SUPPORTING INFORMATION FOR

"Bifunctional Inhibitors as a new tool to reduce cancer cell invasion by impairing MMP-9 homodimerization"

Elisa Nuti,[1] Lea Rosalia,[1] Doretta Cuffaro,[1] Caterina Camodeca,[1] Chiara Giacomelli,[1] Eleonora Da Pozzo,[1] Tiziano Tuccinardi,[1] Barbara Costa,[1] Claudia Antoni,[1,2] Laura Vera,[2] Lidia Ciccone,[1] Elisabetta Orlandini,[1] Susanna Nencetti,[1] Vincent Dive,[2] Claudia Martini,[1] Enrico A. Stura,[2] Armando Rossello[1,*]

[1]Dipartimento di Farmacia, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy

[2]CEA, iBiTec-S, Service d'Ingenierie Moleculaire des Proteines, CE-Saclay, 91191 Gif sur Yvette Cedex, France

Table of Contents

I. Supplementary Figures

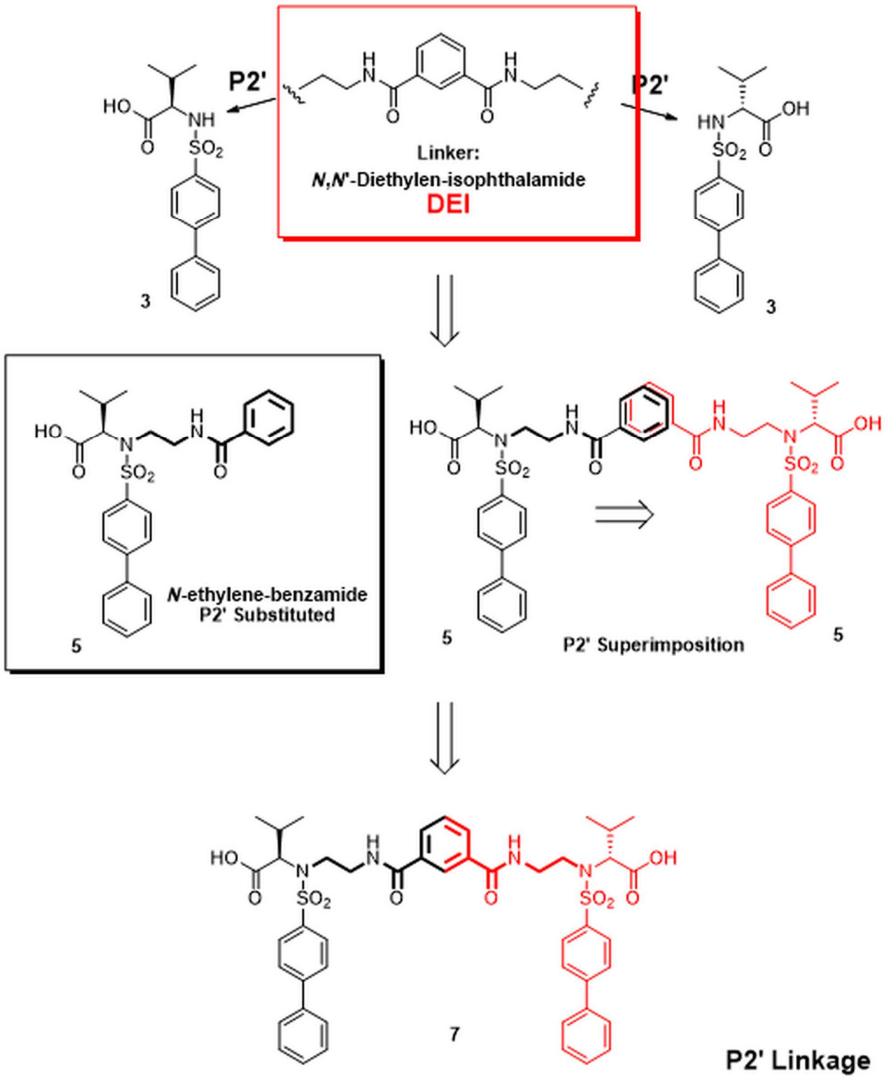


Figure S1. Structural relationship between the dimeric inhibitor 7 and its monomers 3 and 5.

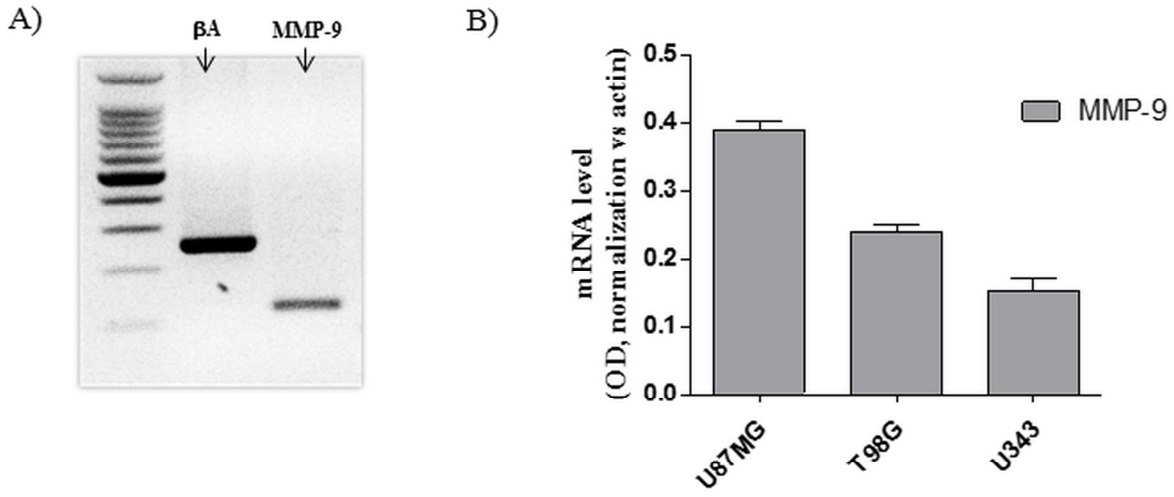


Figure S2. MMP -9 expression levels. (A) Representative samples of the electrophoretic separation of RT-PCR products (ladder 100 bp, β-actin (βA) and metalloprotease 9 (MMP-9)) in human glioblastoma cell line U87MG. Cells from each line were collected and total RNA was extracted and amplified using RT-PCR. The resultant product was separated by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. Optical density (OD, arbitrary units) was measured by ImageJ Software and the mean normalized by β-actin. The Histogram (B) represents the MMP-9 mRNA expression levels in human GBM cell lines (U87MG, T98G and U343MG).

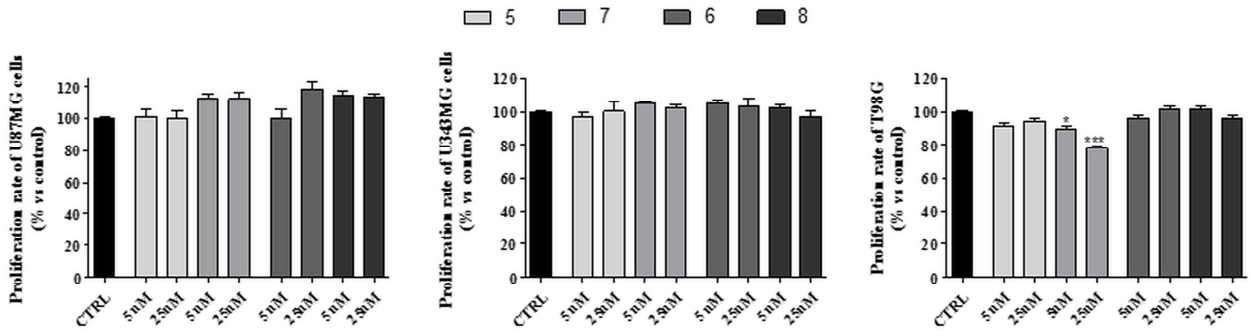


Figure S3. MMP inhibitors effects on cell proliferation. The adherent cells (U87MG, U343MG and T98G) were maintained in the absence (DMSO, control) or presence of different concentration of compounds **5-8**. After 24 h the active mitochondrial metabolism as index of proliferation rate were evaluated using the MTS assay, by the means of spectrophotometer technique. Data are presented as the mean+/- SEM, derived from at least three independent experiments done in duplicate.

