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Synthesis, radiosynthesis, in vitro and preliminary in vivo evaluation of biphenyl carboxylic and hydroxamic matrix metalloproteinase (MMP) inhibitors as potential tumor imaging agents[1]

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Abstract

Excess matrix degradation is one of the hallmarks of cancer and is an important factor in the process of tumor progression. It is implicated in invasion, metastasis, growth, angiogenesis and migration. Many characteristics of matrix metalloproteinases (MMPs) make them attractive therapeutic and diagnostic targets. MMP expression is upregulated at the tumor site, with localization of activity in the tumor or the surrounding stroma, providing a target for medical imaging techniques. Radioiodinated carboxylic and hydroxamic MMP inhibitors 2-(4'-[^{123}I] iodo-biphenyl-4-sulfonylamino)-3-methyl-butyric acid (9) and 2-(4'-[^{123}I] iodo-biphenyl-4-sulfonylamino)-3-methyl-butyramide (11), their unlabelled standards and precursors were synthesized. Radioiodination was conducted by electrophilic aromatic substitution of the tributylstannyl precursors and resulted in radiochemical yields of 70 ± 5% (n = 6) and 60 ± 5% (n = 4), respectively. In vitro zymography and enzyme assays showed for both hydroxamic acid and carboxylic acid compounds a good inhibition activity and a high selectivity for MMP-2. In vivo biodistribution in NMRI mice showed no long-term accumulation in organs and the possibility to accumulate in the tumor in a later phase of this study.

Keywords: Radiolabeled MMP inhibitors; Iodine-123; In vitro enzyme assay; SPECT; Tumor imaging

1. INTRODUCTION

Matrix metalloproteinases (MMPs) are zinc and calcium dependent enzymes which are synthesized as zymogens in connective tissue (Murphy et al., 1991; Woessner, 1991). Matrix metalloproteinases or matrix-ins have been linked with the accelerated breakdown of connective tissue associated with pathological diseases such as arthritis, tumor metastasis, multiple sclerosis and Alzheimer's disease (Docherty et al, 1992; Peress et al., 1995). Excess matrix degradation is one of the hallmarks of cancer and is an important factor in the process of tumor progression. It is implicated in invasion, metastasis, growth, angiogenesis and migration (McCawley and Matrisian, 2000; Matrisian, 1990). MMPs comprise a large family of over 20 proteins that can degrade all the known components of the extracellular matrix such as collagens, proteoclycans and glycoproteins (Nagase and Woessner, 1999; Stetler-Stevenson et al., 1993).

Many characteristics of MMPs make them attractive therapeutic targets as MMP expression is upregulated at the tumor site, whith localization of activity in the tumor or the surrounding stroma. A therapy targeted to the site of the pathology would imply minimal side effects. The anticipated efficacy of MMP inhibitors in the disease arresting process has led to the identification of numerous broad spectrum and specific inhibitors of MMPs, of which several are currently under clinical evaluation (Broadhurst et al., 1997; Morphy et al., 1995). Among the subfamilies, gelatinases (MMP-2 and MMP-9) have become attractive targets for cancer research and development of anticancer drugs (Bremer et al., 2001; Curran and Murray, 1999; Fang et al., 2000; Furumoto et al., 2002) due to their increased expression and activity in human malignant tumor tissue of various organs such as breast, colon and lung (Cai et al., 2002; Furumoto et al., 2003; Liabakk et al., 1996; Passlick et al, 2000).

The catalytic core-domain of MMPs responsible for the active protein-degrading ability includes a metal-binding site for Zn^{2+} , the binding of which is essential for proteolytic activity. Matrix metalloproteinase activity is modulated through interactions with their natural inhibitors, the tissue specific inhibitors of MMPs (TIMPs); TIMPs are able to form complexes with the inactive form of MMP and, thus, have the ability to control MMP

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activation (McCawley and Matrisian, 2000). Because TIMPs are probably not suitable for pharmacologic applications, due to their short half-life in vivo (Wojtowicz et al., 1997), synthetic inhibitors were designed to inhibit MMP activity. These synthetic inhibitors interfere with the catalytic mechanisms in the active site of the enzyme (McCawley and Matrisian, 2000) and contain an effective zinc binding group (e.g. hydroxamic acid, carboxylic acid, or sulfhydryl group) (Fei et al., 2003). To improve selectivity, MMP inhibitors are substituted with side chains that interact with specific sub-sites within the active site of the MMP and which determine the selectivity and binding capacities for a particular subtype (Fei et al., 2003). The S1', mainly hydrophobic, sub-site, for the gelatinases is located more deeply in the molecule than for other subtypes and is targeted to obtain highly selective inhibitors for this subgroup of MMPs. Therefore, a sulfonamide group is incorporated to improve enzyme-inhibitor binding, not only by forming hydrogen bonds but also by properly directing the hydrophobic substituent to the S1' sub-site and enabling it to penetrate deeply (Kiyama et al., 1999).

