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Inhibition of Invasion and Capillary-like Tube Formation by Retrohydroxamate-based MMP Inhibitors

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Matrix metalloproteinases (MMPs), a family of zinc-containing endopeptidases, participate in many normal processes such as embryonic development and wound repair, and in many pathological situations such as cancer, atherosclerosis, and arthritis. Peptidomimetic MMP inhibitors were designed and synthesized with *N*-formylhydroxylamine (retrohydroxamate) as a zinc-binding group and various side chains on the α, P1', and P2' positions. Using *in vitro* MMP assays with purified MMPs (MMP-1, MMP-2, MMP-3, MMP-9, and MMP-14) and fluorogenic peptide substrates, it was found that compounds **2d** and **2g** selectively inhibit gelatinases (MMP-2 and MMP-9) and interstitial collagenase (MMP-1). They also inhibited the chemo-invasion of fibrosarcoma HT-1080 cells and tube formation of human umbilical vascular endothelial cells in a dose-dependent manner. Our results suggest that retrohydroxamate-based MMP inhibitors, especially compounds **2d** and **2g**, have the potential to be used as therapeutic drugs for cancer and other MMP-related diseases.

Key Words : Inhibitor, Invasion, Matrix metalloproteinase (MMP), *N*-Formylhydroxylamine, Retrohydroxamate

Introduction

Matrix metalloproteinases (MMPs) are endopeptidases that can cleave virtually any component of the extracellular matrix. Under normal physiological conditions, the activities of MMPs are precisely regulated by the level of transcription, by activation of precursor zymogens, by inhibition of endogenous inhibitors, and by interaction with specific matrix components. Deregulation of MMP activity is involved in many disastrous diseases such as tumor invasion, metastasis, atherosclerosis, aneurysms, arthritis, tissue ulcers, and fibrosis.[1,2]

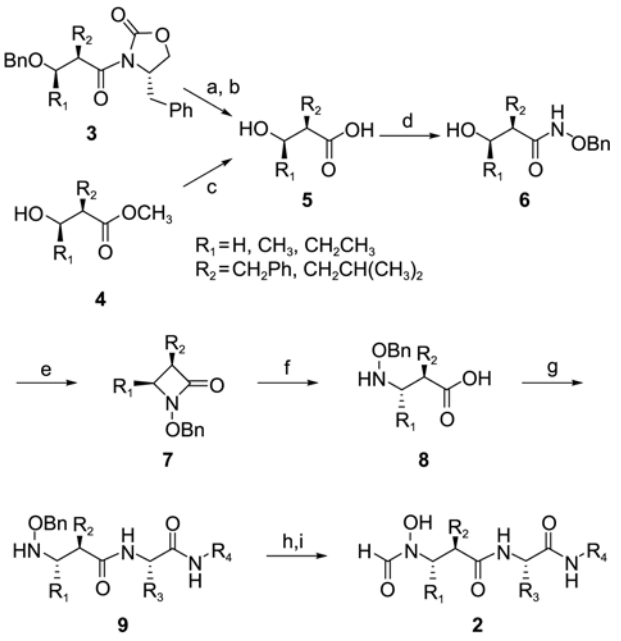
MMP inhibitors have been considered as potential anti-invasive and anti-angiogenic therapies for the treatment of cancer.[3,4] Many of the inhibitors mimic the peptide sequences around the cleavage site of the substrates and contain a chelating group, such as hydroxamate, that binds to the catalytic zinc. For example, hydroxamate-based batimastat (BB-94; British Biotech, Oxford, UK) has broad-spectrum inhibition for many MMPs but has poor oral availability. Marimastat (BB-2516; British Biotech), a chemically modified form of batimastat, has a similar broad-spectrum MMP inhibition and is orally active. However, broad-spectrum MMP inhibitors have been disappointing in clinical trials against cancer.[5,6] Nevertheless, MMP inhibitors, especially with narrow-spectrum inhibition, still hold medical promise as chemo-therapeutic and chemo-preventive drugs for malignant diseases and other MMP-related diseases.[4,5,7]

To show selective inhibition to a small group of MMPs, it was hypothesized that the MMP inhibitors should have a lower affinity for the zinc-binding group. *N*-formylhydroxylamine (retrohydroxamate) was reported to have approximately 10 times less zinc-binding affinity than hydroxamate and to provide long elimination half-life and oral bioavailability.[8] Retrohydroxamate was thus chosen as the zinc-binding group in this study. The MMP inhibitory effect of synthetic retrohydroxamates (**2**) containing various side chains at the α, P1', and P2' positions using the hydroxamate inhibitor BB-94 (batimastat) (**1**) as a reference was analyzed.

Experimental Section

Synthesis of Retrohydroxamate-Based Compounds (2a-2f). The general procedure for the synthesis of retrohydroxamate-based compounds is described in Scheme 1. NMR spectra were recorded on JEOL LA300 and Bruker Avance II 400. High-resolution mass spectra were recorded on a 4.7 Tesla IonSpec ESI-FTMS or a Micromass LCT ESI-TOF mass spectrometer.