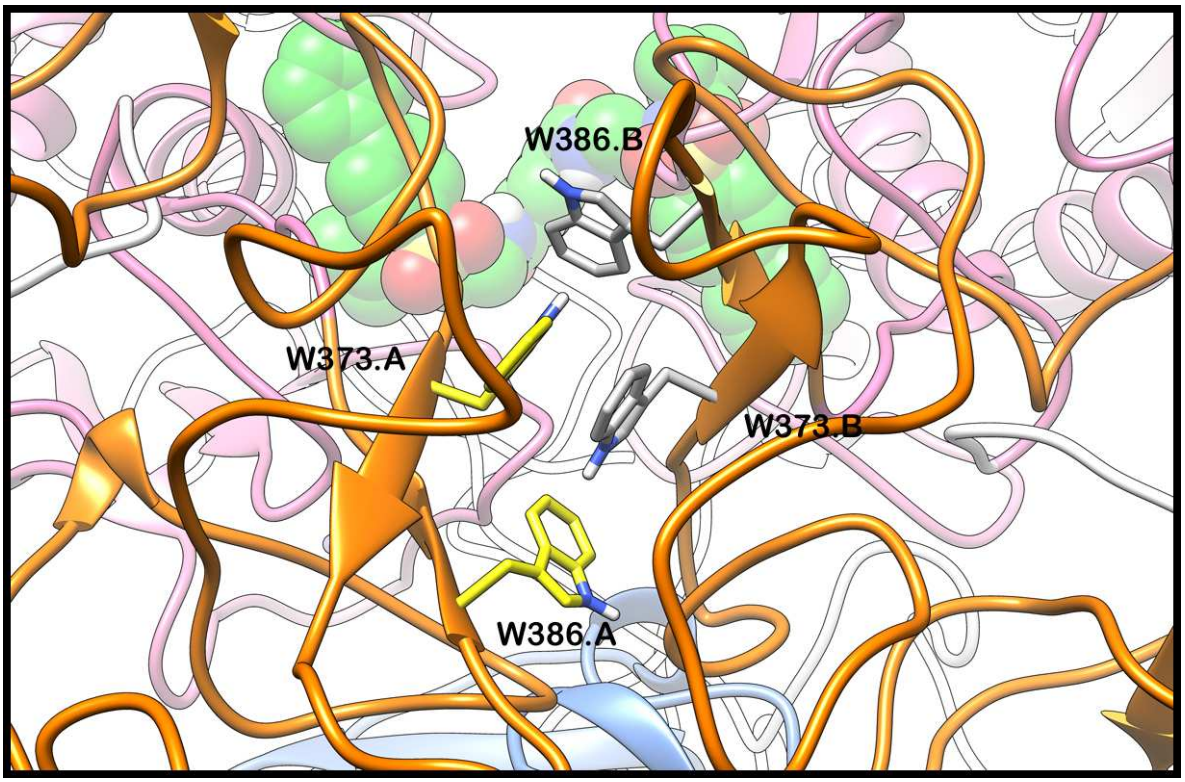


Figure S4. Tryptophan network at the intermolecular contact between the third fibronectin domain of the two MMP-9 proteins.

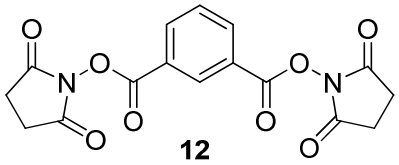
II. General methods for the synthesis of compounds 5-10.

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were determined with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references. Coupling constants *J* are reported in hertz; ^{13}C NMR spectra were fully decoupled. The following abbreviations are used: singlet (s), doublet (d), triplet (t), double-doublet (dd), broad (br) and multiplet (m). Chromatographic separations were performed on silica gel columns by flash column chromatography (Kieselgel 40, 0.040–0.063 mm; Merck) or using Isolute Flash Si II cartridges (Biotage). Reactions

were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F254) sheets that were visualized under a UV lamp and hydroxamic acids were visualized with $FeCl_3$ aqueous solution. Evaporation was performed *in vacuo* (rotating evaporator). Sodium sulfate was always used as the drying agent. Where indicated, the elemental compositions of the compounds agreed to within ±0.4% of the calculated value. Commercially available chemicals were purchased from Sigma-Aldrich. Compound **11** has been prepared as previously described. [1]

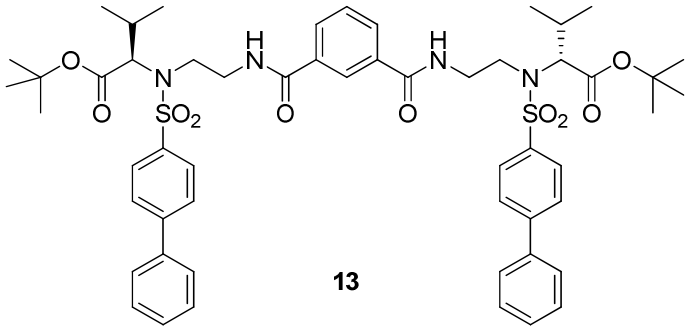
III. Synthetic details and characterization of compounds

Synthesis of P2'dimers 7-10 (SCHEME 1)

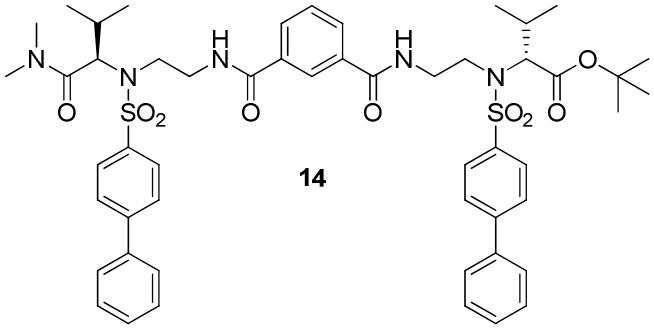


12

1,1'-(1,3-phenylenedicarbonyl)bis-2,5-Pyrrolidinedione (12). Triethylamine (1.5 mL, 11 mmol) was added to a solution of *N*-hydroxy succinimide (1.27 g, 11 mmol) in THF (30 mL). The solution was cooled to 0 °C and isophthaloyl dichloride (1.02 g, 5.0 mmol) was added dropwise. After 2 h of stirring at RT, the solvent was removed in vacuo and the residue was dissolved in CH_2Cl_2 (50 mL), washed with water, dried over Na_2SO_4 and evaporated. The crude product was purified by recrystallization with isopropanol to give **12** as a white solid (1.6 g, 40% yield). ^{1}H-NMR ($CDCl_3$) δ: 2.91 (s, 8H); 7.71 (t, *J*= 8 Hz, 1H); 8.42 (d, *J*= 7.8 Hz, 2H); 8.88 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 25.81; 126.39; 129.83; 132.44; 136.37; 160.68; 168.92.