This report describes the synthesis and in vitro evaluation of 2-(4'-iodo-biphenyl-4-sulfonylamino)-3-methyl-butyric acid, 2-(4'-iodo-biphenyl-4-sulfonylami-no)-3-methyl-butyramide and their iodine-123 analogues. These compounds were selected based upon low nanomolar IC_{50} potencies for gelatinases of related compounds (O'Brien et al., 2000; Tamura et al., 1998).

2. METHODS

2.1. General

The biphenyl sulfonamide derivatives were synthesized via modified literature methods (O'Brien et al, 2000; Hanessian et al, 2001) and are described briefly. All commercially available reagents were purchased from Sigma-Aldrich (St. Louis, USA) unless otherwise specified and used without further purification. Dichlor-omethane, ethylacetate, hexane and methanol were purchased from Lab-Scan analytical sciences (Dublin, Ireland). Ethanol was purchased from VWR International (Leuven, Belgium). [^{123}I] Sodium iodide (in 0.05M NaOH) was purchased from Bristol-Myers Squibb Pharma (Brussels, Belgium). The fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, was purchased from Bachem AG (Bubendorf, Switzerland). A549, lung carcinoma, cells were obtained from ATCC (LGC Promochem, Teddington, UK). Media and other cell products and equipment were purchased from BioWhittaker (Verviers, Belgium). Solutions containing intermediate products were dried over anhydrous magnesium sulphate ($MgSO_4$). All moisture-sensitive reactions were performed under nitrogen atmosphere. Analytical thin layer chromatography (TLC) was carried out using F_{254} precoated silica gel plates (Polygram Sil G/UV254 Machery-Nagel, Düren, Germany). Purification of intermediate or final products was performed by column chromatography using silica gel (silica, 230-400 mesh, Sigma-Aldrich, St. Louis, USA) or preparative TLC using pre-coated silica gel plates (Sil G-200 UV_{254} Machery-Nagel, Germany). Melting points were measured on an Electrothermal IA9100 (Analis, Namen, Belgium) digital melting point apparatus. ^{1}H-NMR spectra were recorded on a 300MHz NMR spectrometer (Varian Mercury 300, Palo Alto, USA) using $(CD_3)_2CO$-d_6, $CDCl_3$-d_1, CD_3OD-d_4 or DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. Chemical shift data for the proton resonances were reported in parts per million *(δ)* relative to the internal standard TMS (*δ*0.0). Abbreviations used are: s, singulet, d, doublet, t, triplet, m, multiplet, br.s, broad signal. If necessary, assignment of the signals was confirmed by COSY (Correlation Spectroscopy). Mass spectra and exact masses were obtained using a time of flight (Q-Tof-2, Micromass, Manchester, UK) mass spectrometer equiped with a standard electrospray ionisation (ESI) interface (KUL, Rega Institute, Belgium). Radiochromatography was performed using an Alltech Alltima C_{18} column (250 × 4.6 mm, 5µm; Lokeren, Belgium) and a Gilson 307 pump (Villiers-le-Bel, France). The effluent was monitored with a UV-VIS detector at λ = 254 nm (2487 dual λ absorbance detector, Waters, Belgium) and an NaI detector (Ludlum, Texas, USA).

2.2. Synthesis of standards and precursors

2.2.1. 4'-Bromo-biphenyl-4-sulfonic acid (1)

To a stirred solution of 4'-bromo-biphenyl (8 g, 34.5mmol) in chloroform (60 ml), chlorosulfonic acid (4.8g, 41.4mmol) was added dropwise. A white solid precipitated during the addition. The mixture was stirred for 4h at room temperature (RT). The precipitate was collected by filtration and washed with cold chloroform. The white solid was oven-dried (40 °C), yielding 1 (98%) which was used without further purification. mp 140-142°C. ^{1}H-NMR ($(CD3)_2CO$-d_6): *δ* 7.95 (d, 2H, Ar*H*), 7.85 (d, 2H, Ar*H*), 7.70 (s, 4H, Ar*H*), 2.10 (s, 1 H, -SO_3*H*). Exact mass (ESI-MS) $[M-H]^-$ calculated for $C_{12}H_{10}SO_3Br$ 310.9378-312.9378, found 310.9381-312.9381.

