Design, Synthesis and Characterization of Potent, Slow-Binding Inhibitors that Are Selective for Gelatinases

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Running Title: slow-binding inhibitors of gelatinases
LIST OF ABBREVIATIONS

The abbreviations used are: APMA, p-aminophenylmercuric acetate; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; MMP, matrix metalloproteinase; DMEM, Dubelcco’s modified Eagle’s medium; Me₂SO, dimethyl sulfoxide; MT-MMP, membrane type MMP; pAB, polyclonal antibody; pfu, plaque-forming units; PBS, phosphate buffer saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TIMP, tissue inhibitor of metalloproteinase.
SUMMARY

Gelatinases have been shown to play a key role in angiogenesis and tumor metastasis. Small molecular weight synthetic inhibitors for these enzymes are highly sought for potential use as anti-metastatic agents. Virtually all of the known inhibitors for MMPs are broad-spectrum. We report herein the synthesis and kinetic characterization of two compounds, 4-(4-phenoxyphenylsulfonyl)butane-1,2-dithiol (1) and 5-(4-phenoxyphenylsulfonyl)pentane-1,2-dithiol (2), which are potent and selective gelatinase inhibitors. These compounds are slow, tight-binding inhibitors of gelatinases (MMP-2 and MMP-9) with $K_i$ values in the nanomolar range. In contrast, competitive inhibition of the catalytic domain of membrane-type 1 metalloproteinase (MMP-14cat) with comparable $K_i$ values ($K_i \sim 200$ nM) was observed. Binding to stromelysin (MMP-3) was substantially weaker, with $K_i$ values in the micromolar range ($K_i \sim 10$ µM). No binding to matrilysin (MMP-7) and collagenase 1 (MMP-1) was detected at inhibitor concentrations up to 60 µM. We have previously shown that synthetic MMP inhibitors work synergistically with TIMP-2 in the promotion of pro-MMP-2 activation by MT1-MMP in a process that depends on the affinity of the inhibitor towards MT1-MMP. It is shown herein that the dithiols are significantly less efficient (> 100 fold) than marimastat, a broad-spectrum MMP inhibitor, in enhancing pro-MMP-2 activation in cells infected to express MT1-MMP, consistent with the lower affinity of the dithiols towards MT1-MMP. Thus, in contrast to broad-spectrum MMP inhibitors, the dithiols are less likely to promote MT1-MMP-dependent pro-MMP-2 activation in the presence of TIMP-2, while maintaining their ability to inhibit active MMP-2 effectively.
INTRODUCTION

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases known to play key roles in normal and pathological conditions involving remodeling and turnover of extracellular matrix (ECM), such as embryonic development, wound healing, angiogenesis, arthritis, cardiovascular diseases and cancer. In cancer, MMPs are known to be required at all stages of tumorigenesis, including tumor establishment and growth, neovascularization, intravasation, extravasation and metastasis (1,2). MMPs are expressed as zymogenic latent enzymes. The zymogenic form has a propeptide that achieves coordination to the catalytic zinc ion by a strictly conserved cysteine residue (for a comparative review of MMP structures see (3)). MMP activation occurs when the cysteine thiolate–zinc ion coordination is broken, usually as a consequence of two proteolytic cleavages of the propeptide, either by autolysis or by hydrolytic action of other proteinases (4-6). MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs), a family of four proteins that are the natural inhibitors of MMPs.

