Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that play important roles in physiological and pathological conditions. Both gelatinases (MMP-2 and -9) and membrane-type 1 MMP (MMP-14) are important targets for inhibition, since their roles in various diseases, including cancer, have been well established. We describe herein a set of mechanism-based inhibitors that show high selectivity to gelatinases and MMP-14 (inhibitor 3) and to only MMP-2 (inhibitors 5 and 7). These molecules bind to the active sites of these enzymes, initiating a slow binding profile for the onset of inhibition, which leads to covalent enzyme modification. The full kinetic analysis for the inhibitors is reported. These are nanomolar inhibitors ($K_i$) for the formation of the noncovalent enzyme-inhibitor complexes. The onset of slow binding inhibition is rapid ($k_{on}$ of $10^4$ to $10^5$ M$^{-1}$ s$^{-1}$), and the reversal of the process is slow ($k_{off}$ of $10^{-3}$ to $10^{-4}$ s$^{-1}$). However, with the onset of covalent chemistry with the best of these inhibitors (e.g. inhibitor 3), very little recovery of activity (<10%) was seen over 48 h of dialysis. We previously reported that broad spectrum MMP inhibitors like GM6001 enhance MT1-MMP-dependent activation of pro-MMP-2 in the presence of tissue inhibitor of metalloproteinases-2. Herein, we show that inhibitor 3, in contrast to GM6001, had no effect on pro-MMP-2 activation by MT1-MMP. Furthermore, inhibitor 3 reduced tumor cell migration and invasion in vitro. These results show that these new inhibitors are promising candidates for selective inhibition of MMPs in animal models of relevant human diseases.

Extracellular proteolysis is an essential aspect of both physiological and pathological processes. Several enzyme families have been implicated in extracellular proteolysis, of which the matrix metalloproteinases (MMPs)$^3$ constitute an important group. The MMPs are zinc-dependent endopeptidases that play key roles in embryonic development, neurological processes, wound healing, angiogenesis, arthritis, cardiovascular diseases, and cancer. Just to mention a few examples. In cancer, for instance, MMPs are implicated at all stages of tumor progression, including tumor growth, angiogenesis, and metastasis (1). Two MMPs, gelatinases A and B (MMP-2 and MMP-9, respectively), are highly expressed in human cancer, and a direct relationship between cancer progression and gelatinase expression and activity has been well established in many studies (2). As tumors manifest high levels of gelatinase activity, inhibitors specific for the gelatinases are highly sought.

In the past 8 years, there have been numerous approaches aimed at targeting MMP activities in tumors, and several clinical trials were carried out to test the efficacy of various inhibitors. Unfortunately, the results of these trials were disappointing due to the lack of an objective clinical response and undesired side effects. Many reasons have been postulated for these effects, but at the core of the problem remains the issue of inhibitor selectivity (3, 4). Indeed, virtually all MMP inhibitors tested so far have been broad-spectrum inhibitors, designed around chelation of the active site zinc ion (5), and their spectrum of inhibition includes, in addition to MMPs, other metalloenzymes. Because targeting gelatinases remains of great promise in cancer therapy (6), efforts aimed at developing better and selective gelatinase inhibitors continue.

A mere handful of selective inhibitors for MMPs have been reported in the literature (for a review, see Ref. 7). We previously described the design and properties of inhibitor 1 (Fig. 1), which is a selective mechanism-based inhibitor for gelatinases. This compound binds to the active sites of MMP-2 and MMP-9, with the thirane moiety coordinating with the zinc ion. This coordination to the active site metal ion activates the thirane ring for opening by the nucleophilic attack of the active site glutamate in these enzymes (Fig. 2). A unique property of this inhibitor is that on binding to the active site zinc ion, a pattern of slow binding for inhibition sets in, leading to a rapid process for the onset of inhibition with an attendant slow recovery from slow binding at the noncovalent stage of inhibition. This noncovalent inhibited species leads to covalent inhibition by modification of the glutamte.

Whereas inhibitor 1, the prototype of this type of novel mechanism-based inhibitor for gelatinases, is showing promise in mouse models for diseases involving gelatinases (8, 9), the poor solubility of this inhibitor in aqueous medium is a limitation of the molecule, which we have attempted to remedy. Furthermore, we have been interested in exploiting the concept behind the inhibitor design in targeting other MMPs. A computational model of the inhibitor bound in the active site of MMP-2 within the constraints of the data from x-ray absorption spectroscopy has been generated (10) (Fig. 2B). This model for inhibition of inhibitor 1 led the way in exploration of the next generation of this type of MMP inhibitor. The possibility for specific electrostatic interactions near the terminal phenyl group in inhibitor 1 bound to the active site of MMP-2 was anticipated for judiciously designed chemical functionalities into the molecular template of compound 1. We have introduced three functional groups, the methylsulfonylamide (compounds 2 and 3), the nitro (compounds 4 and 5), and the acetamide (compounds 6 and 7), at the terminal phenyl ring system to exploit these electrostatic interactions. It was expected that these molecules would improve the solubility in aque-
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To a stirred solution of 9 (3.46 g, 20.8 mmol) in NaN-dimethylformamide (100 ml) were added cesium carbonate (10.2 g, 31.2 mmol) and 1-fluoro-4-nitrobenzene (10) (2.94 g, 20.8 mmol) at room temperature, and the mixture was stirred at the same temperature for 2 days. After dilution with water, the mixture was extracted into hexane (3×). The combined organic layer was washed with water and brine, dried over Na₂SO₄, and concentrated under reduced pressure to give 11 (5.32 g, 89%) as a pale yellow oil. ¹H NMR (300 MHz, CDCl₃): δ 3.55 (dt, 2H, J = 6.9, 1.2 Hz), 5.10 (dt, 1H, J = 10.2, 1.2 Hz), 5.13 (dt, 1H, J = 7.1, 1.2 Hz), 5.88 (ddt, 1H, J = 17.1, 10.2, 6.9 Hz), 6.98–7.04 (m, 4H), 7.38–7.43 (m, 2H), 8.18–8.22 (m, 2H), 13C NMR (125 MHz, CDCl₃): δ 37.8, 117.1, 117.9, 120.9, 126.0, 132.3, 132.5, 133.4, 142.7, 153.4, 163.1; HRMS (FAB) calcd for C₁₇H₁₇NO₂S (M⁺) 287.0616, found 287.0593.

