both tumor types (Fig. 2A).

Gelatin zymography of MCF7-β3/MT and MCF7-β3/ΔPEX tumor extracts supported these observations. Although the normal mammary tissue specimens did not activate the 68 kDa MMP-2 proenzyme, the 62 kDa MMP-2 mature enzyme was readily observed in MCF7-β3/ΔPEX xenografts and, especially, in MCF7-β3/MT tumors. The presence of the human MT1-MMP and Ki-67 transcripts in MCF7-β3/MT and MCF7-β3/ΔPEX xenografts were also confirmed by RT-PCR (Fig. 2B).

We concluded that PEX is essential for both the efficient MT1-MMP proteolysis and the tumor growth. Naturally, if PEX was absent in the MT1-MMP structure, the proteolytically competent MT1-MMP ΔPEX mutant was largely incapable of degrading COL-I and promoting tumor growth. Our results support and extend the findings by others who suggested that PEX was essential for cleaving COL-I fibers at the cell surface (22).

**Small-molecule compounds targeting PEX**

We hypothesized that it would be exceedingly difficult to identify small-molecule inhibitors that would directly bind the PEX dimerization interface and that would interfere with the multicontact PEX homodimerization interface. Instead, we attempted to identify allosteric rather than competitive inhibitors of homodimerization. For this purpose, we selected a druggable pocket-like site in the center of the PEX structure. This site is largely formed by β-strands 1 of blades I to IV and it is distinct from the PEX dimerization interface (Fig. 3A).

The diverse 275,000 compound DTP/NCI database was screened using the Q-MOL software against the docking site using the X-ray structure of MT1-MMP (PDB 3C7X). As a result, top ranking 19 hits were identified, ordered from the DTP/NCI, and analyzed further. To select the most efficient compound(s), we used multiple enzyme and cell-based tests, which are summarized in Fig. 3B and shown in more detail in Figs. 4 and 5.

First, we determined whether the compounds were capable of interacting with CAT and inhibiting its catalytic activity. On the basis of our measurements, we discarded compounds 2, 3, 6–8, 10, 11, 18, and 19 the IC50 values of which were below 20 μmol/L (Fig. 4). Compounds 1, 5, 9, and 12–17, which did not affect the cleavage activity were studied further.

Next, we determined whether the compounds affected cell viability. Because normal cells are more sensitive relative to cancer cells, we used normal mammary epithelial 184B5-MT cells in our tests. The original 184B5 cells were obtained from...
normal augmentation mammoplasty material and immortalized by exposure to benzo(a)pyrene (40). These normal cells are neither malignant nor tumorigenic in immunodeficient mice (30). Compounds 16 and 17 exhibited cell toxicity and were then discarded. Compounds 1, 5, 9, and 12–15 were analyzed further (Fig. 5A).

We then established if the compounds were capable of affecting cell migration on COL-I. Nonmigratory MCF-7 cells could not be rationally used in these experiments. Instead, we used migratory 184B5-MT cells that stably expressed high levels of MT1-MMP (Fig. 5B). Original 184B5 cells do not produce MT1-MMP and do not migrate on COL-I. Following transfection with MT1-MMP, the resulting 184B5-MT cells expressed high levels of the protease and acquired an ability to migrate on COL-I. Among the 7 compounds we tested, compounds 5, 9, and 14 repressed migration of 184B5-MT cells on COL-I in a dose-dependent manner (Fig. 5C). We next retested the inhibitory potency of compounds 5, 9, and 14 using breast carcinoma MDA-MB-231 cells. These cells are naturally invasive regardless of minor levels of MT1-MMP (Fig. 5B). Compounds 9 and 14, as expected, did not show any inhibitory effect in MDA-MB-231 cells. Compound 5, however, inhibited migration of MDA-MB-231 cells, suggesting an off-target effect (Fig. 5D).

We next determined the ability of compounds 5, 9, and 14 to inhibit adhesion of 184B5-MT on the Matrigel and COL-I substrates. Although compounds 9 and 14, as expected, did not affect cell adhesion, compound 5, because of its off-target effect, inhibited adhesion of cells on both substrates (Fig. 5E). The data in MDA-MB-231 cells were similar (not shown).
on these results, we discarded compound 5. Compounds 9 and 14 were studied further.