A sub-group of the MMP family, gelatinases A and B (MMP-2 and MMP-9, respectively), has been shown to play a key role in angiogenesis and tumor metastasis (7,8). MMP-2 has been the subject of intense investigation since the observation that its activation correlates with tumor spread and poor prognosis was made (9). Latent MMP-2 (pro-MMP-2) is activated by membrane type-MMPs (MT-MMPs) (10,11), a unique MMP subfamily currently comprising six members (MT1- to MT6-MMP). Contrary to the other MMPs, which are secreted into the extracellular milieu, the MT-MMPs are tethered to the plasma membrane either by a transmembrane domain (TMD) (MT1-, MT2-, MT-3 and MT5-MMP) or by a glycosylphosphatidylinositol (GPI) anchor (MT4- and MT6-MMP). MT1-MMP (MMP-14) (12) is known to be the primary pro-MMP-2 physiological activator through a highly regulated mechanism (13) involving TIMP-2 (14). It has been shown that TIMP-2 binds active MT1-MMP through the N-terminal cysteine residue (15,16), while the C-terminal domain of TIMP-2 interacts with the hemopexin-like domain of pro-MMP-2 (17), forming a ternary complex (18) that efficiently concentrates pro-MMP-2 on the cell surface, thereby promoting its interaction with a neighboring TIMP-2-free MT1-MMP. MT1-MMP cleaves pro-MMP-2 at the Asn^{37}-Leu^{38} peptide bond, generating an inactive 64-kDa intermediate. Subsequently, a second autolytic cleavage at Asn^{80}-Tyr^{81} bond achieves the full activation of pro-MMP-2 (19). This process requires protonation of the coordinated cysteine thiolate before its dissociation from the active site.
The key role that MMPs play in neovascularization and metastasis has made these enzymes major targets for therapeutic intervention, and therefore synthetic small molecular weight MMP inhibitors are highly sought. Several orally active MMP inhibitors were synthesized and tested in animal models yielding encouraging results (20,21). Some of these MMP inhibitors, including the broad-spectrum hydroxamate-based batimastat and marimastat (22,23), reached phase 3 of clinical trials. However, administration of these inhibitors, either alone or in combination with standard chemotherapeutic agents, to cancer patients produced severe side effects and showed no clinical efficacy. The current view is that these trials were misguided, since the subjects had pre-existing metastasis and improved survival rates should not have been anticipated (24). On the other hand, a recent study (25) demonstrated that batimastat administration led to liver metastasis in mice inoculated with human breast carcinoma cells. Results from our laboratories (26) showed that both batimastat and marimastat, in concert with TIMP-2, enhanced MT1-MMP-dependent pro-MMP-2 activation when compared with the activation observed with TIMP-2 alone. On the cell membrane, active MT1-MMP (57 kDa) undergoes autolysis and shedding of the catalytic domain, yielding a major membrane-bound species of 44 kDa, which is devoid of proteolytic activity (27). Binding of TIMP-2 or of a synthetic MMP inhibitor to active MT1-MMP prevents autolysis, resulting in accumulation of enzyme on the cell surface. We have shown that in the presence of TIMP-2 accumulation of active MT1-MMP by synthetic MMP inhibitors can generate MT1-MMP/TIMP-2 complexes on the cell surface, which then promote pro-MMP-2 activation (26). Thus, under certain conditions, synthetic MMP inhibitors with high affinity towards MT1-MMP can enhance the effects of TIMP-2 in pro-MMP-2 activation by MT1-MMP. We have recently reported the first mechanism-based inhibitor (“suicide substrate”) for MMPs, SB-3CT (28,29), which exhibits high selectivity for the gelatinases, but poor affinity for MT1-MMP. Consistently, SB-3CT does not enhance the MT1-MMP-mediated activation of pro-MMP-2 in the presence of TIMP-2. Thus, selective inhibitors for gelatinases may have clinical advantages by reducing side effects and improving efficacy. We report herein the synthesis and kinetic characterization of two novel inhibitors, 4-(4-phenoxyphenylsulfonyl)butane-1,2-dithiol (1) and 5-(4-phenoxyphenylsulfonyl)pentane-1,2-dithiol (2) that also exhibit selectivity towards gelatinases. However, their mechanism of inhibition is different. A comparative study of these compounds on activation of pro-
MMP-2 in cells expressing MT1-MMP shows that, when compared to marimastat, significantly higher dithiol concentrations are required for active MT1-MMP binding and enhancement of pro-MMP-2 activation in the presence of TIMP-2. Moreover, at higher concentrations both marimastat and the dithiols inhibit MMP-2, leading to accumulation of the 64-kDa MMP-2 inactive intermediate and preventing further MMP-2 generation.

**EXPERIMENTAL PROCEDURES**

*Synthesis* - $^1$H and $^{13}$C NMR spectra were recorded on either a Varian Gemini-300, a Varian Mercury-400 or a Varian Unity-500 spectrometer. Chemical shifts are reported in ppm from tetramethylsilane on the $\delta$ scale. Infrared spectra were recorded on a Nicolet 680 DSP spectrophotometer. Mass spectra were recorded on a Kratos MS 80RFT spectrometer. Melting points were taken on an Electrothermal melting point apparatus and are uncorrected. Thin-layer chromatography was performed with Whatman 0.25 mm silica gel 60-F plates. All other reagents were purchased from either Aldrich Chemical Company or Across Organics. 4-(4-Phenoxyphenylsulfonyl)ethylthiirane and 4-(4-phenoxyphenylsulfonyl)propylthiirane were prepared according to the method reported earlier (28).

**4-(4-Phenoxyphenylsulfonyl)butane-1,2-dithiol** - A solution of sodium hydrosulfide (13 mg, 0.18 mmol) in methanol (0.5 ml) was cooled to 0 °C. Hydrogen sulfide gas was then bubbled slowly through the solution for 5 minutes. To this solution was added 2-(4-phenoxyphenylsulfonyl)ethylthiirane (39 mg, 0.12 mmol) in chloroform/methanol (1:2, 0.8 ml) over a period of 10 minutes, with continuous bubbling of hydrogen sulfide. This mixture was stirred at 0 °C for a further 30 minutes and then at room temperature for 2 hours. The reaction mixture was poured into water (20 ml) and then acidified with a few drops of sulfuric acid (1 M), before extraction with ether (3 x 10 ml). The combined organic extracts were dried over magnesium sulfate and concentrated to give the desired product as a waxy white solid 42 mg, (99%).