2.2.2. 4'-Bromo-biphenyl-4-sulfonyl chloride (2)

A catalytic amount of anhydrous *N*,*N*-dimethylformamide (*N*,*N*-DMF) was added dropwise under an inert atmosphere to a solution of 1 (10g, 32.1mmol) in thionylchloride (32 ml) and the resulting mixture was refiuxed at 80 °C for 4 h. After cooling to RT, the solvent was removed under reduced pressure and residual thionylchloride was removed by co-evaporation with toluene (2 × 30 ml) yielding a yellow solid (90%) which was used without further purification, mp 122-124°C. ^{1}H-NMR ($CDCl_3$-d_1): δ 8.1 (d, 2H, Ar*H),* 7.8 (d, 2H, Ar*H*), 7.65 (d, 2H, Ar*H*), 7.5 (d, 2H, Ar*H*).

2.2.3. 2-(4'-Bromo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid t-butyl ester (3)

To a mixture of L-valine *tert*-butyl ester hydrochloride (3.8g, 18.2mmol) and sulphonyl chloride 2 (6g, 18.2mmol) in aqueous tetrahydrofuran (1:1, THF:H_2O, 60 ml), 2 equivalents triethylamine (3.7g, 36.4mmol) were added dropwise . The reaction mixture was stirred at RT for 1 h, followed by addition of ethylacetate (60 ml) and aqueous HC1 (60ml, 1M), respectively. The organic phase was separated, dried over anhydrous magnesium sulphate and concentrated in vacuo. The residue was analysed with TLC (hexane/ethylacetate: 60/ 40) and one newly formed product 3 (R_f 0.6) was observed. Purification by column chromatography (silica, 200 × 30 mm) using a gradient system (hexane/ ethylacetate 90/10 → 85/15) yielded 3 (80%) as a white solid, mp 133-134. ^{1}H-NMR (DMSO-d_6): δ 8.13 (d, 1 H, -SO_2N*H*-), 7.88-7.58 (m, 8H, Ar*H*), 3.45 (dd, 1 H, -NHC*H*-), 1.94 (m, 1H, -C*H*(CH_3)$_2$), 1.13 (s, 9H, t-butyl), 0.82 (app. t, 6 H, *CH*(C*H*$_3$)$_2$). Exact mass (ESI-MS) [M-H]$^-$ calculated for $C_{21}H_{26}SO_4NBr$ 466.0688-468.0688, found 466.0677-468.0662.

2.2.4. 2-(4'-Bromo-biphenyl-4-sulphonylamino) -3-methyl-butyric acid (4)

3 (4g, 8.6mmol) was added to a stirred solution of anisol (0.9g, 8.6mmol) in TFA (17.1ml, 0.22mol). The reaction mixture was stirred at RT for 4h and poured over ice. The resulting precipitate was collected by filtration. The crude product was washed with ice-cold water and dried to a constant weight. TLC evaluation (hexane/ethylacetate: 60/40) showed the starting product *(Rf* 0.6) and one newly formed product 4 (R_f 0.15). The mixture was purified by column chromatography (silica, 200 × 30 mm) using a gradient system (hexane/ethylacetate (90/10 → 85/15), yielding 4 (70%) as a white solid, mp 181-183. 'H-NMR (DMSO-d_6): δ 12.6 (br.s, 1 H, COO*H*), 8.03 (d, 1 H, -SO_2R*H*-), 7.88 (s, 4H, Ar*H*), 7.64 (s, 4H, Ar*H*), 3.60 (br.s, 1 H, NHC*H*), 1.98 (m, 1 H, C*H*(CH_3)$_2$), 0.82 (dd, 6 H, C*H*(C*H*$_3$)$_2$). Exact mass (ESI-MS) [M-H]$^-$ calculated for $C_{17}H_{18}SO_4NBr$ 410.0062-412.0062, found 410.0024-412.0009.

2.2.5. 2-(4'-Bromo-biphenyl-4-sulphonylamino) -3-methyl-N-trityloxy-butyramide (5)