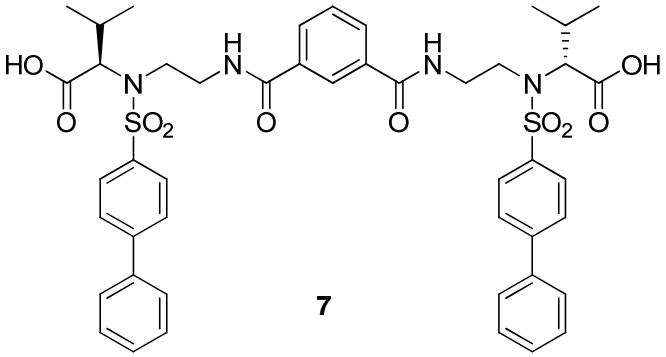
13



14

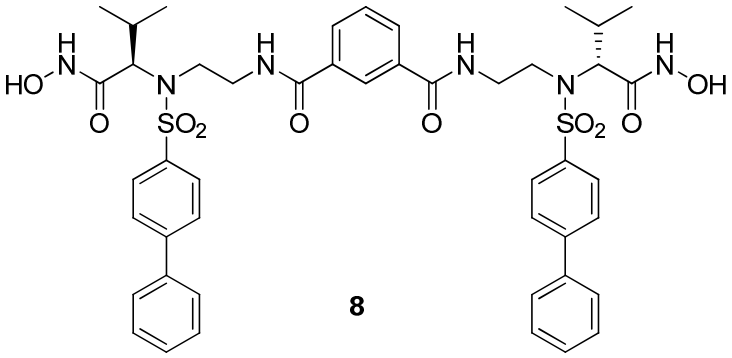
***N,N′*-bis[N-(biphenyl-4-sulfonyl)-(*R*)-[2-(1-tert-butoxycarbonyl-2 methylpropyl)-aminoethyl]]-benzene-1,3-dicarboxamide (13) and 2-{(Biphenyl-4-sulfonyl)-(*R*)-[2-(3-{2-[(biphenyl-4-sulfonyl)-(1-dimethylcarbamoyl-2-methyl-propyl)-amino]-ethylcarbamoyl}-benzoylamino)-ethyl]-amino}-(*R*)-3-methyl-butyric acid tert-butyl ester (14).** A solution of **12** (106 mg, 0.29 mmol) and *N*,*N*-diisopropylethylamine (0.41 mL, 2.35 mmol) in dry DMF (2.1 mL) was slowly added to a solution of the salt **11** (212 mg, 0.49 mmol) in dry DMF (1.0 mL) cooled to 0 °C under argon atmosphere. The resulting solution was stirred overnight at RT and the solvent was removed in vacuo. The crude product was purified by flash chromatography (*n*-hexane/EtOAc 3:2) using a Isolute Flash Si II cartridge to give the di-*tert*-butyl ester **13** (R_f=0.22) as a green oil (59 mg, 21% yield) and **14** (R_f=0.15) as a yellow oil (69 mg, 25% yield). Compound **13**: ^{1}H-NMR ($CDCl_3$) δ: 0.95 (d, *J*= 6.4 Hz, 6H); 1.02 (d, *J*= 6.4 Hz, 6H); 1.23 (s, 18H); 2.09-2.25 (m, 2H); 3.43-3.57 (m, 4H); 3.70-3.75 (m, 4H); 3.99 (d, *J*= 10.2 Hz, 2H); 7.37-7.57 (m, 11H); 7.61-7.65 (m, 4H); 7.87-7.91 (m, 4H); 8.00 (d, *J*= 8.6 Hz, 2H); 8.36 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 19.49; 20.25; 27.90; 29.49; 40.94; 44.14; 66.74; 82.40; 125.48; 127.30; 127.76;

128.10; 128.56; 129.10; 130.27; 134.57; 138.12; 139.21; 145.80; 166.70; 170.02. Compound **14**: ^{1}H-NMR ($CDCl_3$) δ: 0.95 (d, *J*= 6.4 Hz, 6H); 1.01 (d, *J*= 6.4 Hz, 6H); 1.22 (s, 18H); 2.88 (s, 6H); 3.43-3.57 (m, 4H); 3.69-3.77 (m, 4H); 4.02 (d, *J*= 10.2 Hz, 2H); 7.37-7.56 (m, 10H); 7.63-7.67 (m, 4H); 7.75 (d, *J*= 5 Hz, 1H); 7.86-7.91 (m, 4H); 8.12-8.19 (m, 2H); 8.58 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 19.47; 20.22; 25.81; 27.85; 29.49; 40.98; 44.03; 66.70; 82.51; 125.52; 127.25; 127.72; 127.99; 128.56; 129.07; 129.21; 133.02; 133.40; 135.08; 138.15; 139.08; 145.78; 165.65; 169.22; 170.18.



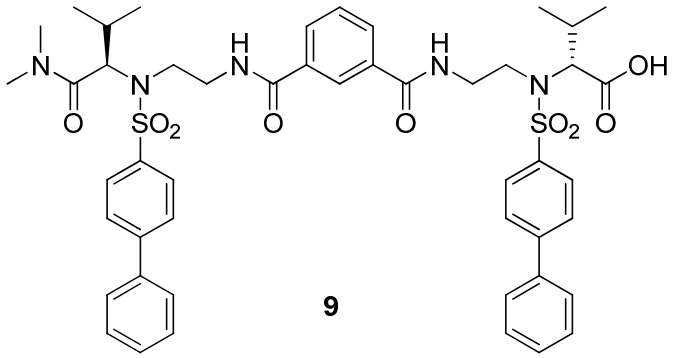
7

***N,N′*-bis(2-[(biphenyl-4ylsulfonyl)[(2*R*)-1-hydroxy-3-methyl-1-oxobutan-2-yl]-amino]ethyl)benzene-1,3-dicarboxamide (7).** Trifluoroacetic acid (0.63 mL, 8.2 mmol) was added dropwise to a stirred solution of *tert*-butyl ester **13** (59 mg, 0.06 mmol) in freshly distilled CH_2Cl_2 (3.6 mL), cooled to 0 °C. The solution was stirred for 5 h at 0 °C and the solvent was removed in vacuo to give **7** as a white solid (58 mg, 98% yield). Mp: 103-105 °C; ^{1}H-NMR ($CDCl_3$) δ: 0.85 (d, *J*= 6.4 Hz, 6H); 0.96 (d, *J*= 6.4 Hz, 6H); 2.02-2.18 (m, 2H), 3.42-3.78 (m, 4H); 3.95-4.00 (m, 4H); 4.14 (d, *J*= 10.2 Hz, 2H); 7.15-7.41 (m, 11H); 7.54-7.78 (m, 4H), 7.69-7.86 (m, 6H); 8.02 (s, 1H); 9.48 (s, 2H). ^{13}C-NMR ($CDCl_3$) δ: 19.62; 20.02; 28.52; 40.16; 43.76; 66.14; 127.23; 127.59; 127.72; 128.63; 129.03; 130.60; 133.24; 138.06; 138.79; 145.71; 167.58; 174.70. Elemental Analysis for $C_{46}H_{50}N_4O_{10}S_2$. Calculated: %C, 62.57; %H, 5.71; %N, 6.34; Found %C, 62.73; %H, 5.89; %N, 6.11.