(*R*)-2-((Benzyloxy)methyl)-4-methylpentanoic Acid. To a stirred solution of (*S*)-4-benzyl-3-((*R*)-2-((benzyloxy)-methyl)-4-methylpentanoyl) oxazolidin- 2-one (**3g**, 26.8 g, 67.9 mmol) in THF/H_2O (4/1, 420 mL), 30% hydrogen peroxide (56 mL, 272 mmol) and lithium hydroxide (4.55 g,



Scheme 1. Reagents and conditions: (a) LiOH, H_2O_2, THF/H_2O; (b) H_2, Pd/C, EtOH; (c) NaOH, H_2O, MeOH; (d) $BnONH_2$·HCl, DMAP, EDCI, CH_2Cl_2; (e) PPh_3, DEAD, THF; (f) LiOH, H_2O_2, THF/MeOH/H_2O; (g) HOBt, EDCI, NMM, L-amino acid-*N*-methylamide·HCl, DMF; (h) HCO_2H, Ac_2O, CH_2Cl_2; (i) H_2, Pd/C, EtOH.

110 mmol) were added at 0 °C, and the mixture was stirred for 3 hours at 0 °C. The reaction mixture was treated with aqueous Na_2SO_3 solution (34.3 g in 200 mL H_2O) and concentrated under reduced pressure. The residue was washed with dichloromethane, and the aqueous layer was acidified to pH 2.5 with 2 N HCl and extracted with ethyl acetate. The organic solution was dried over magnesium sulfate and concentrated under reduced pressure to yield the product (15 g, 94%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 7.36-7.25 (m, 5H), 4.54 (s, 2H), 3.66-3.51 (m, 2H), 2.85-2.78 (m, 1H), 1.67-1.53 (m, 2H), 1.36-1.20 (m, 1H), 0.92 (d, *J* = 4.96 Hz, 3H), 0.90 (d, *J* = 4.77 Hz, 3H).

(*R*)-2-(Hydroxymethyl)-4-methylpentanoic Acid (5g). To a stirred solution of (*R*)-2-((benzyloxy)methyl)-4-methylpentanoic acid (14 g, 59.24 mmol) in ethanol (500 mL), 10% Pd/C (2.0 g) was added and the mixture was stirred for 20 h at room temperature. The reaction mixture was filtered through Celite and the filtrate was concentrated under reduced pressure to yield the product (8.6 g, 99.3%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 6.68 (s, 1.6H), 3.76 (d, 2H), 2.74-2.65 (m, 1H), 1.73-1.54 (m, 2H), 1.37-1.23 (m, 1H), 0.94 (d, *J* = 4.21 Hz, 3H), 0.92 (d, *J* = 4.42 Hz, 3H).

(*R*)-*N*-(Benzyloxy)-2-(hydroxymethyl)-4-methylpentanamide (6g). To a stirred solution of **5g** (8.0g, 55 mmol) in dichloromethane (200 mL), *O*-benzylhydroxylamine (9.2 g, 57 mmol), 4-dimethylaminopyridine, (DMAP, 13 g, 110 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 11 g, 57 mmol) were added at 0 °C and the mixture was stirred for 24 hours at room temperature. To the reaction mixture, 1 N hydrochloric acid (220 mL) was added and the solution was extracted with dichloromethane. The organic layer was washed with water and saturated aqueous sodium chloride solution and then dried over magnesium sulfate and concentrated under reduced pressure to yield the product (11 g, 80%) as a white crystalline solid. ^{1}H-NMR (CD_3OD): δ 7.44-7.32 (m, 5H), 4.86 (s, 2H), 3.66-3.43 (m, 2H), 3.31-3.29 (m, 1H), 2.23-2.24 (m, 1H), 1.52-1.38 (m, 2H), 1.18-1.11 (m, 1H), 0.89 (d, *J* = 9.16 Hz, 3H), 0.85 (d, *J* = 6.22 Hz, 3H).

(*R*)-1-(Benzyloxy)-3-isobutylazetidin-2-one (7g). To a stirred solution of **6g** (11 g, 44 mmol) in dried tetrahydrofuran (THF, 400 mL) under nitrogen, triphenylphosphine (14 g, 52 mmol) and diethyl azodicarboxylate (DEAD, 9.2 g, 52 mmol) were added at 0 °C and the mixture was stirred for 18 hours at 0 °C. The reaction was quenched by adding water (400 mL) and the aqueous solution was extracted with ethyl acetate. The organic layer was washed with saturated aqueous sodium chloride solution and then dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified by silica-gel column chromatography to yield the product (8.7 g, 85%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 7.43-7.36 (m, 5H), 4.98 (s, 2H), 3.37 (t, 1H), 2.91-2.81 (m, 2H), 1.73-1.57 (m, 2H), 1.34-1.23 (m, 1H), 0.87 (d, *J* = 1,26 Hz, 3H), 0.85 (d, *J* = 1.29 Hz, 3H).