1-Allylthio-4-(4-nitrophenoxy)benzene—To a stirred solution of 11 (636 mg, 2.21 mmol) in tetrahydrofuran (22 ml) were added acetic acid (2.54 ml, 44.2 mmol) and zinc powder (5.80 g, 88.4 mmol) at room temperature, and the suspension was stirred for 30 min (an exothermic reaction). After dilution with ethyl acetate, the mixture was filtered through Celite. The filtrate was washed with saturated NaHCO₃ and brine, dried over Na₂SO₄, and concentrated under reduced pressure to give a crude 12 (577 mg) as an orange oil, which was employed in the next reaction without purification.

To a stirred solution of 12 (577 mg) in CH₂Cl₂ (10 ml) were added pyridine (894 µl, 11.1 mmol) and methanesulfonyl chloride (205 µl, 2.65 mmol) at ice-water temperature. After 15 min, the mixture was warmed to room temperature, and the stirring was continued for an additional 2 h. Subsequent to the addition of saturated NaHCO₃, the mixture was extracted with ethyl acetate (3×). The combined organic layer was washed with 1 M aqueous HCl, saturated NaHCO₃ solution, and brine, dried over Na₂SO₄, and concentrated under reduced pressure. The resultant residue was purified by silica gel column chromatography (CH₂Cl₂) to give 13 (662 mg, 89% from 11) as a pale red solid. Compound 12: ¹H NMR (300 MHz, CDCl₃): δ 3.45 (br d, 2H, J = 7.2 Hz), 3.59 (br s, 2H), 5.01–5.06 (m, 2H), 5.84 (ddt, 1H, J = 17.1, 9.6, 6.9 Hz), 6.66–6.70 (m, 2H), 6.83–6.88 (m, 4H), 7.29–7.32 (m, 2H). Compound 13: ¹H NMR (300 MHz, CDCl₃): δ 3.01 (s, 3H), 3.50 (dt, 2H, J = 7.2, 1.2 Hz), 5.04–5.11 (m, 2H), 5.86 (ddt, 1H, J = 16.8, 10.2, 6.9 Hz), 6.67 (br s, 1H), 6.90–6.95 (m, 2H), 6.96–7.01 (m, 2H), 7.20–7.26 (m, 2H), 7.32–7.37 (m, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 38.4, 39.3, 117.5, 119.3, 119.9, 123.8, 130.2, 132.0, 132.9, 133.8, 155.2, 156.1; HRMS (FAB) calcd for C₁₇H₁₅NO₂S (M⁺) 335.0650, found 335.0639.

4-(4-Acetamidophenoxy)-1-allylthiobenzene—To a stirred solution of 13 (794 mg) in CH₂Cl₂ (10 ml) were added pyridine (577 mg) in CH₂Cl₂ (10 ml) and acetic anhydride (292 µl, 3.09 mmol) at ice-water temperature. After 15 min, the mixture was warmed to room temperature, and the stirring was continued for an additional 2 h. Subsequent to the addition of saturated NaHCO₃, the mixture was extracted with ethyl acetate (3×). The combined organic layer was washed with 1 M aqueous HCl, saturated NaHCO₃ solution, and brine, dried over Na₂SO₄, and concentrated under reduced pressure. The resultant residue was purified by silica gel column chromatography (CH₂Cl₂) to give 14 (782 mg, 99% from 11) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 2.17 (s, 3H), 3.47 (d, 2H, J = 7.0 Hz), 5.04–5.07 (m, 2H), 5.85 (ddt, 1H, J = 17.0, 10.0, 7.0 Hz), 6.87–6.90 (m, 2H), 6.94–6.97 (m, 2H), 7.31–7.35 (m, 2H), 7.44–7.47 (m, 2H), 7.54 (br s, 1H). ¹³C NMR (75 MHz, CDCl₃): δ 28.4, 38.5, 117.5, 118.7, 119.6, 121.7, 129.0, 132.9, 133.6, 133.7, 153.1, 156.7, 168.4; HRMS (FAB) calcd for C₁₇H₁₅NO₂S (M⁺) 335.0650, found 335.0639.

4-(4-Methanesulphonamido)phenoxymethylthiobenzene—To a stirred solution of 13 (544 mg, 1.62 mmol) in CH₂Cl₂ (20 ml) was...
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added mCPBA (4.2 g, 17.05 mmol) at ice-water temperature, and the mixture was stirred at room temperature for 9 days. With ice cooling, the reaction was quenched with saturated Na₂S₂O₃ solution and the mixture was extracted with ethyl acetate (3 ×). The combined organic layer was washed with saturated Na₂S₂O₃ solution, saturated NaHCO₃ solution, and water, and dried over Na₂SO₄, and concentrated under reduced pressure. The resultant residue was purified by silica gel column chromatography (ethyl acetate/hexane = 3/2) to give 2 (386 mg, 62%) as a white solid. ¹H NMR (300 MHz, CDCl₃): δ 2.51 (dd, 1H, J = 4.5, 2.5 Hz), 2.83 (t, 1H, J = 4.5, 1.5 Hz), 3.26–3.34 (m, 4H), 7.16–7.17 (m, 2H), 7.23–7.25 (m, 2H), 7.99–8.01 (m, 2H), 8.28–8.30 (m, 2H); ¹³C NMR (125 MHz, CDCl₃): δ 45.7, 45.8, 59.6, 119.1, 119.6, 120.8, 121.0, 122.6, 131.0, 134.9, 144.0, 160.2, 160.8; HRMS (FAB) calcd for C₁₅H₁₄NO₆S (M + H⁺) 348.0913, found 348.0916.