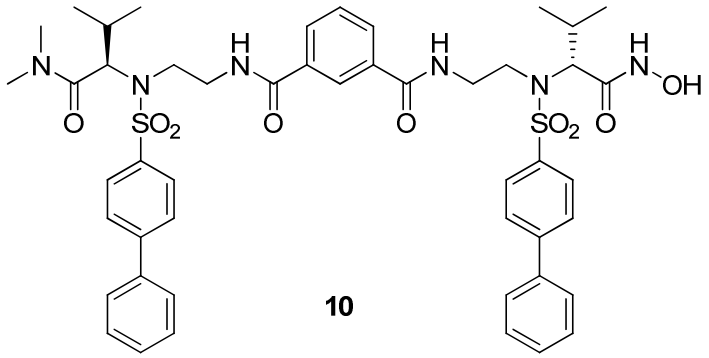
8

***N*,*N*'-bis(2-{(biphenyl-4-ylsulfonyl)[(2R)-1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl]amino}ethyl)benzene-1,3-dicarboxamide (8).** 1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (50 mg, 0.26 mmol) was added portionwise to a stirred and cooled solution (0 ºC) of di-carboxylic acid **7** (94 mg, 0.10 mmol), and *O*-(*tert*-butyldimethylsilyl)hydroxylamine (38 mg, 0.26 mmol) in dry CH_2Cl_2 (10 mL) under nitrogen atmosphere. After stirring at room temperature overnight, the mixture was washed with water and the organic phase was dried and evaporated *in vacuo*. The crude product was purified by flash chromatography (*n*-hexane/EtOAc 1:1) using a Isolute Flash Si II cartridge to give the *O*-silylate derivative as a yellow oil (13 mg). Silyl precursor (13 mg, 0.01 mmol) was then dissolved in dry CH_2Cl_2 (0.6 mL) and TFA (0.12 mL, 1.53 mmol) was added dropwise at 0 °C. After 5 h of stirring TFA was evaporated to afford **8** as a white solid (10 mg, 99% yield). Mp: 121-123 °C; ^{1}H-NMR ($CDCl_3$) δ: 0.86- 0.91 (m, 12 H); 2.08-2.20 (m, 2H); 3.20-3.49 (m, 4H); 3.60-3.94 (m, 6H); 7.43.7.58 (m, 1H); 7.67-7.71 (m, 4H); 7.88-7.92 (m, 4H); 8.04 (d, *J*= 8.4 Hz, 2H); 8.34 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 19.29; 19.65; 27.26; 29.91; 40.48; 43.80; 125.74; 127.34; 127.87; 128.68; 129.10; 130.83; 133.84; 138.32; 138.95; 146.07; 167.81; 169.13. Elemental Analysis for $C_{46}H_{52}N_6O_{10}S_2$. Calculated: %C, 60.51; %H, 5.74; %N, 9.20; Found %C, 60.73; %H, 5.89; %N, 9.01.



9

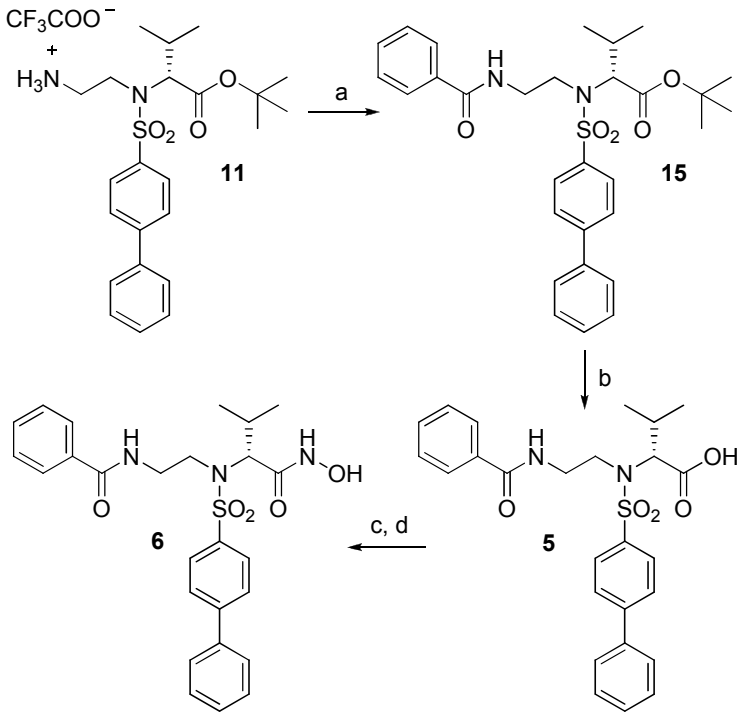
2-{(Biphenyl-4-sulfonyl)-(*R*)-[2-(3-{2-[(biphenyl-4-sulfonyl)-(1-dimethylcarbamoyl-2-methyl-propyl)-amino]-ethylcarbamoyl}-benzoylamino)-ethyl]-amino}-(*R*)-3-methyl-butyric acid (9). Trifluoroacetic acid (0.65 mL, 8.5 mmol) was added dropwise to a stirred solution of *tert*-butyl ester **14** (60 mg, 0.06 mmol) in freshly distilled CH_2Cl_2 (3.7 mL), cooled to 0 °C. The solution was stirred for 5 h at 0 °C and the solvent was removed in vacuo. The crude product was purified by flash chromatography ($CHCl_3$/MeOH 12:1) (R_f=0.27) using a Isolute Flash Si II cartridge to give **9** as a solid (24 mg, 44 % yield). Mp: 118-120 °C; ^{1}H-NMR ($CDCl_3$) δ:0.73-0.87 (m, 6H); 1.11-1.28 (m, 6H); 2.04-2.16 (m, 2H), 2.86 (s, 6H); 3.41 (m, 4H); 3.71 (m, 4H); 4.06-4.17 (m, 2H); 5.40 (m, 2H); 7.26-7.42 (m, 11H); 7.55-7.59 (m, 4H), 7.85-7.89 (m, 4H); 7.98-8.06 (s, 2H); 8.39 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 19.91; 25.94; 28.70; 29.87; 40.39; 44.03; 67.12; 125.41; 127.28; 127.57; 127.88; 128.56; 129.07; 129.41; 133.18; 133.86; 134.77; 138.70; 139.03; 145.53; 161.39; 166.16; 166.49. Elemental Analysis for $C_{48}H_{55}N_5O_9S_2$. Calculated: %C, 63.35; %H, 6.09; %N, 7.70; Found %C, 63.49; %H, 6.30; %N, 7.52.



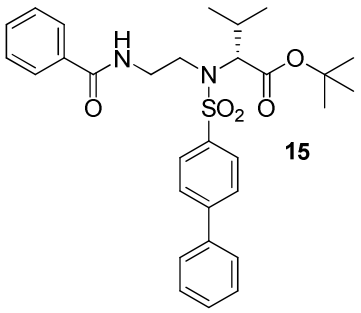
10

2-{(Biphenyl-4-sulfonyl)-(*R*)-[2-(3-{2-[(biphenyl-4-sulfonyl)-(1-dimethylcarbamoyl-2-methyl-propyl)-amino]-ethylcarbamoyl}-benzoylamino)-ethyl]-amino}-(*R*)-3-methyl-N-hydroxy-butanamide (10). 1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (38 mg, 0.2 mmol) was added portionwise to a stirred and cooled solution (0 ºC) of carboxylic acid **9** (150 mg, 0.16 mmol), and *O*-(*tert*-butyldimethylsilyl)hydroxylamine (35 mg, 0.24 mmol) in dry CH_2Cl_2 (5 mL) under nitrogen atmosphere. After stirring at room temperature overnight, the mixture was washed with water and the organic phase was dried and evaporated *in vacuo*. The crude product was purified by flash chromatography (*n*-hexane/EtOAc 1:3) using a Isolute Flash Si II cartridge to give the mono-hydroxamate derivative **10** as a white solid (4 mg, 2.7% yield). Mp: 87-89 °C; ^{1}H-NMR ($CDCl_3$) δ: 0.82-1.01 (m, 6H); 1.43-1.34 (m, 6H); 2.04-2.17 (m, 2H); 2.93 (s, 6H); 3.17 (m, 4H); 3.42-3.90 (m, 4H); 3.96-3.99 (m, 2H); 4.26 (d, *J*= 10.4 Hz, 2H); 7.26-7.47 (m, 10H); 7.57-7.61 (m, 4H); 7.83-7.88 (m, 4H); 8.06-8.11 (m, 4H); 8.36 (s, 2H). ^{13}C-NMR ($CDCl_3$) δ: 19.74; 20.24; 25.95; 29.91; 40.19; 44.25; 65.96; 125.46; 127.32; 127.68; 128.63; 129.09; 129.39; 133.18; 133.98; 134.97; 138.75; 138.97; 145.73; 161.53; 165.70; 169.47; 173.77. Elemental Analysis for $C_{48}H_{56}N_6O_9S_2$. Calculated: %C, 62.32; %H, 6.10; %N, 9.08; Found %C, 62.64; %H, 6.39; %N, 9.01.