$^1$H (400 MHz, CDCl$_3$) $\delta$ 7.86-7.83 (m, 2H), 7.44-7.40 (m, 2H), 7.26-7.22 (m, 1H), 7.10-7.06 (m, 5H), 3.41-3.34 (m, 1H), 3.25-3.18 (m, 1H), 3.02-2.93 (m, 1H), 2.84-2.69 (m, 2H), 2.36-2.26 (m, 1H), 1.90-1.80 (m, 1H), 1.66 (d, $J$ 6 Hz, 1H), 1.64 (t, $J$ 8.8 Hz, 1H); $^{13}$C (100 MHz, CDCl$_3$) $\delta$ 162.94, 155.02, 132.54, 130.55, 130.51, 125.48, 120.70, 117.94, 54.57, 42.44, 34.13, 29.91; $m/z$ (EI) 320 ([M – SH$_2$]$^+$, 30%), 235 (40), 217 (60), 170 (100); HRMS calcd for C$_{16}$H$_{18}$O$_3$S$_3$ – H$_2$S 320.0541, found 320.0540.
5-(4-Phenoxyphenylsulfonyl)pentane-1,2-dithiol - This material was prepared in the same manner as 4-(4-Phenoxyphenylsulfonyl)butane-1,2-dithiol with the exception that 3-(4-phenoxyphenylsulfonyl)propylthiirane was used in place of 2-(4-phenoxyphenylsulfonyl)ethylthiirane. The desired product was obtained as a waxy white solid (99%). \( ^1H \) (400 MHz, CDCl\(_3\)) \( \delta \) 7.86-7.83 (m, 2H), 7.44-7.40 (m, 2H), 7.09-7.07 (m, 4H), 3.11-3.08 (m, 2H), 2.84-2.82 (m, 1H), 2.75-2.71 (m, 1H), 2.04-1.47 (m, 4H), 1.66 (d, \( J \) 7.2 Hz, 1H), 1.63 (t, \( J \) 8 Hz, 1H); \( ^{13}C \) (100 MHz, CDCl\(_3\)) \( \delta \) 162.85, 155.08, 132.60, 130.58, 130.50, 125.43, 120.68, 117.93, 56.16, 43.34, 35.19, 34.07, 20.99; \( m/z \) (ESI) 391 ([M +Na\(^+\)], 100%); HRMS calcd for C\(_{17}\)H\(_{20}\)O\(_3\)S\(_3\) – H\(_2\)S 334.0697, found 334.0695.

Cell Culture – HeLa S3 cells were obtained from American Type Culture Collection (ATTC, Manassas, VA) (CCL-2.2) and grown in suspension in MEM Spinner medium (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 5% horse serum. Nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) cells were obtained from the ATTC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. All tissue culture reagents were purchased from Gibco BRL (Grand Island, NY).

Recombinant Vaccinia Viruses – Recombinant vaccinia viruses expressing either bacteriophage T7 RNA polymerase (vTF7-3) or MT1-MMP (vTF-MT1) under the T7 promoter were produced by homologous recombination, as previously described (27,30-32).

Recombinant Proteins, Enzymes and Protein Inhibitors – Human recombinant pro-MMP-2, pro-MMP-9, TIMP-1 and TIMP-2 were expressed in HeLa S3 cells infected with the corresponding recombinant vaccinia viruses and purified to homogeneity as previously described (17). Heat activated human stromelysin 1 (MMP-3) was generously provided by Dr. Paul Cannon (Center for Bone and Joint Research, Palo Alto, CA). Pro-MMP-2 and pro-MMP-9 were activated by incubation with \( p \)-aminophenylmercuric acetate (APMA) and heat-activated stromelysin 1, respectively, as previously described (28). The recombinant catalytic domain of human MT1-MMP (MMP-14\(_{cat}\)) encompassing residues Ile\(^{114}\) to Ile\(^{318}\), expressed in Escherichia coli (33), was a generous gift from Dr. Harald Tschesche (University of Bielefeld, Bielefeld, Germany). Recombinant human active matrilysin (MMP-7) was obtained from CHEMICON (Temecula, CA). Human recombinant interstitial collagenase (pro-MMP-1) was a generous gift from Dr. William Parks (Washington University, St. Louis, MO). Pro-MMP-1 was
activated by incubation with APMA (1 mM) in a buffer consisting of 0.1 M Tris, 10 mM CaCl$_2$, pH 7.5. The concentrations of heat-activated stromelysin 1 and MMP-9 were determined by active-site titration with recombinant TIMP-1, whereas MMP-2, MMP-7 and MT1-MMP$_{cat}$ concentrations were obtained by titration with recombinant TIMP-2. The hydroxamate-based inhibitors marimastat (BB-2516) and batimastat (BB-94) were obtained from British Biotech (Annapolis, MD). Stock solutions of marimastat, batimastat and of the dithiol compounds were prepared in Me$_2$SO, in the millimolar concentration range.