[4-(4-Acetamidophenyl)phenylsulfonfonylmethylthiirane—This material was prepared in the same manner as described for 3, with the exception that 6 was used in place of 2. The crude material was purified by silica gel column chromatography (ethyl acetate/hexane, 3/2 to 1/2) to give 7 (76%) as a white solid. ¹H NMR (300 MHz, CDCl₃): δ 2.16 (dd, 1H, J = 5.1, 1.8 Hz), 2.20 (s, 3H), 2.34 (dd, 1H, J = 6.3, 1.5 Hz), 3.06 (m, 1H), 3.19 (dd, 1H, J = 14.1, 7.8 Hz), 3.52 (dd, 1H, J = 14.1, 5.7 Hz), 7.03–7.08 (m, 4H), 7.52 (br s, 1H), 7.56–7.59 (m, 2H), 7.84–7.87 (m, 2H); ¹³C NMR (125 MHz, CDCl₃): δ 24.1, 24.4, 26.0, 62.6, 117.4, 121.0, 121.8, 130.7, 131.6, 135.2, 150.7, 163.1, 168.6; HRMS (FAB) calcd for C₁₃H₁₂NO₆S₃ (M + H⁺) 364.0677, found 364.0651.

Assessment of Inhibitor Solubility—Aliquots (10 μl) of the solutions of the thiorane compounds 1, 3, 5, and 7 in Me₂SO (e.g. 10 mM, 12 mM, 14 mM and higher concentrations) were added to 990 µl of buffer R (50 mM HEPES (pH 7.5), 0.15 mM NaCl, 5 mM CaCl₂, 0.01% Brij-35, 1% Me₂SO) at 37 °C. Each mixture was inspected for clarity (or turbidity) to calculate the approximate upper limit of solubility.

Enzymatic Activity Assays—Enzymatic activity was monitored with synthetic peptide, fluorescence-quenched substrates from Peptides International, Inc. (Louisville, KY). The activities of MMP-2, MMP-9, MMP-7, and MMP-14 were monitored with MOCACPLGLA pr(Dnp)AR-NH₂ at excitation and emission wavelengths of 328 and 393 nm, respectively, in buffer R. MOCACRPKPVF-norvalyl (Nva)WRK(Dnp)NH₂ was the fluorogenic substrate used to measure MMP-3 at 325 and 393 nm in buffer R. MMP-1 was assayed with (Dnp)P cyclohexanoylanil (Cha)GC(Me)-HAKN*-methylanthranoyl (NMa)NH₂ at 340 and 440 nm, in a buffer consisting of 50 mM Tris (pH 7.6), 200 mM NaCl, 5 mM CaCl₂, 20 mM ZnSO₄, 0.05% Brij-35. Less than 10% substrate hydrolysis was monitored (12). Fluorescence was measured using a Photon Technology International spectrofluorometer, equipped with RadioMaster™ and FelixTM hardware and software, respectively. The excitation and emission band passes were 1 and 3 nm, respectively. An integration time of 4 s was used for data acquisition. The assays were carried out at 25 °C, and the cuvette holder was kept at the same temperature. Quartz or disposable acrylic micro- or semimicrocuvettes from Sarstedt (New York, NY) and Perfection Scientific (Atascadero, CA), respectively, were used.

Enzyme Inhibition Studies—Slow binding enzyme inhibition was monitored continuously for 20–60 min by adding the enzyme (0.5–1 nM) to a solution of buffer R containing the appropriate fluorogenic substrate and increasing concentrations of the inhibitor (final volume 2 ml) in acrylic cuvettes with stirring. The progress curves were nonlinear least squares-fitted to Equation 1 (13).

\[ F = v_o + (v_o - v_i) \left(1 - \exp(-kt)\right)/k + F_o \]  

where \( v_o \) represents the initial rate, \( v_i \) is the steady state rate, \( k \) is the apparent first order rate constant characterizing the formation of the steady-state enzyme-inhibitor complex, and \( F_o \) is the initial fluorescence, using the program Scientist (MicroMath Scientific Software, Salt Lake City, UT). Association and dissociation rate constants (\( k_{\text{on}} \) and \( k_{\text{off}} \), respectively) were obtained from the slope and intercept, respectively, of plots of the apparent first order rate constant \( k \) versus the inhibitor concentration according to Equation 2.

\[ k = k_{\text{off}} + k_{\text{on}}/[I](1 + [S]/K_{\text{m}}) \]  

describing a one-step association mechanism (Scheme 1), where \( S \) is the fluorogenic peptide substrate used, and the \( EI^* \) is the product of slow binding inhibition. The expression for \( k_{\text{on}} \) includes the requisite conformational change necessary for the formation of \( EI^* \). The \( K_m \) values used for the reaction of MMP-2, MMP-9, and MMP-14 with the fluorogenic substrate MOCACPLGLA pr (Dnp)AR-NH₂ were 2.46 ± 0.34, 3.06 ± 0.74 (14), and 6.9 ± 0.6 μM (15), respectively. The inhibition constant,
$K_i$ was given by $k_{\text{on}}/k_{\text{off}}$. Alternatively, $K_i$ values were obtained by plotting $(v_o - v_s)/v_s$ versus the inhibitor concentration, according to Equation 3.

\[
\frac{(v_o - v_s)}{v_s} = \frac{1}{K_i(1 + [S]/K_m)} 
\]  
(Eq. 3)

For analysis of simple linear competitive inhibition, reaction mixtures containing the enzyme ($\sim 1$ nM) and increasing concentrations of the inhibitor, in buffer R (final volume 1 ml), were incubated for $\sim 16$ h at 25 °C in acrylic semimicrocuvettes. The remaining enzymatic activity was measured with the appropriate synthetic peptide fluorogenic substrate for 5–10 min. The initial velocities for the reaction of the enzyme with the substrate were determined by linear regression analysis of the fluorescence versus time traces using FeliX™. These initial rates were fitted to Equation 4 (16),

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

where $v_i$ and $v_o$ represent the initial velocity in the presence and absence of inhibitor, respectively, using the program Scientist.