Carboxylic acid 4 (2.4 g, 5.8 mmol), *N*-(3-dimethyla-minopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) (1.455g, 7.6mmol, 1.3eq.) and 1-hydroxy-1H-benzotriazole hydrate (HOBt) (1.026 g, 7.6 mmol, 1.3 eq.) were brought under an inert atmosphere into a two neck round bottom flask, equipped with a reflux condenser and a gas trap. Anhydrous tetrahydrofuran (60 ml) and *N*-methylmorpholine (NMM) (0.9g, 8.8 mmol, 1.5eq.) were added and after stirring at RT for 20 min, *O*-trityl hydroxylamine (2.4 g, 8.8 mmol, 1.5 eq.) was added to the mixture. After stirring for another 16h at RT, the mixture was diluted with ethylacetate (60 ml) and the organic phase was washed with 0.1 M $NaHCO_3$ (50 ml), 0.1 M HCl (50 ml) and water (50 ml), respectively. The organic phase was dried over anhydrous magnesium sulphate and concentrated in vacuo. TLC analysis (hexane/ethylacetate: 60/40) of the mixture showed a newly formed product (R_f 0.48). Purification by column chromatography using a gradient system (hexane/ethylacetate 95/5→80/20) yielding 5 (50%) as a slightly yellow solid, mp 186-187. ^{1}H-NMR (DMSO-d_6): δ 10.00 (br.s, 1H, CO-N*H*-O), 7.90-7.60 (m, 9H, Ar*H,* -SO_2N*H*-), 7.2 (m, 15H, trityl*H*), 3.56 (br.s, 1H, NHC*H*), 1.42 (m, 1H, C*H*(CH_3)$_2$), 0.44 (d, 6H, CH(C*H*$_3$)$_2$). Exact mass (ESI-MS) [M-H]$^-$ calculated for $C_{36}H_{33}SO_4N_2Br$ 667.1272-669.1272, found 667.1282-669.1271.

2.2.6. 2-(4'-Bromo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (6)

To a solution of 5 (658.8mg, 0.986mmol) in dichloromethane (4 ml), saturated with water, was added 50% TFA in dichloromethane (2 ml). The solution was stirred for 1 h at RT, diluted with dichloromethane (10 ml), washed with 0.5M $NaHCO_3$ (40 ml) and dried over anhydrous magnesium sulphate. The mixture was evaluated with TLC (ethylacetate/ hexane: 80/20) and showed a new-formed product (R_f 0.58). TLC indicated the presence of 6 by spraying with 5% iron (III) chloride in hydrochloric acid (0.5mol/l). Purification was conducted on preparative TLC (hexane/ethylacetate (60/40)) (Rf 0.15). 6 was obtained as a yellow oil (30%). ^{1}H-NMR (DMSO-d_6): δ 10.55 (br.s, 1H, CO-NH-O*H*), 8.82 (s, 1H, CO-N*H*-OH), 8.02 (s, 1H, -SO_2R*H*-), 7.85-7.70 (m, 8H, Ar*H),* 3.25 (s, 1H, NHC*H*), 1.8 (m, 1H, C*H*(CH_3)$_2$), 0.73 (m, 6H, CH(C*H*$_3$)$_2$). Exact mass (ESI-MS)

$[M-H]^-$ calculated for $C_{17}H19SO_4N_2Br$ 425.0171-427.0171, found 425.0166-427.0140.

2.2.7. Synthesis of iodine analogues

All steps in the synthesis of the iodine molecules were similar to those described for bromine analogues with more or less the same yields. 4'-iodo-biphenyl was purchased from Daniels Fine Chemicals Ltd. (Edmonton, Alberta, Canada)

2.2.7.1. 2-(4'-Iodo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (4').

TLC evaluation of the white solid (hexane/ethylacetate: 60/40) showed one spot 4' (R_f 0.15, yield 70%). mp .222-224°C ^{1}H-NMR (DMSO-d_6): δ 12.55 (s, 1H, COO*H*), 8.03 (d, 1H, -SO_2N*H*-), 7.78 (s, 4H, Ar*H*), 7.50 (s, 4H, Ar*H*), 3.54 (t, 1H, NHC*H*), 1.93 (m, 1H, C*H*(CH_3)$_2$), 0.82 (s, 6H, CH(CH_3)$_2$). Exact mass (ESI-MS) $[M-H]^-$ calculated for $C_{17}H_{18}SO_4NI$ 457.9924, found 457.9904.

2.2.7.2. 2-(4'-Iodo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (6').

TLC evaluation of the yellow oily substance (ethylacetate/hexane: 80/20) showed one spot 6' (R_f 0.58, 30% yield). ^{1}H-NMR (CD_3OD-d_4): δ 10.6 (s, 1H, CO-NH-O*H*), 8.8 (s, 1H, CO-N*H*-OH), 8.02 (d, 1H, -SO_2N*H*-), 7.9-7.4 (4d, 8H, Ar*H*), 3.36 (d, 1H, NHC*H*), 1.85 (m, 1H, C*H*(CH_3)$_2$), 0.85 (m, 6H, CH(CH_3)$_2$). Exact mass (ESI-MS) $[M-H]^-$ calculated for $C_{17}H_{19}SO_4N_2I$ 473.0032, found 473.0038.