**Fluorescence Enzymatic Activity Assays** – Fluorescence was measured with a Photon Technology International (PTI) spectrofluorometer, interfaced to a Pentium computer, equipped with the RadioMaster™ and FeliX™ hardware and software, respectively. The assays were carried out at 25.0 °C and the cuvette holder was thermostatted at the same temperature. Excitation and emission band passes of 1 and 3 nm, respectively, were used. Fluorescence measurements were taken every 4 s. MMP-2, MMP-9, MMP-7 and MT1-MMP activities were monitored with the fluorescence-quenched substrate MOCAcPLGLA$_3$pr(Dnp)AR-NH$_2$ at excitation and emission wavelengths of 328 and 393 nm, respectively. MOCAcRPKPVE(Nva)WRK(Dnp)NH$_2$ was the fluorogenic substrate used to monitor MMP-3 enzymatic activity at 325 and 393 nm. MMP-1 activity was assayed with the fluorogenic synthetic substrate (Dnp)P(Cha)GC(Me)HAK(NMa)NH$_2$, at 340 and 440 nm. All fluorogenic substrates were obtained from Peptides International, Inc. (Louisville, KY). Less than 10% hydrolysis of the substrates was monitored, as described by Knight (34). The assays were performed as previously described (35).

**Enzyme Inhibition Studies** – Progress curves for slow-binding inhibition analysis were obtained by adding enzyme (0.5-1 nM) to a mixture of MOCAcPLGLA$_3$pr(Dnp)AR-NH$_2$ (7 µM) and varying concentrations of the dithiol inhibitors in buffer R (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM CaCl$_2$, 0.01% Brij-35, and 5% Me$_2$SO; final volume 2 ml) in acrylic cuvettes with stirring. Hydrolysis of the fluorogenic substrate was monitored for 15-30 min. The progress curves were nonlinear least squares fitted to Eq. 1 (36),

\[ F = v_o t + (v_o - v_s) \frac{(1 - \exp(-kt))}{k} + F_0 \]  

(Eq. 1)

where $v_o$ represents the initial rate, $v_s$, the steady-state rate, $k$, the apparent first-order rate constant characterizing the formation of the steady-state enzyme-inhibitor complex and $F_0$, the initial fluorescence, using the program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT). The obtained $k$
values, $v_0$ and $v_s$ were further analyzed according to Equations 2 and 3 for a one-step association mechanism.

$$k = k_{\text{off}} + k_{\text{on}} [I]/(1 + [S]/K_m) \quad \text{(Eq. 2)}$$

$$\frac{v_0 - v_s}{v_s} = \frac{[I]}{(K_i(1 + [S]/K_m))} \quad \text{(Eq. 3)}$$

The $K_m$ values used for the reaction of MMP-2 and MMP-9 with the fluorogenic substrate were $2.46 \pm 0.34$ and $3.06 \pm 0.74 \mu M$, respectively (17). Intercept and slope values, obtained by linear regression of the $k$ versus inhibitor concentration plot (Eq. 2), yielded the association and dissociation rate constants $k_{\text{on}}$ and $k_{\text{off}}$, respectively, and the inhibition constant $K_i$ ($k_{\text{off}}/k_{\text{on}}$). Alternatively, $K_i$ was determined from the slope of the $(v_o - v_s)/v_s$ vs $[I]$ plot according to Equation 3. The dissociation rate constants were determined independently from the enzyme activity recovered after dilution of a pre-formed enzyme-inhibitor complex.

Thus, 200-400 nM of the enzyme was incubated with ~100 µM of the inhibitor for at least 45 min at 25.0°C. The complex was diluted 400-fold into 2 ml of buffer R containing fluorogenic substrate (10 µM final concentration). Recovery of enzyme activity was monitored for 60 min. The data were analyzed as described elsewhere (26,28). For competitive inhibition, initial rates were obtained by adding enzyme (0.5 nM) to a mixture of fluorogenic substrate (7 µM) and varying concentrations of inhibitor in buffer R (final volume 1 ml), in quartz semi-micro cuvettes, and monitoring the increase in fluorescence with time for 5-10 minutes. The initial velocities were determined by linear regression analysis of the fluorescence versus time traces using FeliX™. The initial rates were fitted to Eq. 4 (37), where $v_i$ and $v_0$ represent the initial velocity in the presence and absence of inhibitor, respectively, using the program SCIENTIST.