**Equilibrium Dialysis**—Mixtures of enzyme (10 nM) in the presence and absence of inhibitor (1 mM) were incubated at room temperature.

**SCHEME 1**

**FIGURE 2.** Mechanism of action of inhibitor 1. A, coordination of the thiirane with the zinc ion is a prerequisite for the inhibition process. B, stereoview of the computational model for the noncovalent binding of inhibitor 1 in the active site of MMP-2. The active site of the enzyme is depicted as a Connolly surface in green. The active site zinc ion is depicted as an orange sphere, with the three coordinating histidine residues depicted in capped sticks. Inhibitor 1 (in capped sticks and colored according to atom types) is shown coordinated to the active site zinc ion via the thiirane sulfur. The loop that constitutes the $S_1$ subsite of the enzyme is drawn as a tube in purple, so the terminal phenyl group of the inhibitor is visible. The site of structure elaboration in arriving at molecules 2–7 is indicated by the white arrow.

**FIGURE 3.** Synthetic scheme for the mechanism-based inhibitors for MMPs. The synthetic details are given under “Experimental Procedures.”
Mechanism-based Inhibition of MMPs

| Enzyme | $k_{on}$ | $k_{off}$ | $K_i$ |
|--------|---------|-----------|-------|
|        | $M^{-1}s^{-1}$ | s$^{-1}$ | $\mu M$ |
| Compound 3 | | | |
| MMP-2 | $(2.1 \pm 0.5) \times 10^4$ | $(3.5 \pm 1.6) \times 10^{-4}$ | 0.016 ± 0.009 |
| MMP-9 | $(4.9 \pm 0.8) \times 10^4$ | $(9.0 \pm 2.0) \times 10^{-4}$ | 0.18 ± 0.05 |
| MMP-14 | $(6.9 \pm 0.8) \times 10^4$ | $(6.4 \pm 0.5) \times 10^{-4}$ | 0.9 ± 0.1 |
| MMP-7 | | | 295 ± 10 |
| MMP-3 | | | 3.6 ± 0.2 |
| MMP-1 | | | NI up to 25 $\mu M$ |
| Compound 5 | | | |
| MMP-2 | $(1.9 \pm 0.6) \times 10^3$ | $(1.3 \pm 0.2) \times 10^{-3}$ | 0.7 ± 0.2 |
| MMP-9 | | | 1.0 ± 0.1 |
| MMP-14 | | | 4.9 ± 0.3 |
| MMP-7 | | | 153 ± 16 |
| MMP-3 | | | 131 ± 9 |
| MMP-1 | | | 67 ± 18 |
| Compound 7 | | | |
| MMP-2 | $(1.2 \pm 0.3) \times 10^3$ | $(1.3 \pm 0.3) \times 10^{-3}$ | 0.11 ± 0.04 |
| MMP-9 | | | 0.13 ± 0.01 |
| MMP-14 | | | 0.68 ± 0.05 |
| MMP-7 | | | 39 ± 3 |
| MMP-3 | | | 12.2 ± 0.9 |
| MMP-1 | | | 5.4 ± 0.4 |

* NI, not inhibiting.

for ~3 h. The remaining enzyme activity was measured with the appropriate fluorogenic substrate, as described above. Part of the reaction mixture (~150 $\mu l$) was dialyzed in either dialysis tubing (Invitrogen) or in a 0.1–0.5-ml capacity Slide-A-Lyzer® dialysis cassette (Pierce), against buffer R (3 x 1 liter) containing no Me$_2$SO, at room temperature, for >4-h periods prior to change of buffer to allow for equilibration, over a 48-h period. The remainder of the inhibition mixture was left on a rotator, at room temperature, over the same period of time. Both the dialyzed and nondialyzed solutions were tested for MMP activity using the proper fluorogenic substrate. Enzyme activity was expressed as a percentage relative to that in the absence of inhibitor.

Cell Culture—Human HeLa S3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) (CCL-2.2) and grown in suspension in a MEM Spinner (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 5% horse serum. Nonmalignant monkey kidney epithelial cells, BS-C-1 (CCL-26), and human fibrosarcoma cells, HT1080 (CCL-121), were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum and antibiotics.

Recombinant Vaccinia Viruses—Recombinant vaccinia viruses encoding for T7 RNA polymerase (vTF7-3) or pro-MMP-2 (vT7-72), pro-MMP-9 (vT7-92), MT1-MMP (vT7-MT1), TIMP-1 (vT7-TIMP-1), and TIMP-2 (vSC59-T2) were produced by homologous recombination as previously described (17).

Recombinant Proteins, Enzymes, and Inhibitors—Human recombinant pro-MMP-2 and 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 from the media as previously described (14). Pro-MMP-2 and pro-MMP-9 were activated by incubation with 1 mM p-aminophenylmercuric acetate (APMA) for ~2 h at 37 °C as previously described (14). APMA was dialyzed out in collagenase buffer (50 mM Tris-HCl (pH 7.5), 5 mM CaCl$_2$, 150 mM NaCl, and 0.02% Brij 35). Human recombinant active MMP-1 and MMP-7 were from R&D (Minneapolis, MN) and Chemicon International (Temecula, CA), respectively, and the recombinant catalytic domains of human MMP-3 and MMP-14 were from EMD Biosciences (La Jolla, CA). Active enzyme concentration was determined by active site titration with solutions of either TIMP-1 or TIMP-2 with known concentration. The hydroxamate-based MMP inhibitor BB-94 was synthesized in the Mobashery laboratory, and GM6001 was purchased from Chemicon. Stock solutions of BB-94, GM6001, and compounds 2–7 were prepared in Me$_2$SO in the millimolar concentration range.