Synthesis of monomeric compounds 5 and 6 (Scheme S1)

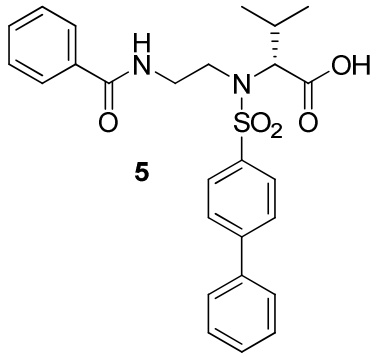


Reagents and conditions: (a) benzoyl chloride, DIPEA, DMF, rt, 18 h; (b) TFA, CH_2Cl_2, 0 °C, 5 h; (c) $TBDMSiONH_2$, EDC, CH_2Cl_2, rt, 16 h; (d) TFA, CH_2Cl_2, 0 °C, 5 h.



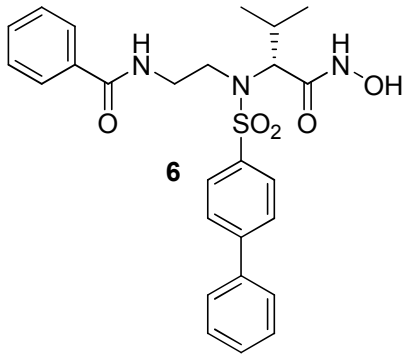
(*R*)-*tert*-Butyl 2-(*N*-(2-benzamidoethyl)biphenyl-4-ylsulfonamido)-3-methylbutanoate (15). A solution of **11** (74 mg, 0.13 mmol) in dry DMF (2 mL) was treated with benzoyl chloride (0.02 mL, 0.16 mmol) and *N*,*N*-diisopropylethylamine (0.05 mL, 0.27 mmol) under nitrogen atmosphere. The

reaction mixture was stirred at RT for 18 h, then was diluted with EtOAc, washed with H_2O, dried over Na_2SO_4 and evaporated. The crude was purified by flash chromatography (*n*-hexane/EtOAc 8:1) using a Isolute Flash Si II cartridge to give **15** as a yellow oil (30 mg, 41% yield). ^{1}H-NMR ($CDCl_3$) δ: 0.96 (d, *J*= 6.4 Hz, 3H); 1.04 (d, *J*= 6.4 Hz, 3H); 1.26 (s, 9H); 2.06-2.25 (m, 1H); 3.50-3.62 (m, 1H); 3.70-3.76 (m, 2H); 4.01-4.09 (m, 2H); 7.37-7.69 (m, 10H); 7.84-7.93 (m, 3H); 8.09-8.13 (m, 1H).



5

(*R*)-2-(*N*-(2-Benzamidoethyl)biphenyl-4-ylsulfonamido)-3-methylbutanoic acid (5). Trifluoroacetic acid (0.24 mL, 3.13 mmol) was added dropwise to a stirred solution of *tert*-butyl ester **15** (30 mg, 0.05 mmol) in freshly distilled CH_2Cl_2 (1.0 mL), cooled to 0 °C. The solution was stirred for 5 h at 0 °C and the solvent was removed *in vacuo*. The crude product was purified by trituration with *n*-hexane/Et_2O to give **5** as a white solid (15 mg, 57% yield). Mp: 64-65 °C; ^{1}H-NMR ($CDCl_3$) δ: 0.91 (d, *J*= 6.2 Hz, 6H); 2.01-2.16 (m, 1H); 3.34-3.53 (m, 1H); 3.58-3.83 (m, 3H); 4.11 (d, *J*= 10.4 Hz, 1H); 5.67 (br s, 1H); 7.33-7.55 (m, 9H); 7.60-7.64 (m, 2H); 7.78-7.88 (m, 4H). ^{13}C-NMR ($CDCl_3$) δ: 19.71; 20.09; 28.89; 40.67; 44.51; 66.19; 127.19; 127.41; 127.67; 128.14; 128.70; 129.16; 131.73; 133.97; 137.64; 139.15; 146.06; 168.03; 172.86. Elemental Analysis for $C_{26}H_{28}N_2O_5S$. Calculated: %C, 64.98; %H, 5.87; %N, 5.83; Found %C, 65.09; %H, 5.98; %N, 5.65.



6

(*R*)-*N*-(2-(N-(1-(hydroxyamino)-3-methyl-1-oxobutan-2-yl)biphenyl-4-ylsulfonamido)ethyl)benzamide (6). 1-[3-(Dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (126 mg, 0.65 mmol) was added portionwise to a stirred and cooled solution (0 ºC) of carboxylic acid **5** (158 mg, 0.32 mmol), and *O*-(*tert*-butyldimethylsilyl)hydroxylamine (97 mg, 0.65 mmol) in dry CH_2Cl_2 (13 mL) under nitrogen atmosphere. After stirring at room temperature overnight, the mixture was washed with water and the organic phase was dried and evaporated *in vacuo*. The crude product was purified by flash chromatography (*n*-hexane/EtOAc 4:1) using a Isolute Flash Si II cartridge to give the *O*-silylate derivative as a yellow oil (79 mg). Silyl precursor (79 mg, 0.12 mmol) was then dissolved in dry CH_2Cl_2 (5 mL) and TFA (5.6 mL, 7.3 mmol) was added dropwise at 0 °C. After 5 h of stirring the solvent was evaporated and the crude product was purified by trituration with *n*-hexane/Et_2O to afford **6** as a white solid (56 mg, 87% yield). Mp: 83-85 °C; ^{1}H-NMR (DMSO-d_6) δ: 0.87-0.94 (m, 6H); 2.16-2.22 (m, 1H); 3.22-3.29 (m, 1H); 3.64-3.76 (m, 3H); 3.99-4.06 (m, 1H); 7.47-7.59 (m, 6H); 7.77-7.99 (m, 8H); 8.64 (s, 1H); 10.88 (s, 1H). ^{13}C-NMR (acetone-d_6) δ: 19.52; 20.05; 41.06; 44.67; 64.46; 127.97; 128.21; 128.68; 129.01; 129.17; 129.77; 131.76; 135.60; 139.66; 139.95; 145.98; 167.14. Elemental Analysis for $C_{26}H_{29}N_3O_5S$. Calculated: %C, 63.01; %H, 5.90; %N, 8.48; Found %C, 63.20; %H, 5.98; %N, 8.30.

IV. Biological Activity on Isolated Enzymes

MMP inhibition assays.

Recombinant human MMP-14 catalytic domain was a kind gift of Prof. Gillian Murphy (Department of Oncology, University of Cambridge, UK). Pro-MMP-1, pro-MMP-2, and pro-MMP-9, were purchased from Calbiochem. Proenzymes were activated immediately prior to use with *p*-aminophenylmercuric acetate (APMA 2 mM for 1 h at 37°C for MMP-2 and MMP-1, 1 mM for 1 h at 37°C for MMP-9). For assay measurements, the inhibitor stock solutions (10 mM in DMSO) were further diluted, at 7 different concentrations (0.01 nM-200 μM) for each MMP in the fluorimetric assay buffer (FAB: Tris 50 mM, pH = 7.5, NaCl 150 mM, $CaCl_2$ 10 mM, Brij 35 0.05% and DMSO 1%). Activated enzyme (final concentration 2.9 nM for MMP-2, 2.7 nM for MMP-9, 1 nM for MMP-14cd, 2.0 nM for MMP-1) and inhibitor solutions were incubated in the assay buffer for 4 h at 25 °C. After the addition of 200 μM solution of the fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 (Bachem) for all the enzymes in DMSO (final concentration 2 μM), the hydrolysis was monitored every 15 sec. for 20 min. recording the increase in fluorescence (λ_{ex} = 325 nm, λ_{em} = 400 nm) using a Molecular Devices SpectraMax Gemini XS plate reader. The assays were performed in triplicate in a total volume of 200 μL per well in 96-well microtitre plates (Corning, black, NBS). Control wells lack inhibitor. The MMP inhibition activity was expressed in relative fluorescent units (RFU). Percent of inhibition was calculated from control reactions without the inhibitor. IC_{50} was determined using the formula: $V_i/V_o = 1/(1 + [I]/ IC_{50})$, where V_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration [I] and V_o is the initial velocity in the absence of the inhibitor. Results were analyzed using SoftMax Pro software[2] and GraFit software.