$$\frac{v_i}{v_0} = \frac{(K_m+[S])/(K_m(1 + [I]/K_i)+[S])}{(K_m+[S])/(K_m(1 + [I]/K_i)+[S])} \quad \text{(Eq. 4)}$$

Cell Infection, Treatment with Synthetic Inhibitors and Pro-MMP-2 Activation – To express MT1-MMP, ~90% confluent BS-C-1 cells cultured in 6-well plates were co-infected with 5 pfu/cell each of vTF7-3 and vTF-MT1 viruses for 45 min in infection medium (DMEM supplemented with 2.5% fetal bovine serum and antibiotics) at 37°C. After infection the cells were rinsed with serum free DMEM supplemented with L-glutamine and antibiotics and incubated in the same medium containing varying concentrations of synthetic inhibitors. After ~16 hours the inhibitor-containing media were aspirated, the cells were rinsed twice with phosphate buffer saline (PBS) and incubated with Opti-MEM without phenol red containing pro-MMP-2 (25 nM). At varying times, media aliquots were removed and MMP-2 activity...
was assayed with MOCAcPLGLA₂pr(Dnp)AR-NH₂, as described (35). After 4-6 hours the remaining media were collected, the cells were rinsed twice with cold phosphate buffer saline (PBS) and lysed with cold lysis buffer (25 mM Tris-HCl (pH 7.5), 1% IGEPAL CA-630, 100 mM NaCl) containing protease inhibitors (1 pellet of Complete Mini, EDTA-free protease inhibitor cocktail from Roche Diagnostics (Mannheim, Germany)/10 ml of buffer). The media and lysate samples were analyzed for pro-MMP-2 cleavage by gelatin zymography. The lysates were also subjected to immunoblot analysis to ascertain the level of MT1-MMP expression and processing as described below.

Gelatin Zymography and Immunoblot Analysis – Gelatin zymography was performed using 8% Tris-glycine SDS-polyacrylamide gels containing 0.1% gelatin, under non-reducing conditions, as previously described (38). The samples for immunoblot analysis were subjected to reducing SDS-PAGE, followed by transfer to nitrocellulose membranes. The transferred proteins were developed using rabbit polyclonal antibody 437 (pAb 437) to MT1-MMP (39). Horseradish peroxidase (HRP) labeled anti-rabbit IgG (ImmunoPure R from Pierce, Iselin, NJ) was the secondary antibody used. Detection was performed using SuperSignal R enhanced chemiluminescence substrate for HRP, with West Pico or Femto sensitivity, according to the manufacturer’s (Pierce) instructions.

RESULTS

Design and synthesis of 4-(4-phenoxyphenylsulfonyl)butane-1,2-dithiol (1) and 5-(4-phenoxyphenylsulfonyl)pentane-1,2-dithiol (2) – Thiolates are the best ligands for zinc ions, a factor that has been used in design of enzyme inhibitors (40-48). A report for a dithiol inhibitor of VanX, a bacterial zinc-dependent D,D-dipeptidase, was published recently (49), which prompted us to consider the issue of chelation of the active site zinc ion in MMP-2 by dithiol inhibitors. The dithiol inhibitors for VanX were slow-binding inhibitors. In slow-binding inhibition, inhibitor binding initiates a conformational change that results in an enzyme-inhibitor complex that resists dissociation. As a result of the interactions that initiate the onset of the conformational change, and the difficulty of its reversal, slow-binding inhibitors can afford excellent selectivity in inhibition of closely related enzymes. This will be of great interest to inhibition of MMPs, since their active sites are extremely similar to one another.

Dithiol inhibitors 1 and 2 were designed based on our knowledge of the binding interactions of the thiirane mechanism-based inhibitors ("suicide substrates") that we had developed for gelatinases (28,29),
though the mechanisms for inhibition of the two classes of inhibitors are very different. The biphenyl moiety in these inhibitors fits well in the S1’ subsite of gelatinase A. The oxygens of the sulfone make hydrogen bonds to the backbone amines of Leu-191 and Ala-192. Sequestration of the inhibitor in the active site brings the thiols close to the coordination environment of the zinc ion.

![Chemical structure](image)

We synthesized thiiranes 3 and 4 in six steps according to reported procedures (28). The thiirane moiety in each case was opened by reaction with sodium hydrosulfide to afford the desired dithiols 1 and 2 in excellent yields.

