Study of a D-proline peptidomimetic inhibitor of melanoma and endothelial cells invasion through activity towards MMP-2 and MMP-9

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Electronic Supplementary Information (ESI)

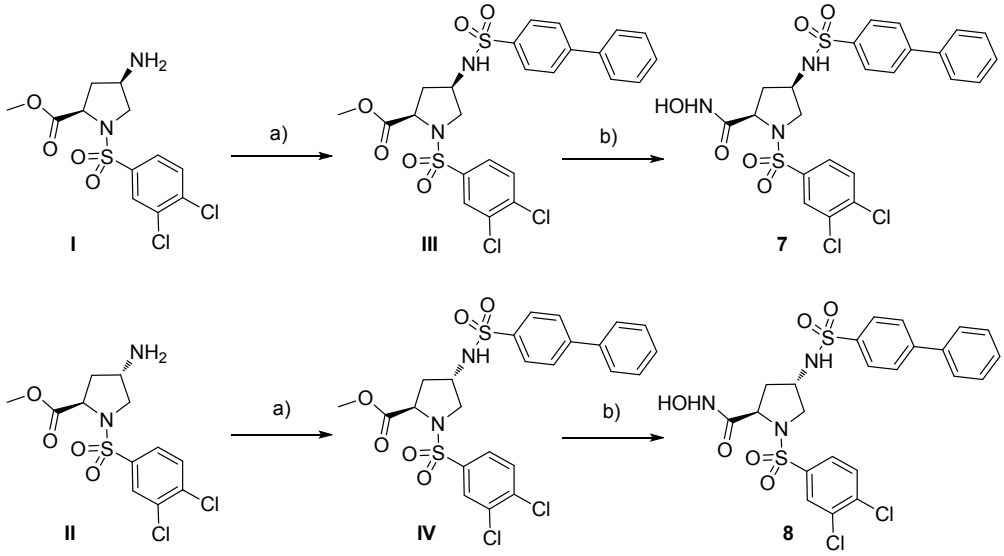
Experimental Section

Synthesis. ^{1}H NMR spectra were recorded on a Varian Mercury 400 (^{1}H: 400 MHz), and ^{13}C NMR spectra on a Varian Gemini 200 (^{13}C: 50 MHz). The chemical shifts (δ) and coupling constants (J) are expressed in ppm and hertz, respectively. Flash column chromatography (FCC) purifications were performed manually using glass columns using Merck silica gel (0.040–0.063 mm). TLC analyses were performed on Merck silica gel 60 F254 plates. Melting points were recorded on a BÜCHI B-540 instrument and are uncorrected. Optical rotations were measured with JASCO DIP-360 digital polarimeter. Elemental analyses were recorded on a Perkin Elmer 240 C,H,N analyzer. ESI mass spectra were recorded on a Thermo LCQ-Fleet. All commercially available reagents and solvents were used as received, unless otherwise specified. Dichloromethane (DCM) was distilled over CaH_2. Analytical HPLC purity tests were performed on a Dionex UltiMate 3000 (columns: Phenomenex Synergi 4µm Fusion-RP 80A, 150×4.6 mm and Phenomenex 4µm Synergi-RP 80A, 150x4.6 mm), using 0.1% TFA-buffered elution gradient CH_3CN:H_2O from 5:95 to 95:5. All tested compounds were >95% pure.

Compounds **1**-**6** and **9**-**11** were prepared as previously described.[1]

Compounds **7** and **8** were prepared from the corresponding *N*-sulfonyl-4-aminoproline methyl ester derivatives I and II using the same chemistry as reported in ref. 1, and as outlined in Scheme S1.

Scheme S1. Synthesis of diastereomeric hydroxamic acids **7** and **8**[i]



[i] Conditions: a) biphenyl-4-sulfonyl chloride, Et_3N, an. CH_2Cl_2, r.t., o.n.; b) NH_2OK/NH_2OH solution in MeOH, r.t., 3 days.

(2*R*,4*R*)-methyl 4-(biphenyl-4-ylsulfonamido)-1-(3,4-dichlorophenylsulfonyl)pyrrolidine-2-carboxylate (III). To a solution of (2*R*,4*R*)-methyl 4-amino-1-(3,4-dichlorophenylsulfonyl)
pyrrolidine-2-carboxylate (**I**)[1] (100 mg, 0.28 mmol) and triethylamine (79 μL, 0.57 mmol) in anhydrous CH_2Cl_2 (1.4 mL) biphenyl-4-sulfonyl chloride (78 mg, 0.31 mmol) was added at 0 °C. The mixture was allowed to reach room temperature, and it was left overnight stirring under a nitrogen atmosphere. Successively, the mixture was washed with $NaHCO_3$, 1N HCl and brine. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Compound **III** was isolated by flash chromatography (petroleum ether/EtOAc 1:1) as a white solid (71 mg, 45%).