Pro-MMP-2 Activation on Cells—Confluent BS-C-1 cells in 12-well plates were co-infected with vTF7-3 and vT7-MT1 viruses for 45 min in infection medium (DMEM supplemented with 2.5% fetal bovine serum and antibiotics), at 37 °C, as described (18). The infection medium was removed, and the cells were incubated overnight with serum-free DMEM supplemented with L-glutamine and antibiotics containing increasing concentrations (0–5 $\mu M$) of the synthetic MMP inhibitors. The cells were washed twice with phosphate-buffered saline and incubated for 6 h with serum-free DMEM containing pro-MMP-2 (10 nM). The cells were rinsed twice with cold phosphate-buffered saline and lysed with cold lysis buffer (25 mM Tris-HCl (pH 7.5), 1% IGEPAL CA-630, 100 mM NaCl) containing protease inhibitors (one pellet of Complete Mini, EDTA-free protease inhibitor mixture from Roche Applied Science/10 ml of buffer). The lysates were then subjected to gelatin zymography to monitor pro-MMP-2 activation and to immunoblot analysis to detect MT1-MMP expression and processing.

Gelatin Zymography and Immunoblot Analysis—Gelatin zymography was performed using 10% Tris/glycine SDS-polyacrylamide gels, containing 0.1% gelatin, as previously described (19). The samples for immunoblot analysis were subjected to reducing SDS-PAGE followed by transfer to nitrocellulose membranes. MT1-MMP was probed with rabbit polyclonal antibody 815 to MT1-MMP from Chemicon.


Migration and Invasion Assays—For migration assays, HT1080 cells were cultured in 6-well plates in complete medium until they reached confluence. Prior to the migration assay, the cells were treated with serum-free medium containing mitomycin C (25 μg/ml), in the presence and absence of concanavalin A (25 μg/ml, 30 min) to induce pro-MMP-2 activation (20). Scratch wounds were then carefully made in the confluent monolayer using a disposable plastic pipette tip. After gentle rinsing twice with phosphate-buffered saline to remove detached cells, serum-free media containing increasing concentrations of inhibitor 3 were added, and the cells were incubated at 37 °C for various times. Photographs were taken using an Olympus model DF 12–2 camera connected to a Nikon TMS-F microscope at ×10 magnification, at the indicated time points. The extent of wound closure in the presence or absence of inhibitor was quantified by measuring the width of the wound with a ruler using an amplified PowerPoint figure.

Tumor cell invasion was carried out in 8-μm pore Transwell inserts (BD Biosciences) coated with 50 μg of Matrigel/insert. HT1080 cells suspended in serum-free DMEM containing 0.1% bovine serum albumin and various doses of inhibitor 3 (0.1–10 μM) or 1% Me2SO (vehicle) were seeded (2 × 105 cells/insert) on the Matrigel-coated inserts. The lower compartment was filled with DMEM containing 5% fetal bovine serum. After an 18-h incubation at 37 °C in a humidified atmosphere with 5% CO2, the upper surface of the membrane in each insert was wiped off with a cotton swab to remove all of the noninvading cells. The cells that migrated to the lower side of the Matrigel-coated filter were fixed and stained with Diff-Quik® (Dade Behring Inc., Newark, DE) and counted under a microscope in three different fields. Each treatment was assayed in quadruplicate.

Chemosensitivity Assay—Cell viability after exposure of the cells to the inhibitors was assessed by 2-(4-iiodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1, Roche Applied Science) staining. Briefly, HT1080 cells (2 × 104 cells/well) were seeded in 96-well culture plates in complete medium. After overnight culture, the medium was replaced with serum- and phenol-free media containing 0.1% bovine serum albumin and supplemented with or without inhibitor 3 (0–10 μM final concentrations). Control medium was supplemented with 1% Me2SO. After an 18-h incubation, WST-1 (10 μl/well) was added, and the difference in absorbance at 450 and 655 nm (reference filter) was measured using a Bio-Rad Benchmark microplate reader. Data were collected using the Microplate Manager software. The absorbance of blank wells containing control medium but no cells (typically <5%) was subtracted. Each treatment was assayed in quadruplicate.

RESULTS AND DISCUSSION

Design and Synthesis of MMP Inhibitors—The computational model for binding of inhibitor 1 to the active site of MMP-2 is shown in Fig. 2B. The site for substitution at the para position of the terminal phenyl ring of the inhibitor is indicated by an arrow. Fig. 3 shows the synthetic route for oxiranes 2, 4, and 6, and thiranes 3, 5, and 7. Chemoselective allylation of commercially available 4-hydroxythiophenol (8) provided phenol 9 (11) in 70% yield, which was subsequently coupled with 1-fluoro-4-nitrobenzene (10) in the presence of cesium carbonate to afford the diphenyl ether 11 in 89% yield. The nitro group of 11 was reduced over elemental zinc, and the resulting amine 12 was treated with methanesulfonyl chloride or acetic anhydride to give the corresponding amides 13 and 14, respectively, in high yields. Oxidation of 13, 11, and 14 to their corresponding oxiranes 2, 4, and 6 required an excess of mCPBA (10–12 eq.) and long reaction times (9–10 days), due to the low reactivity of the olefin moieties; the isolated yields of the oxiranes were moderate (34–62%). Finally, the conversion of compounds 2, 4, and 6 to the corresponding thiiranes 3, 5, and 7 was accomplished by the treatment with thiourea in good yields. Compounds 3 and 7 had improved solubility in aqueous solution compared with the prototypic inhibitor 1. Solubility was investigated in 50 mM HEPES (pH 7.5), 0.15 M NaCl, 5 mM CaCl2, 0.01% Brij 35, 1% Me2SO, which was the buffer that we used for all of the kinetic experiments (see below). The maximal solubility for the samples was as follows: 1, 80 μM; 3, 340 μM; 5, 60 μM; 7, 540 μM.