(*R*)-2-(((Benzyloxy)amino)methyl)-4-methylpentanoic Acid (8g). To a stirred solution of **7g** (8.0 g, 34 mmol) in THF/H_2O/MeOH (3/1/1), lithium hydroxide (14 g, 0.34 mol) was added, and the mixture was stirred for 18 hours at room temperature. To the reaction mixture, water (200 mL) was added and the pH of the solution was adjusted to 2.5 by adding 3 N hydrochloric acid. The solution was extracted with ethyl acetate, and the organic layer was dried over magnesium sulfate and concentrated under reduced pressure to yield the product (8.5 g, 99%) as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 7.38-7.27 (m, 5H), 4.72 (d, 2H), 3.15-3.03 (m, 2H), 2.85-2.76 (m, 1H), 1.71-1.58 (m, 2H), 1.35-1.25 (m, 1H), 0.93 (d, *J* = 6.07 Hz, 3H), 0.91 (d, *J* = 6.04 Hz, 3H).

(*R*)-2-((*N*-Hydroxyformamido)methyl)-4-methyl-*N*-((*S*)-1-(methylamino)-1-oxo-3-phenylpropan-2-yl)pentanamide (2g). Compound **8g** (100 mg, 0.40 mmol) was dissolved in dichloromethane (3.0 mL) and the solution was cooled to 0 °C. To the solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 92 mg, 0.48 mmol), 1-hydroxybenzotriazole (HOBt, 64 mg, 0.48 mmol), and *N*-methylmorpholine (NMM, 0.44 mL, 4.0 mmol) were added and the mixture was stirred for 45 min at room temperature. To the reaction mixture, (*S*)-2-amino-*N*-methyl-3-phenylpropanamide (110 mg, 0.60 mmol) was added and the mixture was stirred for 24 hours at room temperature. The reaction mixture was diluted with ethyl acetate and washed with 1 N hydrochloric acid, saturated aqueous sodium bicarbonate solution, and saturated aqueous sodium chloride solution. The organic layer was dried over magnesium sulfate and concentrated under reduced pressure followed by silica-gel column chromatography to yield (*R*)-2-((benzyloxyamino)methyl)-4-methyl-*N*-((*S*)-1-(methylamino)-1-oxo-3-phenylpropan-2-yl)pentanamide (**9g**, 101 mg) as a white

solid. Compound **9g** (101 mg) was dissolved in dichloromethane (2 mL) and cooled to 0 °C. To the solution, formic acid (1.3 mL) and acetic anhydride (0.26 mL, 2.81 mmol) were added and the mixture was stirred for 3 hours at 0 °C. The reaction mixture was concentrated under reduced pressure and dissolved in dichloromethane. The solution was washed with saturated aqueous sodium chloride solution and then dried over magnesium sulfate and concentrated under reduced pressure to yield a white solid (96 mg) which was directly used without further purification. The solid was dissolved in EtOH (5 mL) and 10% Pd/C (10 mg) was added. The mixture was stirred for 4 hours under hydrogen (1 atm) at room temperature and concentrated under reduced pressure followed by silica-gel column chromatography to yield the product (65 mg, 52%) as a white solid. ^{1}H-NMR (CD_3OD): δ 8.13 (s, 0.42H), 7.79 (s, 0.48H), 7.29-7.19 (m, 5H), 4.53-4.48 (m, 1H), 3.68-3.29 (m, 2H), 3.06-2.75 (m, 3H), 2.63 (d, 3H), 1.49-1.23 (m, 2H), 1.20-1.08 (m, 1H), 0.91-0.80 (m, 6H). HRMS *m/z* calcd for $C_{18}H_{27}N_3O_4$ $[M+Na]^+$ 372.1899, Found 372.1895.

(*S*)-2-[(*R*)-2-Benzyl-3-(formyl-hydroxy-amino)-propionylamino]-3,3-*N*-trimethyl-butyram-ide (2a): ^{1}H-NMR ($CDCl_3$): δ 8.41 (s, 0.4H), 7.83 (s, 0.6H), 7.29-7.19 (m, 5H), 6.65 (d, 0.4H), 6.55 (d, 0.6H), 4.91-4.83 (m, 1H), 4.03-3.95 (m, 0.4H), 3.84-3.74 (m, 0.6H), 3.62-3.43 (m, 1H), 3.16 (s, 1H), 3.13 (s, 2H), 2.89-2.79 (m, 0.6H), 2.76-2.71 (m, 0.4), 1.69-1.34 (m, 1.8), 1.29-1.20 (m, 1.2H), 1.01-0.95 (m, 9H). HRMS *m/z* calcd for $C_{18}H_{27}N_3O_4$ $[M+Na]^+$ 372.1899, Found 372.1896.