**Compound 9 affects the PEX function**

We next determined whether compounds 9 and 14 affected homodimerization of MT1-MMP. For this purpose, we used MCF7-MT-V5 cells that were transiently transfected with the MT1-MMP-FLAG construct. MCF7-MT cells that expressed MT1-MMP without a V5 tag served as a control. In 24 hours posttransfection, the cells were coincubated with compounds 9 and 14 (100 μmol/L) or DMSO (1%) and then lysed. The MT1-MMP-FLAG construct was pulled down with anti-FLAG beads. The levels of coprecipitated MT1-MMP-V5 were then measured in the pull down samples using Western blotting with the V5 antibody. Compound 9, but not compound 14, significantly reduced the levels of coprecipitated MT1-MMP-V5 in the pull down samples (Fig. 5F). We concluded that compound 9 directly interacted with PEX in the full-length MT1-MMP structure and that these interactions affected PEX homodimerization.

To confirm that the catalytic activity of cellular full-length MT1-MMP was not affected by compound 9, we coincubated 184B5-MT cells with both the MMP-2 proenzyme (23) and compound 9. The activation level of MMP-2 was then observed with gelatin zymography. This test confirmed that compound 9 did not inhibit the catalytic function of cellular MT1-MMP (Fig. 5G). In addition, compound 9 did not inhibit the catalytic activity of MMP-2 (not shown). As a result of our tests, compound 9 was selected over 14 for our subsequent xenograft studies in mice.

**Compound 9 affects protumorigenic MT1-MMP in vivo**

The in vivo effects of compound 9 were assessed with MCF7-b3/MT cells. Cells (4 × 10⁶) were embedded in Matrigel and xenografted into immunodeficient mice (n = 5). On day 29, when the tumors reached a 40 to 80 mm³ size, a low, 0.5 mg/kg,
amount of compound 9 (from a 400 μmol/L stock in PBS) was injected intratumorally. Because of its limited solubility, higher concentrations of compound 9 in aqueous solutions could not be reached. Control mice received vehicle alone. Injections were continued 3 times per week for an additional 2 weeks. A significant reduction in tumor size (366/123 mm³) was observed in the experimental group, while the tumor size reached 933/C6 169 mm³ in the control. We also showed that compound 9 (400 μmol/L) did not show any cytotoxicity in MCF7-b3/MT cells, suggesting that cancer cell death did not affect tumor growth (Fig. 6A). At the end of the experiments, MCF7-b3/MT xenografts were excised and analyzed.

Gelatin zymography of tumor extracts confirmed that compound 9 did not affect the ability of MT1-MMP to activate MMP-2 (Fig. 6B). H&E staining did not reveal any significant effect of compound 9 on xenograft angiogenesis when compared with control mice (Fig. 6C). There was no physical damage and necrosis in the tumors following the compound 9 injections. Similar levels of the MT1-MMP and Ki-67 immunoreactivity were observed in the experimental and control tumors. In turn, both the immunoreactivity and levels of COL-I were significantly enhanced in the experimental group (Fig. 6D). Notably, the collagen staining pattern in mice treated with compound 9 became comparable with that in MCF7-b3/APEX tumors (Fig. 2A and 6D) suggesting that compound 9 repressed the collagenolytic function of PEX.

To corroborate these results, we used a COL-I degradation assay and MCF7-b3/MT cells to assess an inhibitory potency of compound 9. As additional controls, we used MCF7-b3/zeo cells that do not express MT1-MMP and MCF7-b3/APEX cells. Both MCF7-b3/zeo and MCF7-b3/APEX cells were incapable of COL-I degradation. In turn, MCF7-b3/MT cells efficiently degraded the COL-I matrix. Compound 9 dramatically reduced the collagenolytic activity in MCF7-b3/MT
Additional distinctions were observed in the homodimerization of cellular MT1-MMP. The affected the conformation and flexibility of blades I to IV of the β-propeller and, as a result, decreased the PEX-dependent homodimerization of cellular MT1-MMP.

PEX-ligand complex modeling

To visualize the interactions of compound 9 with PEX, we modeled the compound-PEX complex using the PDB 3C7X as a template (Fig. 7). Our results suggested that compound 9 is in a vicinity of Met-328, Arg-330, Asp-376, Met-422, and Ser-470 of the druggable pocket. The latter is formed by the internal portions of the 4 polypeptide chains the external portions of which shape blades I to IV. Based on our modeling, it is tempting to hypothesize that the binding of compound 9 affected the conformation and flexibility of blades I to IV of the β-propeller and, as a result, decreased the PEX-dependent homodimerization of cellular MT1-MMP.