M.p. 71.8-72.9°C. $[\alpha]^{22}_D$ = +22.8 (*c* = 0.4, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, *J* = 8.2 Hz, 2H), 7.69 (d, *J* = 7.3 Hz, 2H), 7.59 (d, *J* = 8.2 Hz, 2H), 7.51-7.47 (m, 2H), 7.42-7.31 (m, 4H), 6.04 (d, *J* = 9.2 Hz, 1H), 4.13 (dd, *J* = 3.4, 9.7 Hz, 1H), 3.82-3.79 (br, 1H), 3.63 (s, 3H), 3.26-3.17 (m, 2H), 2.15-2.08 (m, 1H), 1.93-1.79 (m, 1H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ 173.0, 145.6, 138.9, 136.8, 133.3, 132.4, 129.2 (2C), 129.0 (2C), 128.5, 127.7 (2C), 127.4 (2C), 127.3 (2C), 127.2 (2C), 58.6, 54.1, 53.1, 52.7, 36.5 ppm. elemental analysis calcd (%) for $C_{24}H_{22}Cl_2N_2O_6S_2$: C 50.62, H 3.89, N 4.92; found C 50.60, H 3.86, N 4.92. MS: *m/z* (ES^+) 499 $[M+H-2Cl]^+$. elemental analysis calcd (%) for $C_{24}H_{22}Cl_2N_2O_6S_2$: C 50.62, H 3.89, N 4.92; found C 50.61, H 3.89, N 4.90.

(2*R*,4*S*)-methyl 4-(biphenyl-4-ylsulfonamido)-1-(3,4-dichlorophenylsulfonyl)pyrrolidine-2-carboxylate (IV). To a solution of (2R,4S)-methyl 4-amino-1-(3,4-dichlorophenylsulfonyl)
pyrrolidine-2-carboxylate (**II**)[1] (70 mg, 0.20 mmol) and triethylamine (41 μL, 0.28 mmol) in anhydrous CH_2Cl_2 (1 mL) biphenyl-4-sulfonyl chloride (60 mg, 0.24 mmol) was added at 0 °C. The mixture was allowed to reach room temperature, and it was left overnight stirring under a nitrogen atmosphere. Successively, the mixture was washed with $NaHCO_3$, 1N HCl and brine. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Compound **IV** was isolated by flash chromatography (petroleum ether/EtOAc 1:1), as a white solid (50 mg, 46%).

M.p. 64.2-65.2°C. $[\alpha]^{23}_D$ = +20.9 (*c* = 0.25, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (d, *J* = 8.2 Hz, 2H), 7.81 (d, *J* = 7.3 Hz, 2H), 7.69 (d, *J* = 8.2 Hz, 2H), 7.62-7.54 (m, 2H), 7.51-7.40 (m, 4H), 5.41 (d, *J* = 7.8 Hz, 1H), 4.41 (t, *J* =

5.8 Hz, 1H), 4.01-3.96 (m, 1H), 3.62 (s, 3H), 3.53 (dd, *J* = 5.8, 10.2 Hz, 1H), 3.23 (dd, *J* = 5.3, 10.2 Hz, 1H), 2.14-2.10 (m, 2H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ 171.5, 145.8, 138.9, 138.2, 137.5, 133.2, 132.5, 129.1 (2C), 129.0 (2C), 128.5, 127.8 (2C), 127.5 (2C), 127.4 (2C), 127.2, 58.7, 53.1, 52.7, 52.2, 37.1 ppm. MS: *m/z* (ES^+) 499 $[M+H-2Cl]^+$. elemental analysis calcd (%) for $C_{24}H_{22}Cl_2N_2O_6S_2$: C 50.62, H 3.89, N 4.92; found C 50.60, H 3.86, N 4.92.

General Procedure for the synthesis of hydroxamic acids[1]

Preparation of NH_2OK/NH_2OH solution: $NH_2OH{\cdot}HCl$ (8 eq) was solubilized in MeOH (0.4 mL/mmol) by heating to reflux. Most, but not all of the salt dissolved. The solution was cooled to <40 °C, and a solution of KOH (12 eq) in MeOH (0.2 mL) was added in one portion. The resulting suspension was cooled to room temperature and used without prior removal of precipitated material. A solution of the ester (1 eq) in previously prepared NH_2OK/NH_2OH solution was stirred at room temperature for 3 days. The reaction mixture was taken up in 1N HCl, extracted with EtOAc, dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/MeOH 4:1) to afford hydroxamic acids. Absence of epimerization at the C-2 atom of the pyrrolidine ring was assessed by HPLC, resulting in stereoisomeric purity >90%.