(2*R*,3*S*)-2-Benzyl-*N*-[(*S*)-2,2-dimethyl-1-methylcarbamoyl-propyl]-3-(formyl-hydroxy-amino)-butyramide (2b): ^{1}H-NMR ($CDCl_3$): δ 8.44 (s, 0.4H), 7.81 (s, 0.6H), 7.27-7.19 (m, 5H), 6.59 (d, 0.4H), 6.49 (d, 0.6H), 4.90-4.83 (m, 1H), 4.01-3.95 (m, 0.4H), 3.81-3.74 (m, 0.6H), 3.15 (m, 1H), 3.09 (s, 2H), 2.92-2.79 (m, 0.6H), 2.76-2.71 (m, 0.4), 1.70-1.34 (m, 1.8), 1.30-1.20 (m, 1.2H), 1.16-1.12 (m, 3H), 1.01-0.95 (m, 9H). HRMS *m/z* calcd for $C_{19}H_{29}N_3O_4$ $[M+Na]^+$ 386.2056, Found 386.2057.

(2*R*,3*S*)-2-Benzyl-3-(formyl-hydroxy-amino)-pentanoate [(*S*)-2,2-dimethyl-1-methylcarbamoyl-propyl]-amide (2c): ^{1}H-NMR ($CDCl_3$): δ 8.42 (s, 0.4H), 7.80 (s, 0.6H), 7.28-7.16 (m, 5H), 6.64 (d, 0.4H), 6.54 (d, 0.6H), 4.89-4.83 (m, 1H), 4.02-3.95 (m, 0.4H), 3.83-3.74 (m, 0.6H), 3.18 (m, 1H), 3.12 (s, 2H), 2.89-2.79 (m, 0.6H), 2.75-2.71 (m, 0.4), 1.69-1.34 (m, 1.8), 1.28-1.20 (m, 1.2H), 1.16-1.12 (m, 2H), 1.01-0.95 (m, 12H). HRMS *m/z* calcd for $C_{20}H_{31}N_3O_4$ $[M+Na]^+$ 400.2212, Found 400.2212.

(*R*)-2-[(Formyl-hydroxy-amino)-methyl]-4-methyl-pentanoate [(*S*)-2,2-dimethyl-1-methylcarbamoyl-propyl]-amide (2d): ^{1}H-NMR ($CDCl_3$): δ 8.47 (s, 0.4H), 7.89 (s, 0.6H), 6.66 (d, 0.4H), 6.51 (d, 0.6H), 4.90-4.85 (m, 1H), 4.01-3.95 (m, 0.4H), 3.83-3.74 (m, 0.6H), 3.64-3.43 (m, 1H), 3.17 (s, 1H), 3.13 (s, 2H), 2.89-2.79 (m, 0.6H), 2.76-2.71 (m, 0.4), 1.69-1.34 (m, 1.8), 1.29-1.20 (m, 1.2H), 1.01-0.95 (m, 9H), 0.93-0.88 (m, 6H). HRMS *m/z* calcd for $C_{15}H_{29}N_3O_4$ $[M+H]^+$ 316.2236, Found 316.2236.

(*S*)-2-[(*R*)-2-Benzyl-3-(formyl-hydroxy-amino)-propionyl-amino]-4-methyl-pentanoate methylamide (2e): ^{1}H-NMR ($CDCl_3$): δ 8.39 (s, 0.4H), 7.81 (s, 0.6H), 7.28-7.18 (m, 5H), 6.63 (d, 0.4H), 6.45 (d, 0.6H), 4.90-4.81 (m, 1H), 4.03-3.94 (m, 0.4H), 3.82-3.74 (m, 0.6H), 3.61-3.43 (m, 1H), 3.15 (s, 1H), 3.13 (s, 2H), 2.87-2.79 (m, 0.6H), 2.74-2.71 (m, 0.4), 1.67-1.34 (m, 1.8), 1.25-1.20 (m, 1.2H), 1.02-0.95 (m, 9H). HRMS *m/z* calcd for $C_{18}H_{27}N_3O_4$ $[M+Na]^+$ 372.1899, Found 372.1897.

(*R*)-2-Benzyl-3-(formyl-hydroxy-amino)-*N*-[(*S*)-1-methylcarbamoyl-2-phenyl-ethyl]-propion amide (2f): ^{1}H-NMR (CD_3OD): δ 8.15 (s, 0.42H), 7.82 (s, 0.48H), 7.31-7.18 (m, 10H), 4.55-4.47 (m, 1H), 3.67-3.31 (m, 2H), 3.04-2.75 (m, 3H), 2.62 (d, 3H), 1.48-1.25 (m, 1H), 1.20-1.08 (m, 1H). HRMS *m/z* calcd for $C_{21}H_{25}N_3O_4$ $[M+Na]^+$ 406.1743, Found 406.1744.