Enzyme Inhibition Kinetics—The mechanism of action of this class of MMP inhibitors stipulates that the thiolate sulfur would coordinate with the active site zinc ion (Fig. 2). Consistent with this expectation, the three synthetic thiiranes of this study (compounds 3, 5, and 7) are excellent inhibitors of gelatinases (and of MT1-MMP in the case of inhibitor 3) (TABLE ONE), whereas the corresponding oxiranes (compounds 2, 4, and 6) are either poor inhibitors or not inhibitory at all toward all of the tested MMPs (TABLE TWO).

In slow binding inhibition, upon binding of the inhibitor to the enzyme, the complex undergoes a requisite conformational change that is not readily predisposed for the reversal of the inhibition (21–23). The slow binding inhibitor shows a unique profile for the onset of inhibition that is discerned by nonlinear progress curves. We see slow binding behavior for inhibitor 3 (with MMP-2, MMP-9, and MMP-14), for inhibitor 5 (only with MMP-2), and for inhibitor 7 (only with MMP-2) (Fig. 4).

The second order rate constants for the onset of slow binding inhibition (k_on) are rapid (10^3 to 10^5 M⁻¹ s⁻¹), and the first order rate constants for the reversal of the process from the noncovalent enzyme-inhibitor complexes (k_off) are slow (10⁻⁴ to 10⁻¹ s⁻¹; e.g. the t_1/2 values for reversal for inhibitor 3 with MMP-2 and MMP-9 are 34 and 13 min, respectively). The dissociation constants for the noncovalent

| Enzyme | K_i (μM) |
|-------|---------|
| Compound 2 | 2.4 ± 0.5 |
| MMP-2 | 2.4 ± 0.5 |
| MMP-9 | 1.5% up to 26 μM |
| MMP-14 | 2.4 ± 1000 μM |
| MMP-7 | 379 ± 29 |
| MMP-3 | 3.4 ± 9 |
| MMP-1 | 13 ± 1 |
| MMP-9 | 25 ± 5 |
| MMP-14 | 76 ± 13 |
| MMP-7 | 130 ± 15 |
| MMP-3 | 190 ± 100 μM |
| MMP-1 | 100 ± 50 μM |

TABLE TWO

Kinetic parameters for MMP inhibition by compounds 2, 4, and 6

The enzymes (0.5–1 nm) were incubated with increasing concentrations of inhibitor in buffer R. The remaining activity was measured with the appropriate synthetic fluorogenic substrate. The kinetic parameters for rapid, competitive inhibition were evaluated as described under “Experimental Procedures.”
valent complexes ($K_i$) that result from slow binding inhibition are computed from the ratios of $k_{off}/k_{on}$. Compounds 3, 5, and 7 are clearly selective for gelatinases, with 3 showing the slow binding behavior with MMP-14 as well. The $K_i$ values of the slow binding component for inhibition by 3 (16 ± 9 nM, 180 ± 50 nM, and 900 ± 100 nM for MMP-2, MMP-9, and MMP-14, respectively), 5 (700 ± 200 nM for MMP-2), and 7 (110 ± 40 nM for MMP-2) are listed in TABLE ONE. It is noteworthy that the inhibition profiles for inhib-
itors $1, 3, 5,$ and $7$ as mechanism-based inhibitors are different from one another, despite the similar structural template for the class. Briefly, inhibitor $1$ can inhibit both MMP-2 and -9 (the gelatinases), inhibitor $3$ inhibits the gelatinases plus MMP-14, and most interestingly, inhibitors $5$ and $7$ are mechanism-based inhibitors only for MMP-2. Furthermore, these are nanomolar inhibitors for their targeted enzymes and exhibit comparable values for the $k_{on}$ and $k_{off}$ parameters for the slow binding components of their kinetics.

**Covalent Versus Noncovalent Inhibition of MMPs**—The thiirane class of MMP inhibitors was designed to be covalent enzyme inhibitors. Upon formation of the noncovalent enzyme-inhibitor complex, the ubiquitous active site glutamates of MMPs (Glu$^{404}$ for MMP-2, for example) were expected to be covalently modified by the inhibitor with the requisite thiirane ring opening (Fig. 2). The kinetics of inhibition indicate two components, a noncovalent stage (slow binding) and a subsequent stage that may be attributed to the covalent modification of the active site glutamate, as will be outlined.

The covalent component of inhibition results in modification of the glutamate as an ester on its side chain carboxylate. The earlier x-ray absorption spectroscopy analysis with inhibitor $1$ (10) had provided evidence for the covalent bond formation, in that upon the onset of inhibition, the method revealed the formation of a thiolate from the thiirane of the inhibitor (ring opening), coordinated to the active site zinc ion.

Whereas a slow binding step need not necessarily be a prerequisite for covalent chemistry, both the mechanism-based process leading to covalent enzyme modification and the slow binding behavior produce time dependence for the loss of activity seen with these inhibitors (Fig. 4).

Our experience with inhibitor $1$ had shown that slow binding led to covalent chemistry, with a longevity for the final inhibited species substantially exceeding the duration that would have been anticipated from 4 times the $t_{1/2}$ for recovery of activity from the slow binding component of inhibition (in other words, four half-lives leading to an anticipated 94% recovered of activity due to the noncovalent component). This is the case with inhibitors $3, 5,$ and $7$ as well. The slowest $t_{1/2}$ calculated for recovery of activity from the noncovalent slow binding species for the best inhibition (compound 3 with MMP-2) is 34 min. Yet, a mere 1% of activity recovery was seen for MMP-2 inhibited by inhibitor $3$ after 48 h of dialysis. Four half-lives for recovery from inhibition (94% anticipated recovered activity) with this inhibitor and MMP-2 should be achieved in just under 2.5 h (136 min) were it merely the slow binding event that accounted for MMP-2 inhibition. This is clearly not the case, and the inhibi-
Mechanism-based Inhibition of MMPs

Mechanism-based inhibition of MMPs is more stable than the $k_{\text{off}}$ (from which $k_{\text{on}}$ is evaluated) indicates. The results of dialyses for inhibitors 3, 5, and 7 are given in Fig. 5.