(2*R*,4*R*)-4-(biphenyl-4-ylsulfonamido)-1-(3,4-dichlorophenylsulfonyl)-*N*-hydroxypyrrolidine-2-carboxamide (7). Prepared from **III** using the general procedure**.** Yellow solid, 64% yield. M.p. 161.2-163.5°C. $[\alpha]^{22}_D$ = -25.7 (*c* = 0.7, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77-7.67 (m, 4H), 7.51-7.24 (m, 8H), 5.49 (br, 1H), 4.35-4.09 (m, 2H), 3.72-3.25 (m, 2H), 2.31-1.86 (m, 2H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ 179.0, 145.0, 139.2, 138.7, 136.2, 133.0, 132.2, 129.2, 128.9 (3C), 128.6, 128.2, 127.6 (3C), 127.2 (3C), 61.6, 54.2, 52.2, 36.2 ppm. MS: *m/z* (ES^+) 592,01 $[M+Na]^+$. elemental analysis calcd (%) for $C_{23}H_{21}Cl_2N_3O_6S_2$: C 48.42, H 3.71, N 7.37; found C 48.44, H 3.72, N 7.34.

(2*R*,4*R*)-4-(biphenyl-4-ylsulfonamido)-1-(3,4-dichlorophenylsulfonyl)-*N*-hydroxypyrrolidine-2-carboxamide (8). Prepared from **IV** using the general procedure**.** White solid, 45% yield. M.p. 134.4-136.9°C. $[\alpha]^{22}_D$ = +57.2 (*c* = 0.65, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.70-7.61 (m, 4H), 7.41-7.28 (m, 8H), 5.60 (br, 1H), 4.33-4.10 (m, 2H), 3.37 (m, 1H), 3.06 (m, 1H), 2.19-1.87 (m, 2H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ 177.5, 145.3, 138.9, 138.0, 135.3, 133.4, 132.1, 129.1, 128.8 (3C), 128.3, 127.4 (2C), 127.3, 127.2, 127.1 (3C), 62.1, 55.1, 52.2, 36.8 ppm. MS: *m/z* (ES^+) 592,01 $[M+Na]^+$. elemental analysis calcd (%) for $C_{23}H_{21}Cl_2N_3O_6S_2$: C 48.42, H 3.71, N 7.37; found C 48.43, H 3.75, N 7.35.

Molecular modeling calculations. Automated docking studies were performed using the AutoDock 4.0.1 program, an automated docking suite that employs a Lamarckian genetic algorithm (LGA) as search engine. The Autodocktools 1.4.5 (ADT) graphical interface was used to prepare the enzyme and the ligands PDBQT files.[2] The 3D structures of the ligand was generated using Spartan version 5.147, and then energy-minimized with the same program. The equilibrium geometry was calculated through the semi-empirical method AM1. The coordinates of MMP-2 were retrieved from the Protein Data Bank (PDB code: 1QIB). The ligand-protein complex was unmerged for achieving free enzyme structure and water molecules were removed. Hydrogen atoms were added to the enzyme and the ligands, Gasteiger charges were computed and non-polar hydrogens were merged. Three-dimensional energy scoring grids of 0.375 Å resolution and 40 Å × 40 Å × 40 Å dimensions were computed. The center of the grid was set on the zinc atom. Each docking experiment was derived from a total of 50 runs with a maximum of 2500000 energy evaluations were carried out for the structure of compound **1**, using the default parameters for LGA. Cluster analysis was performed on the docked results using a root-mean-square (rms) tolerance of 1.5 Å. The analysis of the binding mode of the docked conformations was carried out using PyMol Autodock Tools plugin within PyMol software.[3] The docked target-ligand

complexes obtained were optimized with Macromodel by an eMBracE energy minimization. The embrace minimization was carried out using Polak-Ribier conjugate gradient (PRCG). The optimizations were converged to a RMS gradient of the energy less than 0.05 KJ·mol-1, or continued until a limit of 1.000 iterations was reached. The force field used was OPLSA*,[4] with the water GB/SA solvent treatment at a dielectric constant of 1.0.[5] Normal cutoff distances were defined at 7 Å for van der Walls, 12 Å for electrostatics and 4 Å for hydrogen-bonds.

Enzyme inhibition assays. The inhibition potency of hydroxamic acid derivatives against MMP-2 and MMP-9 was assayed through a fluorometric assay using the fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Enzo life science). All the measurements were performed in 96-well plates with a BMG Labtech OptimaStar microplate reader. Excitation and emission wavelengths were 320 and 420 nm, respectively. All incubations were performed at 28 °C in 50 mM TrisHCl, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij35, 1% DMSO at pH 7.5. The inhibitors were pre-incubated with enzymes (1nM) for 5 minutes at room temperature before the reaction was started by the addition of the fluorogenic substrate (3μM). The decrease of fluorescence was monitored over 30 minutes (λ_{ex}=320 nm, λ_{em}=420 nm) at 28 °C. The IC_{50} values were obtained by dose response measurements using inhibitor range of concentrations 0.00001-20 μM and enzyme concentration equal to 1nM. A detergent-based assay was used to determine the presence of promiscuous inhibitors.[6] All the experiments were performed in duplicate and data collected were analyzed using Graphpad 5.0 Software Package (Graphpad Prism, San Diego, CA).