Preparation of MMPs. The catalytic and hinge domains of human MMP-14 (chMMP-14; Tyr-112~Ile-318) were expressed as inclusion bodies in *E. coli* BL21(DE3) cells.[9] The pro-, catalytic, and hinge domains of human MMP-1 (pchMMP-1, Phe-20~Ala-277) were expressed as inclusion bodies in *E. coli* BL21(DE3) cells.[10] To construct the plasmid expressing the catalytic domain of the human MMP-3 (cMMP-3; Phe-100~Pro-273) in *E. coli*, the corresponding cDNA fragment was amplified by PCR using pBacPAK9-MMP-3 (a generous gift from Dr. M.-Y. Kim, AngioLab) as a template with the following set of primers: the 5' primer with an *Nhe*I site, 5'-*GCCGC**GCTAGC*TTCAGAACCTT-TCCTGGC-3' (non-MMP-3 cDNA sequences, italic; restriction enzyme site, bold) and the 3' primer with an *Eco*RI site and termination codon, 5'-*CCGCG**GAATTC**TCA*GGGGG-TCTCAGGGGA-3'. The PCR product was cloned into the *Nhe*I and *Eco*RI site of pGEMEX-1 (Promega, USA). The cMMP-3 was expressed as inclusion bodies in *E. coli* BL21(DE3) pLysS transformed with pGEMEX1-cMMP-3. The functional catalytic domains of MMP-14 and MMP-3, and the dormant pro-catalytic domains of MMP-1 were refolded as previously described.[9] ProMMP-2 and proMMP-9 were expressed in insect cells, Sf9, using the Baculovirus system and purified as previously described.[10,11] The pro-forms of MMPs (100 nM) were activated immediately prior to use by incubating them with 1 mM 4-aminophenyl mercuric acetate at 37 °C in MMP reaction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.5 mM $ZnCl_2$); the reactions were incubated for 30 min for pchMMP-1 and proMMP-2 and 3 h for proMMP-9.[12]

Fluorometric MMP Assays. Enzyme assays were done using the quenched fluorescent substrates, Mca-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 (Bachem California, USA, M1895) for MMP-1, MMP-2, and MMP-14 and Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2 (Bachem California, USA, M2105) for MMP-3 and MMP-9.[13] Each reaction contained 10 nM of an active MMP, 1 μM of a quenched fluorescent substrate, and various concentrations of compounds (dissolved in DMSO and added to 10% of the reaction volume) in 100 μL of MMP reaction buffer.[14] The reaction was incubated at 37 °C for 30 min except for MMP-1 which was incubated for 3 h and was stopped by the

addition of 0.1 M sodium acetate (pH 4.0) at final concentration. Fluorescence was measured using a Kontron SFM25 fluorometer (Milan, Italy) at an excitation wavelength of 328 nm and an emission wavelength of 393 nm.

Cell Culture. Human fibrosarcoma HT-1080 cells were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. HUVECs were isolated from human umbilical cord veins by collagenase treatment as previously described[15] and used in passages 2-7. The cells were grown in M199 medium (Life Technologies, Grand Island, NY, USA) supplemented with 20% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 3 ng/mL basic fibroblast growth factor (Upstate Biotechnology, Lake Placid, NY, USA), and 5 units/mL heparin.

Cytotoxicity Assay. To examine the effect of the compounds on cytotoxicity, sub-confluent HT-1080 cells were incubated for 24 h in DMEM medium supplemented with 10% fetal bovine serum and the indicated concentrations of BB-94, **2d**, and **2g**. The 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the viability of the cells as previously described.[16]

Invasion Assay. The invasiveness of the HT-1080 cells was examined *in vitro* using a Transwell chamber system with 8.0-μm pore-sized polycarbonate filter inserts as previously described.[17] The cells (5×10^4 cells/200 μL) which were depleted in a serum-free medium for 24 h were pre-incubated with the indicated concentrations of BB-94, **2d**, and **2g** in DMEM medium supplemented with 10% fetal bovine serum for 1 h and added to the upper chamber coated with Matrigel (BD Bioscience, NJ, USA). After a 24-h incubation period, cells that invaded to the lower side of the filter coated with gelatin was stained and counted using an optical microscope at 100× magnification.

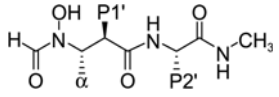
Tube Formation Assay. Capillary-like tube formation assay was done as previously described.[17] Matrigel (250 μL in a concentration of 10 mg/mL) was placed in a 24-well culture plate and polymerized for 30 min at room temperature. HUVECs (5×10^5 cells/mL) were incubated with the indicated concentrations of BB-94, **2d**, and **2g** in 500 μL of M199 medium containing 1% FBS with 10 ng/mL VEGF on the surface of the Matrigel. After 18 h of incubation, the cells were fixed in 3.7% formaldehyde and visualized at 40× magnification.