2.2.8. 2-(4'-Tributylstannyl-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (7)

4 (230 mg, 0.6 mmol) was flushed with nitrogen for 2h and subsequently dissolved in dry toluene (5 ml). Hexabutylditin (974 mg, 1.7 mmol, 3eq.) and a catalytic amount of tetrakistriphenylphosphine-palladium were added and the mixture was refluxed under nitrogen for 15h. The reaction mixture was diluted with ethylacetate (30 ml) and washed with water (50 ml). The organic phase was dried over anhydrous magnesium sulphate and concentrated in vacuo. The mixture was purified on preparative TLC (hexane/ethylacetate/glacial acetic acid (71/28/1)) yielding 7 as a white oily substance (40%). ^{1}H-NMR (CD_3OD-d_4): δ 12.60 (br.s, 1H, COO*H*), 7.95 (d, 2H, Ar*H*), 7.75 (d, 2H, Ar*H*), 7.65 (d, 2H, Ar*H*), 7.55 (d, 2H, Ar*H*), 7.52 (d, 1H, -SO_2NH-), 3.56 (d, 1H, NHCH), 1.97 (m, 1H, C*H*(CH_3)$_2$), 1.80-0.80 (m, 33H, $(Bu)_3$SnH; CH(CH_3)$_2$). Exact mass (ESI-MS) [M-HΓ calculated for $C_{29}H_{45}SO_4NSn$ 622.2018, found 622.2032.

2.2.9. 2-(4'-Tributylstannyl-biphenyl-4-sulphonylamino)-3-methyl-N-trityloxy-butyramide (8)

5 (165mg, 0.247 mmol) was flushed with nitrogen for 2h and then dissolved dry toluene (5 ml). Hexabutylditin (429.8mg, 0.74mmol, 3eq.) and a catalytic amount of tetrakistriphenylphosphine-palladium were added and the mixture was refluxed under nitrogen for 15h. The reaction mixture was diluted with ethylacetate (30 ml) and washed with water (50 ml). The organic phase was dried over anhydrous magnesium sulphate and concentrated in vacuo. Purification using preparative TLC (hexane/ethylacetate (80/20)) yielded 8 as a yellowish substance (40%). ^{1}H-NMR (CD_3OD-d_4): δ 8.80 (s, 1H, CO-N*H*-OH), 7.90-7.54 (4d, 8H, Ar*H*), 7.49 (d, 1H, -SO_2N*H*-), 7.20 (m, 15H, trityl*H*), 3.50 (d, 1H, NHC*H*), 1.80-0.90 (m, 28H, $(Bu)_3$Sn; C*H*(CH_3)$_2$), 0.56 (dd, 6H, CH(CH_3)$_2$). Exact mass (ESI-MS) $[M-H]^-$ calculated for $C_{48}H_{60}SO_4N_2Sn$ 879.3223, found 879.3279.

2.3. Radio synthesis ofiodine-123 labelled carboxylic and hydroxamic MMP inhibitors

2.3.1. 2-(4'-[^{123}I]Iodo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (9)

To a solution of 7 (622 µg, 1 µmol) in ethanol (130 µl), n.c.a. [^{123}I]NaI in sodium hydroxide solution (15 µl 0.05 M), chloramine T (1 µl, 0.1 M solution) and glacial acetic acid (5 µl) were added respectively and the resulting mixture was stirred for 5min at RT. The reaction mixture was quenched by addition of sodium metabisulphite (1 µl, 0.2 M solution). The mixture was purified by HPLC with ethanol/phosphate buffer (0.05M, pH 2) (49/51) as mobile phase at a flow rate of 1 ml/min. The radiolabelled product was collected (R_t 29.97 min.) and analyzed with the same HPLC system. The radiochemical yield was 70±5% (n = 6), the radiochemical purity > 98% and the specific activity >50Ci/µmol.

2.3.2. 2-(4'-[^{123}I] Iodo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (11)

To a solution of 8 (400 µg, 0.45 µmol) in ethanol (130 µl), n.c.a. [^{123}I]NaI in sodium hydroxide solution (15µl

0.05M), chloramine T (1µl, 0.1M solution) and glacial acetic acid (5µl) were added respectively and the resulting mixture was stirred for 5min at RT. The reaction mixture was quenched by addition of sodium metabisulphite (1µl, 0.2M solution). The resulting mixture of radiolabelled product 10 was analysed by HPLC with ethanol/phosphate buffer (0.05 M, pH 6) (70/30) as mobile phase at a flow rate of 1ml/min. *(*R_t 22.5 min.). In a one pot reaction, trifiuoroacetic acid (30µl) was added to compound 10 and stirred for 1 hour to obtain compound 11. This second mixture was purified under the same HPLC conditions as described for product 10 using an ethanol/ phosphate buffer (0.05M, pH 6) (46/54) as mobile phase. The radiolabelled product 11 was collected *(*R_t 20.69 min.) and analysed with the same HPLC system. The radiochemical yield was 60±5% (n = 4), the radiochemical purity > 98% and the specific activity >50Ci/µmol.