V. Biological Activity on GBM Cell lines

Materials

The standard MMP inhibitor belonging to the classical tertiary sulfonamido-based hydroxamates family (CGS_27023A) was synthesized as reported by MacPherson et al.[3] Cell culture media and growth supplements were obtained from Cambrex Bio Science, Walkersville, Inc. (Walkersville, MD, USA). Non-essential amino acids (1%) were from GIBCO (Milan, Italy). Bio-Rad Protein Assays were purchased from Bio-Rad (Hercules, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit was from Promega Italia, Milano, Italy. The RNeasy Mini Kit was from Qiagen, Milano, Italy. The i-Script cDNA synthesis kit was furnished by Biorad s.r.l. and Fluocycle II SYBR was obtained from Euroclone s.p.a. Antibodies anti-MMP-9 (Cod. MAB 13415) and anti-mouse IgG HRP conjugated (Cod. #12-349) were purchased from Merck Millipore, Milano, Italy. Primers for MMP9 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were from standard commercial sources.

Cells and cell culture.

Human glioblastoma U87MG and U343MG cell lines were obtained from the National Institute for Cancer Research of Genoa (Italy) and Cell Lines Service GmbH (Germany), respectively. Each cell line was monitored for DNA profiling. The U87MG cells were cultured in RPMI medium, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 1% non-essential amino acids at 37 °C in 5% CO_2. The U343MG cells were cultured in Minimum essential medium Eagle (MEM) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% non-essential amino acids and 1.0 mM sodium pyruvate at 37 °C in 5% CO_2. The T98G cell line was obtained from American Type Culture Collection (USA). The cell line was monitored for DNA profiling. The T98G cells were cultured in Minimum essential medium Eagle, supplemented with 10%

FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 1% non-essential amino acids at 37 °C and 5% CO_2.

Cell derived MMP-9 mRNA expression levels.

In order to evaluate MMP-9 mRNA levels, a polymerase chain reaction (RT-PCR) was performed. In particular, the relative quantification of MMP-9 gene expression was evaluated using a previously described RT-PCR protocol.[4] In brief, total RNA was extracted using Rneasy® Mini Kit (Qiagen) according to manufacturer's instructions. The concentration and purity of the RNA samples was determined by measuring the absorbance at 260/280 nm. Reverse-transcription was performed with 1 μg of total RNA using i-Script cDNA synthesis kit following manufacturer's instructions. The primers used for the RT-PCR were designed to span intron/exon boundaries to ensure that products did not include genomic DNA (Table S1). The RT-PCR reactions consisted of 25 μL of Fluocycle® II SYBR®, 1.5 μL of both the forward and reverse primers (10 μM), 3 μL of cDNA and 19 μL of water. All reactions were performed for 30 cycles using the following temperature profiles: 98°C for 30 seconds (initial denaturation); 55 °C for 30 seconds (annealing) and 72 °C for 3 seconds (extension). β-Actin was used as housekeeping gene. The RT-PCR products were visualized after electrophoresis migration in a 2% agarose gel and stained with ethidium bromide. Fragment sizes were confirmed by comparison with a 100 pb DNA ladder molecular weight marker (Promega) The bands were viewed under UV light and analyzed by densitometric analysis using the ImageJ (ImageJ Software, version 1.41o; USA).

Table S1. Primers Used for RT-PCR.

Gene	Primer nucleotide sequences	Product size (base pairs)
MMP-9	FOR: 5'-GCACGACGTCTTCCAGTACC-3' REV: 5'-CAGGATGTCATAGGTCACGTAGC-3'	124 pb
β-actin	FOR: 5'-GCACTCTTCCAGCCTTCCTTCC-3' REV: 5'-GAGCCGCCGATCCACACG-3'	254 pb

MMP inhibitors effects on cell proliferation.

Cell proliferation was estimated by the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) conversion assay (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay, Promega), as previously reported.[5] Cells were seeded at 12,000 cells/cm^2 in completed medium at 37 °C and 5% CO_2. Cells were incubated for 24 h with DMSO (control sample) or with test compounds, MTS reagent was added, and the absorbance of individual wells was measured with a microplate reader (Wallac, Victor 2, 1420 Multilabel Counter, PerkinElmer). Each drug concentration was tested in duplicate, and the experiments were repeated at least three times.

MMP inhibitors effects on cell invasiveness.

The chemotaxis and Matrigel invasiveness of the GBM cell lines were evaluated as previously described.[10] Matrigel basement membrane matrix (BD Biosciences, Bedford, MA, USA), and 24 Transwell® (6.5 mm diameter 8.0 m pore size polycarbonate filters, Costar, Corning, NY, USA) were utilized.

Several concentrations of Matrigel were tested to ascertain the best invasive index. The best experimental conditions to evaluate the invasiveness of cells were the following: the surface of the

transwell was coated with Matrigel (50 μL of a solution of 0.32 mg/mL for each well) at room temperature overnight to form a genuine reconstituted basement membrane. The GBM cells were suspended in serum free medium (60,000/400 μL) and added to the upper compartment of the transwell in the absence and in the presence of MMP inhibitors or CGS_27023A, while 200 μL of RPMI (for U87MG) or MEM (for U343MG) containing 10% fetal bovine serum was added to the lower compartment. The cells were allowed to invade through the matrices at 37 °C for 24 h. The number of invading cells was quantified by counting the cells on the lower surface of the transwell membrane after fixing with p-formaldehyde and staining with Crystal Violet. Non-migrating cells on the upper surface were removed with a cotton bud. Pictures of randomly picked light microscope fields were taken (five fields for each filter), and cells were counted using ImageJ (ImageJ Software, version 1.41o; USA). Each drug concentration was tested in duplicate, and the experiments were repeated at least three times.

Data analyses

Statistical analyses were performed by one-way ANOVA (with post hoc Newman–Keuls test) by using the GraphPad Prism computer program (GraphPad Software, version 4.0; San Diego, CA, USA).

VI. MOLECULAR MODELING STUDIES

A high resolution structure of MMP-9 full-length has yet to be elucidated; therefore, prior to assembly the MMP-9 homodimer, a MMP-9 monomer model was developed.

Human MMP-9 full-length model generation. The search for the best template for modeling was carried out by using the BLASTP software.[6] As shown in Figure S5, for the development of the two monomers the following templates were used: a) the crystal structure of compound **7** in complex with

the two MMP-9 catalytic domains (PDB id: 4HMA)[7], b) the crystal structure of the C-terminally truncated human ProMMP-9 (PDB id: 1L6J)[8], c) the published hemopexin domain of MMP-9 (PDB id: 1ITV)[9] and d) the structure of human pro-matrix metalloproteinase-2 (PDB id: 1CK7)[10].