Cell culture. Human melanoma cell lines (A375P) were purchased from ATCC and were grown in Dulbecco's modified Eagle medium containing 4500 mg/L glucose (DMEM 4500) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified incubator containing 10% CO_2. Then 5.0×10^5 cells were seeded in 100 mm Sarstedt dishes and propagated every 3 days by incubation with a trypsin-EDTA solution.

Endothelial precursor circulating cells (EPCs) were obtained accordingly to reported methods.[7] EPC were grown in an endothelial cell growth medium-2 (EGM-2) containing endothelial cell growth supplements and were subcultivated using a trypsin- EDTA solution, 1:3 split ratio. For the use in the experiments, cells (passages 2-4) were grown to confluence in plates coated with 1% bovine gelatin.

Gelatin zymography. 1×10^6 A375P cells or EPCs were seeded in 4 mL volume in a 25 cm^2 culture tissue flask and, after 24 h incubation in standard condition, media were collected. Aliquots of 25 μL were added to the sample buffer in a 1:4 ratio (SDS 0.4%, 2% glycerol, 10 mmol/L Tris-HCl, pH 6.8, 0.001% bromphenol blue). The samples were run on a 8% SDS gel containing 0.1% gelatin. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 and once with incubation buffer [50 mmol/L Tris-HCl (pH 7.5), 200 mmol/L NaCl, 5 mmol/L $CaCl_2$). After SDS-PAGE separation gel lanes were cut into slices and each slice was exposed O/N to standard incubation buffer or to the incubation buffer containing different MMP inhibitors at 20 μM concentration. Lane exposed to incubation buffer only shows the typical bands of digestion corresponding to pro-MMP9 (92kDa), active MMP-9 (86kDa), proMMP-2 (72 kDa) and active MMP-2 (62kDa). After incubation the slices were stained with 0.5% Coomassie brilliant blue and destained with a solution of methanol/acetic acid/water (3:1:6; v/v). After destaining, the slices were immersed in distilled water, scanned immediately and analyzed with Image J for Windows (http://imagej.nih.gov/ij/index.html). Gelatinolytic activity was demonstrated as clear bands on a blue background.

Cell viability assay. A375P melanoma cells were seeded in 60 mm plates in standard conditions. After 24 h, cells were exposed to a control medium and to treatment with compound **1** at conc. 100, 50, 25, 12.5 μM.

After 24 h, cells were harvested, stained with trypan blue and counted under an inverted-phase microscope (quadruplicate haemocytometer counts of duplicate cultures were performed).

Isolation of Endothelial Progenitor cells (EPCs). EPCs were isolated from UCB as reported;[7] human UCB samples (volume > 50 mL) were collected in citrate phosphate dextrose solution from health newborns. We used cord blood units with a number of total nucleated cells < 1.3×10^9 (threshold of suitability for the banking established by the Umbilical Cord Bank of Careggi, Florence, Italy) after maternal informed consent in accordance with the Declaration of Helsinki and in compliance with Italian legislation.[7]

Invasion assay. Human melanoma cells or EPC cells were seeded in their growth medium onto Matrigel-precoated Boyden chamber (8 mm pore size, 6.5 mm diameter, 1.5 μg Matrigel/filter) with or without the MMP inhibitor. In the lower chamber, complete growth medium was added. Following 6 hours of incubation, the inserts were removed and the non-invading cells on the upper surface were removed with a cotton swab. The filters were then stained using the Diff-Quik kit and photographs of randomly chosen fields were taken. Migrated cells were counted by a light microscope (10×) in three random fields per each well. Data were expressed for each treatment as mean values from three wells. The experiment was repeated at least three times and a representative experiment was shown.

In Vivo Angiogenesis Assay. The 5- to 6-weeks-old FVB mice were used. Matrigel solution (BD Biosciences) was mixed with 50 U/mL heparin resuspended in EGM-2 medium and compound **1** at the final concentration of 20 μM. 500 μL Matrigel mixtures were injected subcutaneously, and 5 days after, mice were sacrificed. Matrigel plugs were removed and photographed with the aid of a dissecting microscope. Experiment was repeated three times and a representative picture were shown.

Statistical analysis. Densitometric data were analysed by a multiple-comparison Student–Newman–Keuls test, after demonstration of significant differences among medians by non-parametric variance analysis, according to the Kruskal–Wallis test. Data are expressed as means and standard errors of the mean, depicted by vertical bars, of the indicated number of experiments.

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