2.4. Zymography and enzyme assays to determine inhibition capacities of the compounds on gelatinases

2.4.1. Zymography

Analysis of gelatinolytic activities was performed by gelatin zymography as previously described (Maquoi et al., 2000). Briefly, samples of 10 µl conditionned medium (HT1080) were mixed with equal volumes of sample buffer (62.5mM pH 6.8 Tris-HCl, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) and directly submitted to electrophoresis on 10% acrylamide gels containing 0.1% gelatin. Gels were run at 10 mA, washed with 2% Triton X-100 for 1 h, and incubated in activation buffer (50 mM pH 7.4 Tris-HCl, 200 mM NaCl, 5mM $CaCl_2$, and 0.02% NaN_3) or different concentrations of inhibitor for 16 h at 37°C After staining with Coomassie Brilliant Blue R-250, the gelatinolytic activities were detected as clear bands against a blue background (Hawkes et al., 2001).

2.4.2. In vitro enzyme assays

Enzymes. The recombinant catalytic domain of MT1-MMP(cMTl), MT3-MMP(cMT3) were produced in Liège and described before (Lang et al., 2004). Pro-MMP2 was produced by CHO cells transfected by the pAdG164-plasmid, containing the cDNA of the human pro-MMP2 (kind gift from Dr. K. Tryggvason, University of Oulu, Finland). Four day-conditioned medium from the clone CHO100.11 was collected for the purification of pro-MMP2 using a two-step procedure. First, a batch adsorption on gelatin-sepharose equilibrated in MEM was performed. The resin was then packed into a column and washed with Tris/HCl 50 mM pH 7.5, NaCl 0.15M, $CaCl_2$ 10mM, 0.02% NaN_3 and 1% DMSO. The protein was then eluted with Tris/HCl 50mM pH 7.5, NaCl 0.15M, $CaCl_2$ 10mM, 0.02% NaN_3 and 5% DMSO. In the second step of purification, a Q-sepharose equilibrated in Tris/HCl 50mM pH 8, $CaCl_2$ 1mM and 1% DMSO was used. Elution of pro-MMP2 was achieved by eluting the column with a NaCl gradient from 50 to 300mM. Pro-MMP2 eluted at around 250mM NaCl. Pro-MMP-9 was purchased from R&D Systems. Activation of pro-MMP-2 and pro-MMP-9 was achieved by incubating the enzyme with 1 mM APMA (4-aminophe-nylmercuric acetate) at 25 °C for 2 h for pro-MMP-2 and at 37°C for 16h for pro-MMP-9. The solutions were divided into smaller volumes and frozen at -20 °C until needed.

Inhibition assay. All kinetic measurements were performed in Tris/HCl 50mM pH 7.5, $CaCl_2$ 10mM, Brij 35 0.05%. For each enzyme ([MMP-2] = 0.02nM , [cMT 1] = 0.1nM , [cMT3] = 0.1nM , [MMP-9] = 0.25nM) the initial rate of cleavage of the fluorescent substrate Mca-Pro-Leu-Gly Leu-Dpa-Ala-Arg-NH2 (5 µM for MMP-2 and cMT3 and 3 µM for Cmt1 and MMP-9) was measured over 15 to 20min in the absence and the presence of the inhibitor at different concentration (1-10,000 nM). The percentages of inhibition (% of inhibition) at the different inhibitor concentrations were calculated. IC_{50} values were obtained as described before (Liabakk et al., 1996).

2.5. Biodistributions in NMRI mice

All animals were treated according to the regulations of the Belgian law and the local Ethical Committee.

In vivo evaluation of compounds 9, 11. Approximately, 37kBq (1 µCi) of tracer (9) or tracer (11), dissolved in ethanol/water (200 µl, 5/95), was injected in the tail vein of white mice (NMRI, 20-25 g) of either sex. At 20 and 40 s, 1, 1.5, 2, 3, 5, 10, 20 and 40 min and 1, 2, 3, 6, 9, 15, 24 and 48 h post injection, animals (n = 3) were sacrificed by decapitation after halothane anesthesia. Blood was taken and organs were excised. All tissues were weighed and counted for radioactivity with a Cobra automated gamma counter (Cobra II Series, Canberra Packard, Meriden, CT, USA Cobra). The concentration of radioactivity was expressed as a percentage of the injected dose/g of tissue and decay corrected.