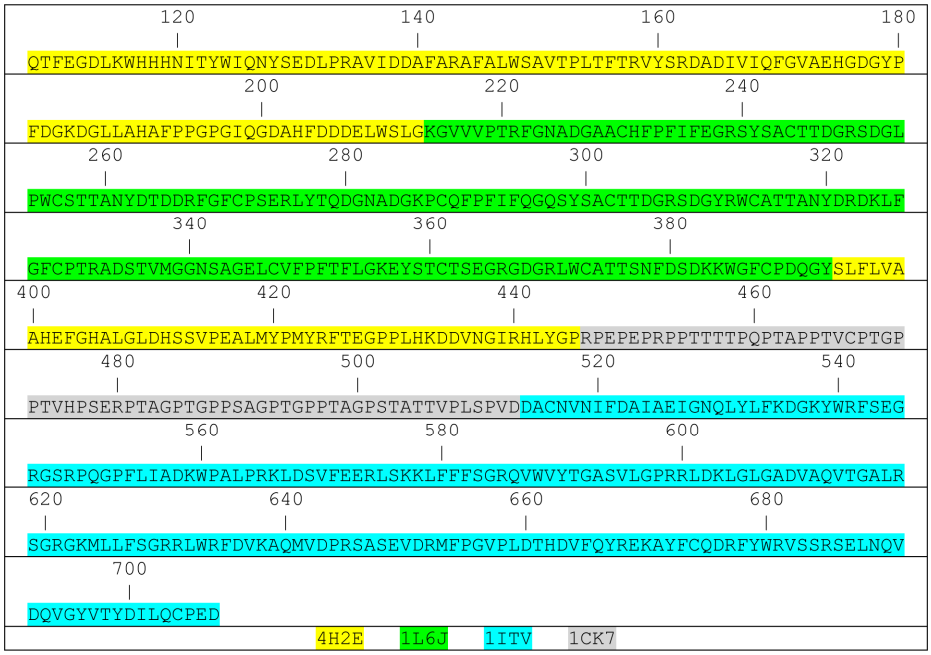


Figure S5. Templates used for the construction of the full-length human MMP-9 monomer.

Once the two monomers were built, they were superimposed to the two chains of the crystal structure of compound **7** in complex with the two MMP-9 catalytic domains (PDB id: 4HMA)[7], also including the ligand. The system was solvated with a 25 Å water cap. Sodium ions were added as counterions to neutralize the system. Prior to MD simulations, two steps of energy minimization were carried out. In the first stage, we kept the homodimer-ligand complex fixed with a position restraint of 100 kcal mol^{-1} $Å^{-2}$ and we just energy minimized the positions of the water molecules. In the second stage, we energy minimized the whole system without applying constraints. The two energy minimization stages consisted of 10000 steps. The first 1000 steps were Steepest Descent, and the last

9000 were Conjugate Gradient (CG). The MD trajectory was run using the energy minimized structure as the input, and particle mesh Ewald electrostatics[11] and periodic boundary conditions were used in the simulation. The time step of the simulations was 2.0 fs with a cutoff of 10 Å for the non-bonded interaction. SHAKE was employed to keep all bonds involving hydrogen atoms rigid. A constant-volume was carried out for 2 ns, during which time the temperature was raised from 0 to 300 K (using the Langevin dynamics method). Then, 98 ns of constant-pressure MD were carried out at 300 K. The final structure was obtained as the average of the last 50 ns of MD energy minimized with the CG method until a convergence of 0.05 kcal mol^{-1} $Å^{-2}$ was reached. The General Amber Force Field (GAFF) parameters were assigned to the ligand. The partial charges were calculated using the AM1-BCC method, as implemented in the Antechamber suite of AMBER 11.[12] As shown in Figure S6, the homodimer-**7** complex seemed to be stable during the simulations. By analyzing the root-mean-square deviation (rmsd) of all the α Carbons from the starting model, we observed an initial increase due to the equilibration of the system, followed, after about 20 ns, by a stabilization of the rmsd value around 4.8 Å. Regarding the geometry of the ligand, we analyzed the rmsd of the position of the ligands with respect to the starting complex structure during the simulation and Figure S6 shows that the ligand demonstrated an rmsd value around 1.5 Å.

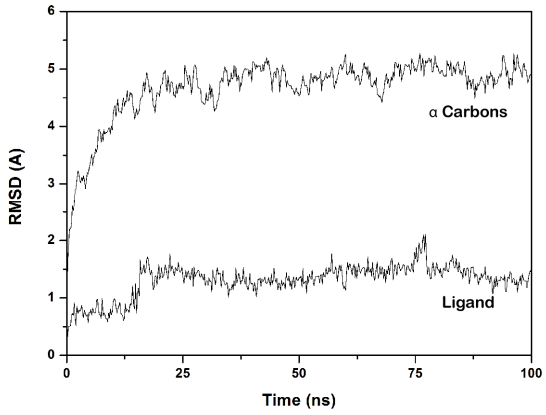


Figure S6. Analysis of the MD simulation for the homodimer-**7** complex. The plot shows the rmsd during the simulations of the α Carbons of the proteins and of the heavy atoms of the ligand.

VII. IN VITRO ADME ASSAYS

Results

Table S2: experimental results of in vitro permeability and thermodynamic water solubility of compound **7**.

Compounds	**PAMPA** [a] Papp × 10^{-6} (cm/s) **(MR%)** [b]	**Water Solub.** (LogS) [c]
7	>0.1 (0.0)	-4.35
Propranolol	9.68 (16.0)	-
Caffeine	>0.1 (0.0)	-

[a] PAMPA see experimental section for details; [b] Membrane Retention; [c] LogS= log mol L^{-1} corresponding at 38,87ug/mL

(Passive Permeability Interpretation: >10 × 10^{-6} cm/s Passive permeability is unlikely to be limiting for passive diffusion; 2 – 10 × 10^{-6} cm/s Permeability may be limiting in case of low solubility, high metabolic turnover rate or active secretion; 0-2 × 10^{-6} cm/s High risk that permeability is limiting for passive diffusion.)

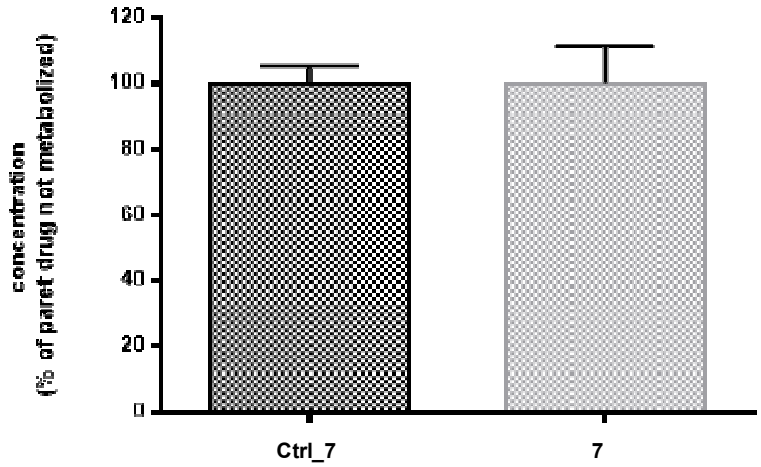


Figure S7. Results of metabolic stability; the metabolized parent drug revealed no significant differences compared to the control not metabolized. Compound **7** is extremely stable towards the cyp-dependent metabolism.

Experimental

Chemicals. All solvents, reagents, were from Sigma-Aldrich Srl (Milan, Italy). Dodecane was purchased from Fluka (Milan, Italy). Pooled Male Donors 20 mg/mL HLM were from BD Gentest-Biosciences (San Jose, California). Milli-Q quality water (Millipore, Milford, MA, USA) was used. Hydrophobic filter plates (MultiScreen-IP, Clear Plates, 0.45 μm diameter pore size), 96-well microplates, and 96-well UV-transparent microplates were obtained from Millipore (Bedford, MA, USA).