Results and Discussion

Chemistry. The general route for the synthesis of retrohydroxamate **2** is outlined in Scheme 1. The chiral auxiliary of **3**[18] was removed with lithium hydroxide/hydrogen peroxide and the hydroxyl group was deprotected under palladium on carbon (Pd/C), followed by coupling with *O*-benzylhydroxylamine hydrochloride to give the protected *N*-hydroxyamide **6**. Alternatively, hydroxy ester **4**[19] was hydrolyzed with aqueous sodium hydroxide, followed by coupling with *O*-benzylhydroxylamine hydrochloride to give the protected *N*-hydroxyamide **6**. This *N*-hydroxyamide **6** was converted into β-lactam **7** through an intramolecular Mitsunobu reaction.[20] Hydrolysis of the β-lactam furnished acid **8**, which was subsequently coupled by standard peptide coupling conditions using L-amino acid-*N*-methylamide to give **9** with a good yield. Hydroxylamine **9** was *N*-formylated using acetic anhydride/formic acid[21] and subsequent removal of the *O*-benzyl protecting group by Pd-catalyzed hydrogenation provided the target compound **2**.

MMP Inhibitory Activity. The synthesized retrohydroxamate compounds were tested on several MMPs (MMP-1, MMP-2, MMP-3, MMP-9, and MMP-14) to evaluate their inhibitory properties. Introduction of alkyl (methyl or ethyl) side chains at the α position enhanced the inhibition of MMP-1 and MMP-3, had little effect on the inhibition of MMP-9, and reduced the inhibition of MMP-2 and MMP-14 (**2a-c**; Table 1). Thus, no substituent at the α position increased the selectivity to MMP-2, MMP-9, and MMP-14 over MMP-1 and MMP-3 (**2a-c**; Table 1). Replacement of the side chain at the P1' position from the benzyl group to the isobutyl group drastically increased the inhibitory ability

Table 1. MMP inhibition by retrohydroxamate derivatives with modified side chains at the α, P1', and P2' positions



Cmpd	α	P1'	P2'	IC_{50} (nM)				
				MMP-1	MMP-2	MMP-9	MMP-3	MMP-14
BB-94[a]	2-thienyl thiomethyl	$CH_2CH(CH_3)_2$	CH_2Ph	2.16 ± 0.21	8.70 ± 0.31	24.4 ± 1.8	19.2 ± 0.26	31.1 ± 0.47
2a	H	CH_2Ph	$C(CH_3)_3$	2,090 ± 162	398 ± 11	809 ± 27	176,000 ± 12,000	5,610 ± 290
2b	CH_3	CH_2Ph	$C(CH_3)_3$	652 ± 130	645 ± 1.3	889 ± 15	158,000 ± 9,200	31,400 ± 300
2c	CH_2CH_3	CH_2Ph	$C(CH_3)_3$	612 ± 54	601 ± 29	837 ± 52	40,200 ± 1,600	18,300 ± 1,000
2d	H	$CH_2CH(CH_3)_2$	$C(CH_3)_3$	30.4 ± 6.4	18.9 ± 1.5	42.7 ± 3.0	22,800 ± 740	200 ± 29
2e	H	CH_2Ph	$CH_2CH(CH_3)_2$	3,870 ± 520	394 ± 19	1,110 ± 71	118,000 ± 4,400	4,860 ± 220
2f	H	CH_2Ph	CH_2Ph	2,270 ± 110	104 ± 8.5	279 ± 14	77,000 ± 4,800	6,230 ± 470
2g	H	$CH_2CH(CH_3)_2$	CH_2Ph	30.4 ± 3.7	6.54 ± 0.27	25.9 ± 2.3	11,400 ± 500	539 ± 42

[a]Zinc-binding group of BB-94 is hydroxamic acid.

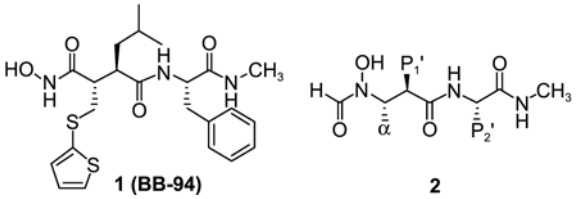


Figure 1. Structural relationship between BB-94 (**1**) and retrohydroxamate-based MMP inhibitors (**2**).

against the tested MMPs (**2a** and **2d**; Table 1). Substitution of the side chain at the P2' position from the *tert*-butyl group to the isobutyl group did not have much of an effect on these MMPs (**2a** and **2e**; Table 1), but a change to a benzyl group increased the inhibitory ability of MMP-2, MMP-9, and MMP-3 2.5~4-fold (**2a** and **2f**; Table 1). Among **2a-f**, compound **2d** exhibited excellent inhibitory selectivity to gelatinases (MMP-2 and MMP-9; also known as 72- and 92-kDa type IV collagenases or gelatinases A and B, respectively) and interstitial collagenase (MMP-1) (Table 1).