**Scheme 1**

![Chemical reaction](image)

*Compounds 1 and 2 as selective gelatinase inhibitors* – As shown in Figure 1 and Table I, compounds 1 and 2 are slow-binding inhibitors of only MMP-2 and MMP-9 with $K_i$ values in the nanomolar range (46-260 nM). These compounds also inhibited MMP-14 with similar affinity ($K_i \sim 200$ nM), although we observed only a competitive mechanism for inhibition in this case. On the other hand, MMP-3 was inhibited at significantly higher inhibitor concentrations ($K_i$ of 10 ± 2 and 4.4 ± 0.8 µM, for compounds 1 and 2, respectively) whereas for the cases of matrilysin (MMP-7) and collagenase-1 (MMP-1), activities remained unchanged in the presence of inhibitor concentrations up to 60 µM. No significant difference in affinity or mechanism of inhibition was detected between the two compounds. Thus, the dithiols exhibit selectivity towards gelatinases and MT1-MMP, and the binding affinity of the enzymes tested is independent of the length of the side chain backbone of the molecules. It is important to note that the values for $k_{off}$ for both inhibitors with either of the gelatinases are slow ($10^{-3}$ s$^{-1}$) and those for $k_{on}$ are very favorable ($10^4$-10$^5$ M$^{-1}$s$^{-1}$), hence the inhibitors are extremely effective in targeting gelatinases.
Effect of Inhibitor 1 on pro-MMP-2 activation by MT1-MMP – We have previously demonstrated (26) that marimastat binds tightly ($K_i = 2.1 \pm 0.5$ nM) (Fig. 2) to a recombinant catalytic domain of MT1-MMP and prevents MT1-MMP autolysis in cells expressing full length MT1-MMP, thus stabilizing the enzyme on the cell surface. In addition, marimastat in concert with TIMP-2 enhances pro-MMP-2 activation mediated by MT1-MMP, a process that is related to its ability to stabilize MT1-MMP. Since the affinities of both the gelatinases and MT1-MMP for the dithiol inhibitors are poorer in comparison to marimastat (Fig. 2 and earlier reports (26,50)), they are expected to be less efficient than marimastat at promoting activation of pro-MMP-2 in the presence of MT1-MMP and TIMP-2. To test this hypothesis, BS-C-1 cells, which express endogenous TIMP-2, were infected to express MT1-MMP and incubated with various concentrations of either inhibitor 1 (0-1000 µM) or marimastat (0-10 µM) to stabilize MT1-MMP on the cell surface. The cells were subsequently examined for their ability to activate exogenously administered pro-MMP-2. As shown in Fig. 3, enhanced pro-MMP-2 activation on cells that were incubated with inhibitor 1 was noticeable at 100 µM of inhibitor. In contrast, a comparable amount of MMP-2 generation was detected at ~100-fold lower marimastat concentrations (1 µM). This effect correlated with the differential affinity of the inhibitors for MT1-MMP since, as shown by immunoblot analysis of the cell lysates (Fig. 3), marimastat promoted the accumulation of the 57 kDa active form of MT1-MMP at ~100-fold lower concentrations than compound 1. Both inhibitor 1 and marimastat appear to bind active MMP-2 thereby inhibiting pro-MMP-2 autolytic activation step as evidenced by the accumulation of the 64 kDa MMP-2 intermediate at the high end of the concentration range used. This effect was apparent at significantly higher concentrations of compound 1 relative to marimastat (~100-fold), which correlates with the inhibitors differential affinities for active MMP-2.

DISCUSSION

We have designed, synthesized and kinetically characterized two novel small molecular weight synthetic inhibitors for MMPs. These compounds were shown to be tight, slow-binding inhibitors for gelatinases, with $K_i$ values in the nanomolar range. The inhibitors contain a dithiol moiety that was expected to interact with the active site zinc ion to give enzyme inhibition. Although the principles that facilitate the manifestation of slow-binding inhibition remain unclear, such inhibition can have important implications for selectivity in targeting closely related enzymes such as MMPs. The rationale is as follows.
The inhibitor needs to bind the enzyme effectively to begin with. On binding, the conformational change that is implicit for the slow-binding behavior would ensue to give the final enzyme-inhibitor complex. Whether this conformational change would take place or not is a function of specific interactions between the inhibitor and the active site. In essence, small changes of the surface features of the active site could make a significant difference in the rate constants for the onset of slow-binding behavior. This is most likely at the root of the selectivity that we observe in inhibition of gelatinases with compounds 1 and 2. Not only are the $K_i$ values for gelatinases in the nanomolar range, it is only with gelatinases that the slow-binding behavior is seen. One observes only simple competitive inhibition of the closely related MMP-3, but with $K_i$ values in the micromolar range. Again, despite high similarities in the active sites of other MMPs, such as MMP-1 and MMP-7, we did not observe inhibition at concentrations of the inhibitors as high as 60 $\mu$M. On the other hand, the catalytic domain of MMP-14 showed affinity for the inhibitors ($K_i$ of approximately 200 nM), but the inhibitors behaved as simple competitive inhibitors of this enzyme.