Discussion

MT1-MMP is essential to pericellular proteolysis and cell invasion (1). MT1-MMP is a multidomain enzyme that exhibits several structural domains. These domains are additional to and distinct from CAT. The role of these domains in the net proinvasive, protumorigenic function of MT1-MMP remained unclear, especially for PEX. To function in the PEX, we designed the MT1-MMP construct lacking PEX and then, for the first time, characterize the ΔPEX mutant in the MT1-MMP–dependent model of tumor growth. In contrast with malignant cells, which expressed the wild-type MT1-MMP and which generated the rapidly growing xenografts, the ΔPEX tumors ceased growing after reaching a 25 to 30 mm³ size. Additional distinctions were observed in the fibrin phenotype, the abundance of COL-I, and the reduced angiogenesis in the ΔPEX tumors compared with the wild-type MT1-MMP xenografts. These observation combined with the results by others (19, 20, 22, 25, 28) led us to suggest that the ΔPEX mutant was largely incapable of COL-I degradation and, hence, that efficient MT1-MMP proteolysis of COL-I was essential for tumor growth in vivo.

It is established that homodimerization of MT1-MMP takes place via PEX (22). The structure of PEX has been solved recently (20). It became apparent that homodimerization involves multiple residue interactions between the PEX monomers. Furthermore, homodimerization occurs in the course of exocytosis of the de novo synthesized MT1-MMP rather than at the cell surface (22). For this reason, we focused on the druggable pocket that was distinct from the PEX dimerization interface.

For this purpose, we used virtual ligand screening using the 275,000 compound DTP/NCI library as a ligand source and the X-ray structure of PEX as a target. Compounds were docked in silico to the druggable pocket-like site. This pocket is distinct from the PEX dimerization interface and localized in the center of PEX at a roughly equal distance from blades I to IV. As a result of our screens, we selected the top-ranking compounds, ordered them from DTP/NCI, and tested using multiple in vitro and cell-based assays. Consequently, we identified a novel inhibitory compound that selectively targeted PEX, reduced PEX homodimerization, and repressed MT1-MMP-mediated cell migration and degradation of COL-I. This compound, however, did not exhibit any observable off-target effects and did not inhibit the cleavage activity of CAT, the efficiency of MMP-2 activation by MT1-MMP and cell adhesion to the COL-I substratum.

The antitumor efficacy of this PEX-targeting compound 9 (NSC405020) was directly shown in the in vivo tests. The low, 0.5 mg/kg, intratumoral injections of the compound significantly repressed tumor growth. Compound 9 injections also caused a fibrotic phenotype and increased the level of COL-I. The fibrotic phenotype caused by the compound was similar to that we observed in the ΔPEX tumors. These findings suggested that the compound affected PEX and the ability of MT1-MMP to function properly in degrading COL-I to support tumor cell invasion. We have already identified additional, potent compounds of the same scaffold (Supplementary Table S1). Based on our ADME studies, we are confident that compound derivatives rather than the original compound 9 will be most suitable for the subsequent systemic administration and the in vivo PK studies.

We believe that our study, especially when combined with the results of others (25, 41, 42), provides proof of concept for a design of those non-CAT inhibitors that will selectively target PEX and that will affect protumorigenic MT1-MMP in a clinically beneficial manner. In addition, as shown by the pioneering work by others (18, 25, 41, 42), targeting PEX seems to be a novel and promising approach to modulating the function of other MMPs, including MMP-9, rather than MT1-MMP alone. Overall, our proof of principle work provides conceptual support to probe MT1-MMP PEX with small-molecule ligands and a rationale for design of selective PEX-targeting drugs.

Figure 7. Modeled PEX-compound 9 structure. Left, structure of a PEX-compound 9 complex. PEX is shown as molecular surface. Middle, cartoon representation of PEX with compound 9 (colored by chemical element type). Right, close-up shows residues proximal to compound 9 (blue). Sodium atom, pink. Chlorine atom, green.
Disclosed Potential Conflicts of Interest
A.V. Chelkov is a consultant on the advisory board of Q-MOL, LLC. No potential conflicts of interest were disclosed by the other authors.

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References
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