Having documented above that mere slow binding behavior cannot be responsible for the complete inhibition that we see, we need to explain why any recovery of activity should be seen if we deal with covalent chemistry. The answer is that the stability of covalent bonds is relative. Esters are among the least stable covalent bonds in aqueous solution (24). This bond would undergo hydrolysis, resulting in recovery of activity. The process accelerates when there is a more significant exposure of the ester bond to water, conditions that can arise when the protein is denatured.

Matrix-assisted laser desorption time-of-flight mass spectrometry analysis, performed on an Applied Biosystems Voyager-DE STR (Framingham, MA) instrument at the Harvard Microchemistry and Proteomics Analysis Facility (Cambridge, MA), was attempted on samples containing MMP-2 (10 μM) in the presence and absence of inhibitor 3 to detect a shift in molecular mass consistent with a complex of active MMP-2 (~62 kDa) with the inhibitor. However, after several attempts with different conditions, we failed to detect a 400-Da addition in molecular mass to the 62-kDa peak. The difficulty is that at this high end of mass detection, the signals are broadened, and the identification of the small incremental increase due to the mass of the inhibitor was not possible within the resolution of the instrument.

Effect of Gelatinase Inhibitors on Pro-MMP-2 Activation by MT1-MMP—MT1-MMP has been identified as the physiological activator of pro-MMP-2 (25). This reaction is regulated at multiple levels, and its rate is significantly enhanced by TIMP-2, which, by binding active MT1-MMP, acts as a "receptor" for pro-MMP-2 on the cell surface (25, 26). The binding of pro-MMP-2 to the MT1-MMP-TIMP-2 complex, facilitates the first pro-MMP-2 cleavage by a neighboring TIMP-2-free MT1-MMP molecule (26). Pro-MMP-2 activation requires a second autolytic cleavage (27), leading to full activation. We have previously shown that broad spectrum synthetic MMP inhibitors (e.g. marimastat) enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2 (15), a process that appears to involve stabilization of mature MT1-MMP at the cell surface by the MMP inhibitor. This enhancing effect on pro-MMP-2 activation was not observed when the cells were exposed to inhibitor 1 (15), which exhibits lower affinity toward MT1-MMP, a feature of its selectivity for inhibition of gelatinases. Therefore, we proposed that nonspecific targeting of MT1-MMP by broad spectrum MMP inhibitors might, under certain conditions, elicit a counterproductive effect by enhancing the activity of the MT1-MMP/gelatinase A axis (28). Because inhibitor 3 is also selective for the gelatinases, we postulated that it might behave like inhibitor 1 in a cellular system of pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2. To this end, B5-C1 cells, which express low levels of endogenous TIMP-2, were infected to express MT1-MMP and incubated with pro-MMP-2 in the presence of either GM6001, a broad spectrum MMP inhibitor, or inhibitor 3, as described (15). Pro-MMP-2 activation was followed by gelatin zymography. As shown in Fig. 6A, exposure of the MT1-MMP-expressing cells to as little as 40 nM GM6001 induced pro-MMP-2 activation, as determined by the appearance of the active form. Higher inhibitor concentrations further enhanced pro-MMP-2 activation, under these conditions. Of note, this enhancing effect of broad spectrum MMP inhibitors such as GM6001 requires the endogenous TIMP-2, as we have previously shown (15). Consistently, GM6001 caused a dose-dependent accumulation of active MT1-MMP (57 kDa) (Fig. 6B). In contrast, when the cells were incubated with inhibitor 3 (up to 4 μM), pro-MMP-2 activation was not observed. Also, the accumulation of active MT1-MMP was not observed with inhibitor 3, consistent with its reduced affinity for this protease when compared with MMP-2 (TABLE ONE). Although inhibitor 3 is also a mechanism-based inhibitor for MT1-MMP, its lower affinity relative to MMP-2 is likely to preclude this inhibitor influencing pro-MMP-2 activation.

FIGURE 5. Equilibrium dialysis of MMP-inhibitor complexes. MMP-2 (A), MMP-9 (B), and MMP-14 (C) (10 nM each) were incubated in the absence (black) and presence of either compound 3 (striped), 5 (light gray), or 7 (dark gray) (1 mM each), in buffer R, for 3 h, at room temperature. The remaining MMP activity was monitored with MOCAcPLGLApr(Dnp)AR-NH$_2$ (0 h). Part of the reaction mixtures was subjected to extensive dialysis against buffer R, containing no Me$_2$SO, as described under "Experimental Procedures," and the remaining solution was placed on a rotator. After 48 h, the enzymatic activity in both the nondialyzed (48 h without dialysis) and dialyzed (48 h with dialysis) solutions was measured with the aforementioned fluorogenic substrate.
under these conditions. It is also possible that covalent inhibition of MT1-MMP, as opposed to a reversible inhibition, alters the availability of the active site of MT1-MMP for TIMP-2 binding, a prerequisite for pro-MMP-2 activation (15). Although more studies are required, these results suggest that the concept behind inhibitor 3 is a promising framework from which to further develop more effective and selective MT1-MMP inhibitors, a key protease in tumor cell invasion. Nevertheless, these studies further demonstrate the selectivity of inhibitor 3 in a live cellular system and lend credit to the hypothesis that selectivity, rather than affinity, may be key to the successful therapeutic application of synthetic MMP inhibitors.