Parallel Artificial Membrane Permeability Assay (PAMPA). Donor solution (0.5 mM) of **7** was prepared by diluting 1 mM dimethylsulfoxide (DMSO) compound stock solution using phosphate buffer (pH 7.4, 0.025 M). Propranolol and caffeine were used as standard references. Filters were coated with 5 μL of a 1% (w/v) dodecane solution of phosphatidylcholine. Donor solution (150 μL) was added to each well of the filter plate. To each well of the acceptor plate were added 300 μL of

solution (50% DMSO in phosphate buffer). All compounds were tested in three different plates on different days. The sandwich was incubated for 5 h at room temperature under gentle shaking. After the incubation time, the plates were separated, and samples were taken from both receiver and donor sides and analyzed using LC with UV detection at 254 nm.

LC analysis were performed by a Varian ProStar 210 system constituted by a binary high-pressure gradient pump and ProStar 340 UV-Vis detector (Varian, Inc.2700 Mitchell DriveWalnut Creek, CA 94598-1675/USA). Chromatographic analysis was performed using a Kinetex EVO C-18 100A column (150 x 4.6 mm, 5 μm particle size) at room temperature. Analysis was carried out using a gradient elution: eluent A being methanol and eluent B consisting of an aqueous solution of formic acid (0.1%). The analysis started with 5% of eluent A, which was rapidly increased up to 95% in 12 min, then slowly increased up to 98% in 20 min. The flow rate was 0.6 mL min^{-1} and injection volume was 20 μL.

Permeability (P_{app}) for PAMPA were calculated according to the following equation, obtained from Wohnsland and Faller[13] and Sugano et al.[14] equation with some modification in order to obtain permeability values in cm s^{-1},

$$P_{app} = \frac{V_D V_A}{(V_D + V_A)At} - \ln(1 - r)$$

where V_A is the volume in the acceptor well, V_D is the volume in the donor well (cm^3), A is the "effective area" of the membrane (cm^2), t is the incubation time (s) and r the ratio between drug concentration in the acceptor and equilibrium concentration of the drug in the total volume (V_D+V_A). Drug concentration is estimated by using the peak area integration.

Membrane retentions (%) were calculated according to the following equation:

$$\%MR = \frac{[r-(D+A)]100}{Eq}$$

where *r* is the ratio between drug concentration in the acceptor and equilibrium concentration, *D, A,* and *Eq* represented drug concentration in the donor, acceptor and equilibrium solution, respectively.

Water Solubility Assay. Each solid compound (1 mg) was added to 1 mL of water. The sample was shaked in a shaker bath at room temperature for 24-36 h. The suspensions were filtered through a 0.45-μm nylon filter (Acrodisc), and the solubilized compound determined by LC-UV method above reported for PAMPA assay. The determination was performed in duplicate. Quantification of the compound was made by comparison with apposite calibration curves realized with standard solutions in methanol.

Microsomal Stability Assay. The compound in DMSO solution was incubated at 37 °C for 60 min in 125 mM phosphate buffer (pH 7.4), 5 μL of human liver microsomal protein (0.2 mg mL^{-1}), in the presence of a NADPH 200 μM at a final volume of 0.5 mL (compounds' final concentration, 50 μM); DMSO did not exceed 2% (final solution). The reaction was stopped by cooling in ice and adding 1.0 mL of acetonitrile. The reaction mixtures were then centrifuged, and the parent drug not metabolized was subsequently determined by LC-UV method previously reported. The percentage of not metabolized compound was calculated by comparison with reference solutions where compound **7** (50 μM) was added at the end of the incubation time.

VIII. Abbreviations

MMP: matrix metalloproteinase

PEX: hemopexin

BEPI: *N,N′*-bis-(3-ethylencarbamoyl-propyl)-isophthalamide

DEI: di-ethylene-isophthalamide

ZBG: zinc-binding group

DIPEA: *N,N*-diisopropylethylamine

TFA: trifluoroacetic acid

TBDMSiONH$_2$: *O*-(*tert*-butyldimethylsilyl)-hydroxylamine

EDC: 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride

RT-PCR: reverse transcription polymerase chain reaction

GBM: glioblastoma multiforme

MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

RMSD: root-mean-square deviation

MD: molecular dynamic

PDB: protein data bank

IX. Supporting Information References

(1) Nuti, E.; Cuffaro, D.; D'Andrea, F.; Rosalia, L.; Tepshi, L.; Fabbi, M.; Carbotti, G.; Ferrini, S.; Santamaria, S.; Camodeca, C.; Ciccone, L.; Orlandini, E.; Nencetti, S.; Stura, E. A.; Dive, V.; Rossello, A. Sugar-based arylsulfonamide carboxylates as selective and water-soluble Matrix Metalloproteinase-12 inhibitors. *ChemMedChem.* **2016**, *11*, 1626-1637.

(2) SoftMax Pro 4.7.1 by Molecular Devices.

(3) MacPherson, L. J.; Bayburt, E. K.; Capparelli, M. P.; Carroll, B. J.; Goldstein, R.; Justice, M. R.; Zhu, L.; Hu, S.; Melton, R. A.; Fryer, L.; Goldberg, R. L.; Doughty, J. R.; Spirito, S.; Blancuzzi, V.; Wilson, D.; O'Byrne, E. M.; Ganu, V.; Parker, D. T. Discovery of CGS 27023A, a non-peptidic, potent, and orally active stromelysin inhibitor that blocks cartilage degradation in rabbits, *J. Med. Chem.* **1997**, *40*, 2525-2532.

(4) Da Pozzo, E.; La Pietra, V.; Cosimelli, B.; Da Settimo, F.; Giacomelli, C.; Marinelli, L.; Martini, C.; Novellino, E.; Taliani, S.; Greco, G. p53 functional inhibitors behaving like pifithrin-β counteract the Alzheimer peptide non-β-amyloid component effects in human SH-SY5Y cells. *ACS Chem. Neurosci.* **2014**, *5*, 390-399.

(5) Gabelloni, P.; Da Pozzo, E.; Bendinelli, S.; Costa, B.; Nuti, E.; Casalini, F.; Orlandini, E.; Da Settimo, F.; Rossello, A.; Martini, C. Inhibition of metalloproteinases derived from tumours: new insights in the treatment of human glioblastoma. *Neuroscience.* **2010**, *168*, 514-522.

(6) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403-410.

(7) Antoni, C.; Vera, L.; Devel, L.; Catalani, M. P.; Czarny, B.; Cassar-Lajeunesse, E.; Nuti, E.; Rossello, A.; Dive, V.; Stura, E. A. Crystallization of bi-functional ligand protein complexes. *J. Struct. Biol.* **2013**, *182*, 246-254.

(8) Elkins, P. A.; Ho, Y. S.; Smith, W. W.; Janson, C. A.; D'Alessio, K. J.; McQueney, M. S.; Cummings, M. D.; Romanic, A. M. Structure of the C-terminally truncated human ProMMP9, a gelatin-binding matrix metalloproteinase. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2002**, *58*, 1182–1192.

(9) Cha, H.; Kopetzki, E.; Hube, R.; Lanzendörfer, M.; Brandstetter, H. Structural basis of the adaptive molecular recognition by MMP9. *J. Mol. Biol.* **2002**, *320*, 1065–1079.

(10) Morgunova, E.; Tuuttila, A.; Bergmann, U.; Isupov, M.; Lindqvist, Y.; Schneider, G.; Tryggvason, K. Structure of Human Pro-Matrix Metalloproteinase-2: Activation Mechanism Revealed. *Science* **1999**, *284*, 1667–1670.

(11) Essmann, U., et al. A smooth particle mesh Ewald method. *J. Chem. Phys.* **1995,** *103*, 8477.

(12) Case, D.A., et al. AMBER, version 11; University of California: San Francisco, CA, 2010.

(13) Wohnsland, F.; Faller, B. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *J. Med. Chem.* **2001**, *44*, 923–930.

(14) Sugano, K.; Hamada, H.; Machida, M.; Ushio, H. High throughput prediction of oral absorption: improvement of the composition of the lipid solution used in parallel artificial membrane permeation assay. *J. Biomol. Screen.* **2001**, *6*, 189–196.