The previously reported mechanism-based inhibitor SB-3CT exhibited a higher degree of selectivity and was capable of discriminating between MMP-2 ($K_i = 14 \pm 4$ nM) and MMP-9 ($K_i = 600 \pm 200$ nM). Contrary to the case of the thiirane inhibitors that showed very strict limitations on structure variation (28), the length of the dithiol side chains (4 or 5 carbons) practically did not affect their binding behavior with gelatinases.

The dithiols exhibit high affinity for gelatinases (MMP-2 and MMP-9), two enzymes that have been associated with pathogenesis of various human pathological conditions. Therefore, selective inhibition of these enzymes with dithiol-like inhibitors represents an advantage over broad-spectrum MMP inhibitors. Like most MMPs, gelatinases are produced in a latent form that is activated by a proteolytic cascade, generally involving the action of other MMPs acting as zymogen activators. For example, MMP-3 and MT1-MMP (9, 12) are the major activators of pro-MMP-9 and pro-MMP-2, respectively. Synthetic MMP inhibitors, depending on their affinities, may influence the process of zymogen activation. Indeed, we have previously shown that high-affinity synthetic MMP inhibitors like marimastat can act synergistically with TIMP-2 in promoting pro-MMP-2 activation by MT1-MMP (26). This effect is mediated at least in part by the stabilization of active MT1-MMP on the cell surface by the action of the inhibitor. Assuming that a fraction of the surface enzyme is free of inhibitor and that the cells continue to
produce and activate pro-MT1-MMP. The accumulated enzyme would form complexes with TIMP-2, which in turn would enhance pro-MMP-2 activation. Alternatively, displacement of MT1-MMP/synthetic inhibitor complexes by TIMP-2 would also generate additional MT1-MMP/TIMP-2 complexes for pro-MMP-2 binding and consequent activation. Such an effect would be undesirable in tissues, since it would favor pericellular proteolysis. It is therefore important to design MMP inhibitors that exhibit selectivity to prevent this potential side effect. In this report, we have shown that both the dithiols and marimastat enhance activation of pro-MMP-2 by MT1-MMP in BS-C-1 cells, which express low amounts of endogenous TIMP-2 (26). However, by exhibiting ~100-fold lower affinity for MT1-MMP than marimastat, the dithiols are significantly less efficient in enhancing the effects of TIMP-2 on pro-MMP-2 activation requiring at least 100 μM to achieve an activation comparable with 1 μM marimastat. This was consistent with the differential effects of the inhibitors on the stabilization of active MT1-MMP on the cell surface. Previous studies have shown that MT1-MMP cleaves pro-MMP-2 at the Asn37-Leu38 peptide bond, present in the propeptide region, generating an inactive 64-kDa intermediate (19). Full pro-MMP-2 activation requires autolytic processing of a second scissile bond at Asn80-Tyr81. The formation of ternary (MT1-MMP/TIMP-2/pro-MMP-2) complexes on the cell surface concentrates pro-MMP-2, thus favoring the second autolytic step. We have herein observed that the dithiols, and marimastat, inhibit the second activation step, inducing accumulation of the inactive MMP-2 intermediate at the highest inhibitor concentrations. However, given the higher affinity of MMP-2 for marimastat, lower concentrations of this inhibitor were required to observe this effect. Accumulation of the intermediate form of MMP-2 is likely a consequence of inhibition of the fully active MMP-2 required for the second autolytic step. The synthetic inhibitors herein characterized and the system used to test their effects in live cells illustrate the complex regulation of MMP proteolytic activity and the different outcomes that different inhibitors may have, depending on their binding affinities and selectivity for the various MMPs. Thus, inhibitors with high affinity for MT1-MMP, like marimastat, may generate at relatively low concentrations, a “pool” of active MT1-MMP, thereby promoting pro-MMP-2 activation. Marimastat is also an effective MMP-2 inhibitor, thus binding the generated active MMP-2 and preventing further pro-MMP-2 full activation and pericellular proteolysis. It is noteworthy that a decline in inhibitor concentration may reverse both of these effects. Thus, while marimastat enhance activation through its effect on MT1-MMP, it will reduce MMP-2
activity by inhibiting the second activation step. Furthermore, by stabilizing MT1-MMP on the cell surface, marimastat may indirectly contribute to the overall activity of this enzyme on the cell surface, under conditions of low inhibitor concentrations. Selective inhibitors for MMP-2, in contrast to broad-spectrum inhibitors, are less likely to bind MT1-MMP and promote pro-MMP-2 activation.