Gelatinases degrade type IV collagen, which is a major component of the basement membrane. Degradation of the basement membrane is essential in invasive growth of tumor cells, metastasis, angiogenesis, and inflammation.[22] In an attempt to develop gelatinase-selective inhibitors, compound **2g** was designed which has no side chain at the α position, an isobutyl side chain at P1' position, and a benzyl side chain at P2' position based on the above results (Table 1) which should show the lowest IC_{50} against MMP-2 and MMP-9. As expected, compound **2g** significantly improved not only the inhibitory potency against MMP-2 and MMP-9 at a level comparable to BB-94, but also improved the selectivity to MMP-2 and MMP-9 over other tested MMPs.

The P1' side chain inserts into the S1' subsite of MMPs which forms a hydrophobic tunnel.[23] Depending on the S1' subsite structure, MMPs can be classified in two groups; those with a deep S1' pocket (MMP-2, 3, 8, 9, 13) and those with a shallow S1' pocket (MMP-1, 7, 11).[24] Interestingly, retrohydroxamate-based narrow-spectrum inhibitors, **2d** and **2g**, have the same isobutyl side chain at the P1' position as a hydroxamate-based broad-spectrum MMP inhibitor BB-94 (Table 1). The P1' isobutyl side chain is hydrophobic but not long. So it binds well to various MMPs without preference for the depth of the S1' pocket. In these regards, **2d** and **2g** still have the potency to inhibit MMP-1 as well as MMP-2 and MMP-9. Thus, it is expected that the introduction of an extended P1' group would provide better selectivity to MMP-2 and MMP-9 over MMP-1.

The S2' subsite is known as a solvent-exposed cleft, showing a general preference for hydrophobic residues.[24] A pan-MMP inhibitor, BB-94, has the same benzyl side chain at the P2' position as our narrow-spectrum inhibitor, **2g**. In our study using retrohydroxamate as a zinc-binding group, the benzyl side chain at the P2' position, compared to other tested hydrophobic side chains, consistently exhibited the improved selectivity to MMP-2, MMP-9, and MMP-3 (**2f** and **2g**; Table 1).

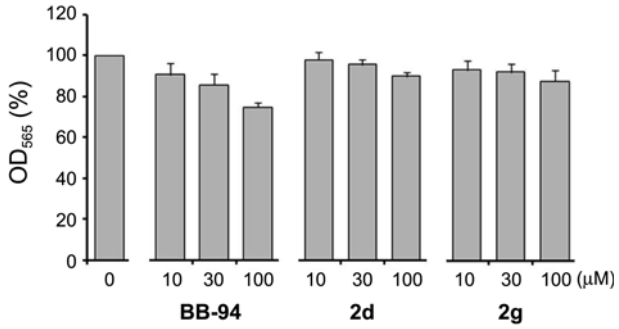


Figure 2. Effect of **2d** and **2g** on the cytotoxicity of HT-1080 cells. Fractions of viable HT-1080 cells determined by MTT assay (OD_{565}) are expressed as a percentage of the control (in the absence of compound). Each value is the mean ± SD of three independent experiments.

BB-94 has a thioethylthiophene chain at the α position which is oriented as a cover over the enzyme.[25] It was known that addition of a side chain to the α position improves the selectivity to MMP-1 and MMP-3 and/or oral availability.[24] We have shown that no substituent at the α position contributes to the negative selectivity to MMP-1 and MMP-3 but the positive selectivity to MMP-2 and MMP-14 (Table 1). Taken together, our data confirm that interactions between inhibitor side chains and enzyme subsites determine the MMP selectivity of inhibitors. Moreover, it seems that a moderate interaction between zinc and retrohydroxamate provides better environment for the best fit between inhibitor side chains and enzyme subsites than a tight zinc-hydroxamate interaction.

Cytotoxicity and Inhibition of Chemo-Invasion and Tube Formation. Compounds **2d** and **2g** were evaluated for cytotoxicity and biological functions including the inhibition of the chemo-invasion of cancer cells and tube-like capillary formation of endothelial cells. Cytotoxicity was analyzed using an MTT-based viability assay of fibrosarcoma HT-1080 cells.[16] Compounds **2d** and **2g** up to 100 μM had little effect on cell viability after a 24-h incubation period, indicating that **2d** and **2g** are much less toxic to cells than BB-94 (Figure 2).

Metastatic tumor cells express higher levels of gelatinolytic MMPs compared to their non-metastatic counterparts. Levels of MMP-2 and MMP-9, therefore, are closely correlated with the metastatic potential.[26-28] Thus, we analyzed the effect of compounds **2d** and **2g** on the chemo-invasion of highly metastatic HT-1080 cells using a Boyden chamber containing Matrigel, mimicking the basement membrane. Invasion of HT-1080 cells was markedly inhibited by **2d** and **2g** in a dose-dependent manner; **2d** (IC_{50} = 39.3 μM) was slightly less potent than BB-94 (IC_{50} = 25.7 μM) but **2g** (IC_{50} = 16.2 μM) was even better (Fig. 3). This result suggests that these compounds could reduce the invasion of tumor cells through the inhibition of MMPs including gelatinases.