Inhibitor 3 Inhibits HT1080 Cell Migration and Invasion—It is well established that tumor cell migration and invasion depend on gelatinase activity. Therefore, we wished to evaluate the effect of inhibitor 3, which is selective for the gelatinases, on the migration and invasion of HT1080 cells as described under “Experimental Procedures.” Cell migration was monitored under conditions of pro-MMP-2 activation, which was achieved by concanavalin A treatment, and inhibition of cell proliferation. As shown in Fig. 7, A and B, exposure of HT1080 cells to various doses (0–20 μM) of inhibitor 3 significantly inhibited (80% at 2 μM) their migration in a scratch wound assay when compared with untreated cells. Likewise, the ability of HT1080 cells to invade Matrigel-coated filters was significantly reduced by inhibitor 3, and as little as 100 nM of inhibitor caused >25% inhibition of HT1080 cell invasion (Fig. 7C). These effects of inhibitor 3 could not be ascribed to cytotoxicity, since no evidence of cell toxicity was detected when HT1080 cells were exposed to inhibitor 3 up to concentrations of 10 μM, as determined using the WST-1 chemosensitivity assay (data not shown). Given the high selectivity exhibited by this compound toward MMP-2 relative to other MMPs (TABLE ONE), the slower migration in the presence of 200 nM inhibitor 3, a concentration too low to inhibit other MMPs, including MT1-MMP, suggests that the observed effect was most likely due to MMP-2 inhibition. These results further demonstrate the ability of inhibitor 3 to act as a selective gelatinase inhibitor in cellular systems and to alter MMP-dependent processes. The new characteristics of inhibitor 3 and its high selectivity make this inhibitor an excellent candidate for future in vivo testing in relevant human disease models in mice.

The thiirane class of mechanism-based inhibitors was conceived, designed, and prepared by us for the first time in our pursuit of selectivity in inhibition of MMPs of importance to several disease processes. We have revealed in the present report that inhibitor 3 targets MMP-2, -9, and -14, whereas inhibitors 5 and 7 are inhibitory only toward...
MMP-2. The activities for these new inhibitors provide a unique opportunity in investigations of the roles of these MMPs in various disease processes.

REFERENCES

1. Egeblad, M., and Werb, Z. (2002) Nat. Rev. Cancer 2, 161–174
2. McCawley, L. J., and Matrisian, L. M. (2000) Mol. Med. Today 6, 149–156
3. Pavlaki, M., and Zucker, S. (2003) Cancer Metastasis Rev. 22, 177–203
4. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002) Science 295, 2387–2392
5. Skiles, J. W., Gonnella, N. C., and Jeng, A. Y. (2004) Curr. Med. Chem. 11, 2911–2977
6. Matrisian, L. M., Sledge, G. W., Jr., and Mohla, S. (2003) Cancer Res. 63, 6105–6109
7. Brown, S., Meroueh, S. O., Fridman, R., and Mobashery, S. (2004) Curr. Top. Med. Chem. 4, 1227–1238
8. Gu, Z., Cui, J., Brown, S., Fridman, R., Mobashery, S., Strongin, A. Y., and Lipton, S. A. (2005) J. Neurosci. 25, 6401–6408
9. Kruger, A., Arlt, M. J., Gerg, M., Kopitz, C., Bernardo, M. M., Chang, M., Mobashery, S., and Fridman, R. (2005) Cancer Res. 65, 3523–3526
10. Kleinfeld, O., Kotra, L. P., Gervasi, D. C., Brown, S., Bernardo, M. M., Fridman, R., Mobashery, S., and Sagi, I. (2001) J. Biol. Chem. 276, 17125–17131
11. Goux, C., Lhoste, P., and Sinou, D. (1994) Tetrahedron 50, 10321–10330
12. Knight, C. G. (1995) Methods Enzymol. 248, 18–34
13. Muller-Steffner, H. M., Malver, O., Hosie, L., Oppenheimer, N. J., and Schuber, F. (1992) J. Biol. Chem. 267, 9606–9611
14. Olson, M. W., Gervasi, D. C., Mobashery, S., and Fridman, R. (1997) J. Biol. Chem. 272, 29975–29983
15. Toth, M., Bernardo, M. M., Gervasi, D. C., Soloway, P. D., Wang, Z., Bigg, H. F., Overall, C. M., DeClerck, Y. A., Tschesche, H., Cher, M. L., Brown, S., Mobashery, S., and Fridman, R. (2000) J. Biol. Chem. 275, 41415–41423
16. Segel, I. H. (1975) Enzyme Kinetics, John Wiley & Sons, Inc., New York
17. Fuerst, T. R., Earl, P. L., and Moss, B. (1987) Mol. Cell. Biol. 7, 2538–2544
18. Hernandez-Barrantes, S., Toth, M., Bernardo, M. M., Yurkova, M., Gervasi, D. C., Raz, Y., Sang, Q. A., and Fridman, R. (2000) J. Biol. Chem. 275, 12080–12089
19. Toth, M., Gervasi, D. C., and Fridman, R. (1997) Cancer Res. 57, 3159–3167
20. Gervasi, D. C., Raz, A., Dehern, M., Yang, M., Kurkinen, M., and Fridman, R. (1996) Biochem. Biophys. Res. Commun. 228, 530–538
21. Duggleby, R. G., Attwood, P. V., Wallace, J. C., and Keech, D. B. (1982) Biochemistry 21, 3364–3370
22. Morrison, J. F. a. W., C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301
23. Szedlacsek, S. E., and Duggleby, R. G. (1995) Methods Enzymol. 249, 144–180
24. Westheimer, F. H. (1987) Science 235, 1173–1178
25. Strongin, A. Y., Marner, R. L., Grant, G. A., and Goldberg, G. I. (1993) J. Biol. Chem. 268, 14033–14039
26. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) J. Biol. Chem. 270, 5331–5338
27. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123
28. Bernardo, M. M., Brown, S., Li, Z. H., Fridman, R., and Mobashery, S. (2002) J. Biol. Chem. 277, 11201–11207