MMP-9 is also inhibited by the dithiols with high affinity and with a slow-binding mode of inhibition, consistent with the high degree of homology that exists between the catalytic sites of the gelatinases. However, in contrast to pro-MMP-2, a cellular mechanism of pro-MMP-9 activation has not been identified yet. Therefore, an analogous effect of the dithiols on pro-MMP-9 activation cannot be envisioned. On the other hand, pro-MMP-9, as a soluble MMP, may be efficiently activated in the extracellular space, for example by MMP-3, among other possible activators. Kinetically, MMP-3 is an extremely efficient pro-MMP-9 activator (35), a process that is accomplished by two consecutive MMP-3 cleavages at the propetide region. As poor inhibitors of MMP-3 (Ki = 4 to 10 µM), the dithiols are not expected to alter the rate of pro-MMP-9 activation, but rather to specifically inhibit active MMP-9, achieving desirable enzyme targeting. Broad-spectrum inhibitors like marimastat are likely to diminish pro-MMP-9 activation by affecting MMP-3 and thus achieve less selectivity but a broader neutralizing effect. Thus, in clinical applications inhibitor selectivity dosage will be crucial in determining the final outcome of their application.

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FIGURE LEGENDS

Fig. 1  Slow-binding behavior for compounds 1 and 2 with MMP-2 and MMP-9. Progress curves were obtained by adding MMP-2 (1 nM) to a solution of substrate MOCAcPLGLA$_2$pr(Dnp)-AR-NH$_2$ (7 µM) containing compound 1 (A), or compound 2 (B), or MMP-9 (0.5 nM) added to a solution of the substrate containing compound 1 (C), or compound 2 (D) in buffer R. The lines represent non-linear least squares fits to Eq. 1 using the program SCIENTIST. Inset: Nonlinear least squares fits of $k$, the apparent first-order rate constant as a function of the inhibitor concentration to Eq. 2, which yields $k_{off}$ and $k_{on}$ from the intercept and the slope, respectively.

Fig. 2  MMP-14$_{cat}$ inhibition by compound 1 and marimastat. MMP-14$_{cat}$ (0.5 nM) was added to mixtures of the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)-AR-NH$_2$ (7 µM) in buffer R containing increasing concentrations of compound 1 (•) and marimastat (■). The lines represent non-linear least-square fits to Eq. 4 using the program SCIENTIST.

Fig. 3  Differential enhancement of MT1-MMP-dependent pro-MMP-2 activation by BS-C-1 cells. BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vTF7-MT1 vaccinia viruses in infection media. *cells were infected with control virus (vTF7-3) only. After 45 min, the media were replaced with serum free DMEM containing the indicated inhibitor concentrations. At 16 h post-infection, the cells were incubated (37 °C, 6 h) in Opti-MEM without phenol red containing pro-MMP-2 (25 nM). The media were collected and analyzed by zymography. The lysates were subjected to gelatin zymography and reducing 12% SDS-PAGE followed by immunoblot analysis using the anti-MT1-MMP pAb 437. Note that in the left lower panel an additional minor MT1-MMP species of ~62 kDa is detected, which represents pro-MT1-MMP with the signal peptide (27).
The enzymes (0.5 nM) were added to a solution of the proper synthetic fluorogenic substrate (5-7 µM) and various concentrations of inhibitor in buffer R. Substrate hydrolysis was monitored for up to 30 min. The kinetic parameters were evaluated as described under “Experimental Procedures”. Analogous results were obtained from at least two independent experiments.

| Enzyme   | $k_{on} \times 10^4$ $\text{M}^{-1}\text{s}^{-1}$ | $k_{off} \times 10^3$ $\text{s}^{-1}$ | $K_i$ $\mu\text{M}$ |
|----------|-----------------------------------|-----------------------------------|------------------|
| Compound 1 |
| MMP-2    | 4.4 ± 0.8                         | 2.04 ± 0.02                       | 0.046 ± 0.008    |
| MMP-9    | 3.9 ± 0.3                         | 3.8 ± 0.6                         | 0.10 ± 0.01      |
| MMP-3    |                                   |                                   | 10 ± 2           |
| MMP-7    | NI$^a$                            |                                   |                  |
| MMP-1    | NI                                |                                   |                  |
| MMP-14$_{cat}$ |                                 |                                   | 0.21 ± 0.03     |
| Compound 2 |
| MMP-2    | 7 ± 1                             | 4 ± 1                             | 0.06 ± 0.02      |
| MMP-9    | 80 ± 20                           | 2.2 ± 0.6                         | 0.26 ± 0.09      |
| MMP-3    | 4.4 ± 0.8                         |                                   | 0.19 ± 0.02      |
| MMP-7    | NI                                |                                   |                  |
| MMP-1    | NI                                |                                   |                  |
| MMP-14$_{cat}$ |                                 |                                   |                  |

$^a$NI for “not inhibiting” at concentrations up to 60 µM.
Figure 2
Figure 3

**Compound 1**

- 72 kDa
- 64 kDa
- 62 kDa

| 0 | 10 | 100 | 1000 |
|---|----|-----|------|

**Marimastat**

- 60 kDa
- 57 kDa

| 0 | 0 | $10^{-2}$ | $10^{-1}$ | 1 | 10 |
|---|---|-----------|-----------|---|----|

(µM)
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