Solid tumors require growth of new blood vessels from pre-existing vessels, called angiogenesis, accompanying ECM degradation for proliferation and metastasis of tumor cells.[29] Thus, many cancer therapies are directed against tumor

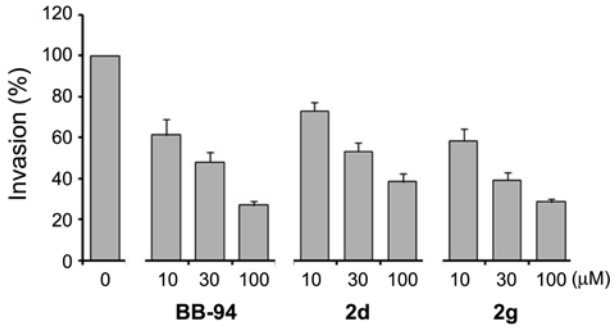


Figure 3. Effect of **2d** and **2g** on chemo-invasion of HT-1080 cells. Fractions of HT-1080 cells that invaded across the Transwell chamber coated with the Matrigel are expressed as percentage of the control (in the absence of compound). Each value is the mean ± SD of three independent experiments.

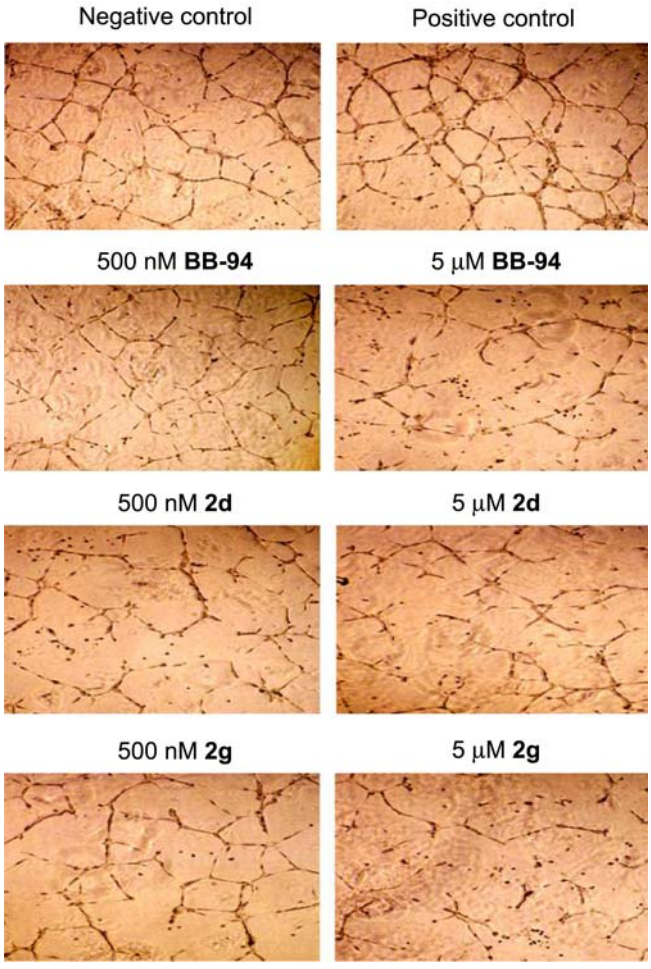


Figure 4. Effects of **2d** and **2g** on the tube formation of HUVECs. HUVECs were incubated on the Matrigel without supplement (Negative control), with 10 ng/mL VEGF only (Positive control), or with VEGF and MMP inhibitors (BB-94, **2d**, or **2g**). Microphotographs (40× magnification) were taken after 18 h of incubation.

angiogenesis. Human umbilical vascular endothelial cells (HUVECs) grown on Matrigel form a capillary-like tube network in an MMP-dependent manner.[30-32] Thus, the effect of **2d** and **2g** on the tube formation of HUVECs was analyzed. Tube formation of VEGF (10 ng/mL)-treated HUVECs was dose-dependently inhibited by **2d** and **2g** similar to the inhibition by BB-94 (Fig. 4) assessed by the total tubule area.

In summary, we have developed the narrow-spectrum MMP inhibitors, **2d** and **2g**. Introduction of retrohydroxamate, a much weaker zinc-binding group than hydroxamate, allowed for the generation of narrow-spectrum MMP inhibitors by optimization of the side chains. Cytotoxicity to fibrosarcoma HT-1080 cells from **2d** and **2g** was negligible. Chemo-invasion of HT-1080 cells and tube formation of HUVECs were effectively inhibited by **2d** and **2g**, similar to the level of inhibition by a hydroxamate-based broad-spectrum MMP inhibitor, BB94. Our studies suggest that **2d** and **2g** have potential as therapeutic drugs to control metastatic cancer and other MMP-related diseases.

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