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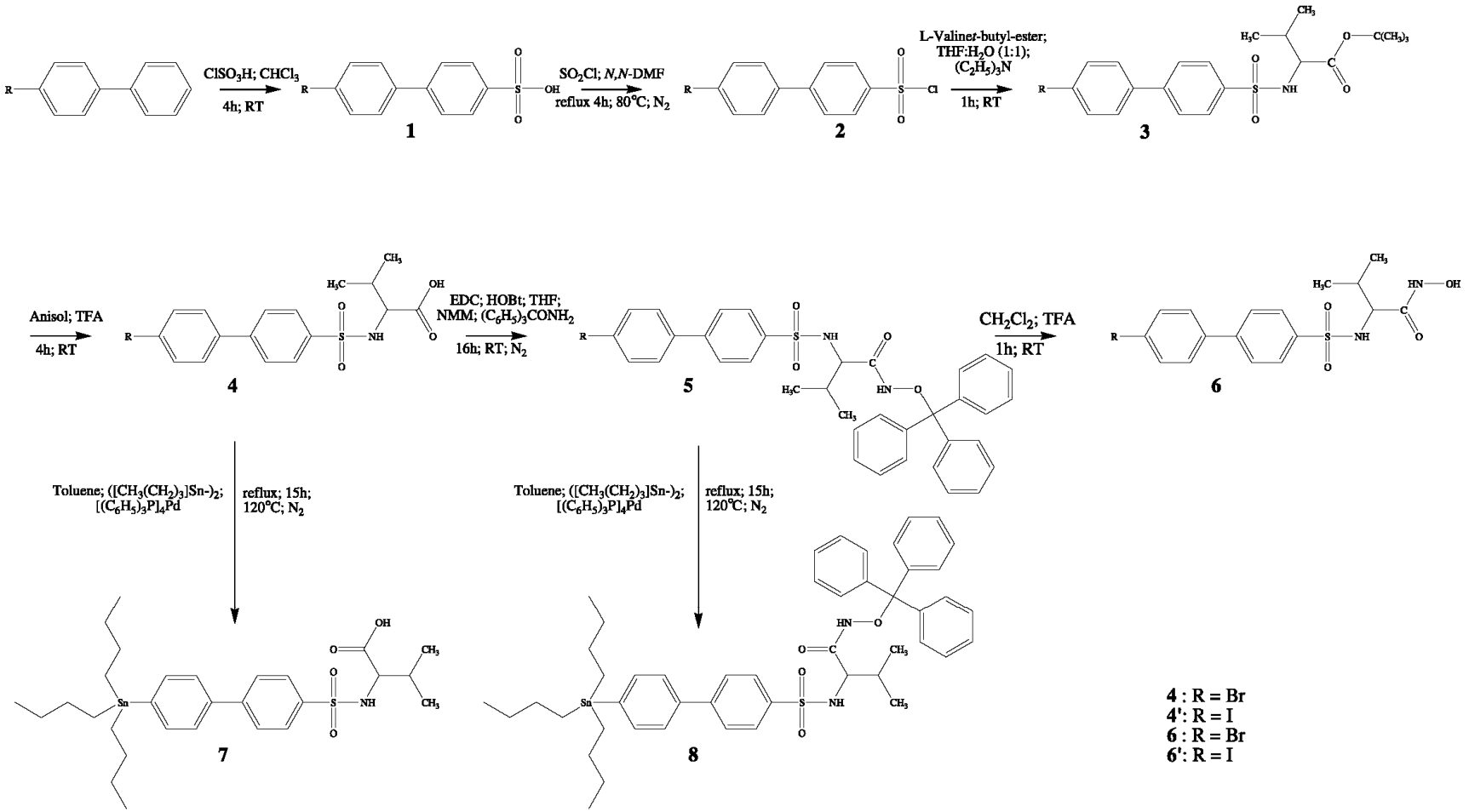
Scheme 1: *Synthesis of standards and precursors.*



3. RESULTS AND DISCUSSION

Standards and precursors were synthesized as shown in Scheme 1. The chemical yields of compounds synthesized were moderate to excellent and are detailed in the materials and methods section. Different published methods of hydroxamic acid synthesis have been described, some of these pathways were attempted but gave insufficient yields. (Tamura et al., 1998; Massa et al., 1990; Natchus et al., 2000; Scozzafava and Supuran, 2000). However, the method described by Hanessian et al. (2001) gave satisfactory results. The radio-iodination was conducted by electrophilic aromatic substitution of the tributylstannyl derivatives (Scheme 2). Mixtures were purified by HPLC on C_{18} column at a flow rate of 1 ml/min. The radiochemical yield for synthesis of radioiodinated compound 9 was 70 ± 5% (n = 6), radiochemical purity of the collected fraction >98% and the specific activity > 50 Ci/µmol. The radiochemical yield for synthesis of radioiodinated compound 11 was 60 ± 5% (n = 4), the radiochemical purity of the collected fraction was >98% and the specific activity > 58Ci/µmol.

Zymography was used to obtain a preliminary estimation of the inhibition capacities of the inhibitors on gelatinases (MMP-2 and MMP-9). The control (1% DMSO in incubation buffer) shows 2 clear bands (proMMP-2 72kDa, proMMP-9 92 kDa) against a blue background of undegraded gelatin whereas the strips incubated in inhibitor (4, 4',6, 6') concentrations (10^{-5}, 10^{-7}, 10^{-9}M) show light bands for MMP-2 at 10^{-5}M and almost no inhibition for MMP-9 (Fig. 1).

Scheme 2: *Synthesis of radiotracers.*

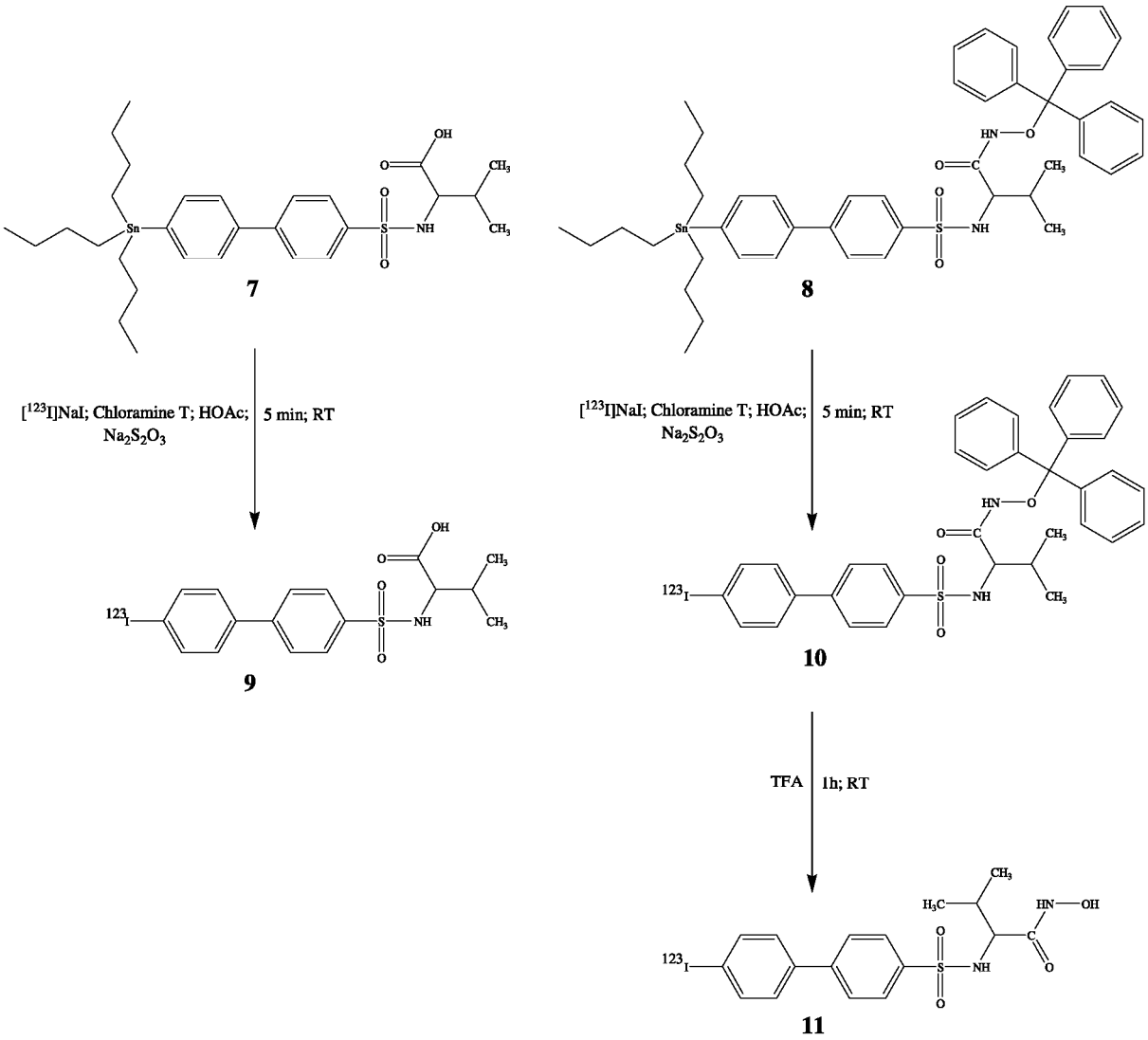


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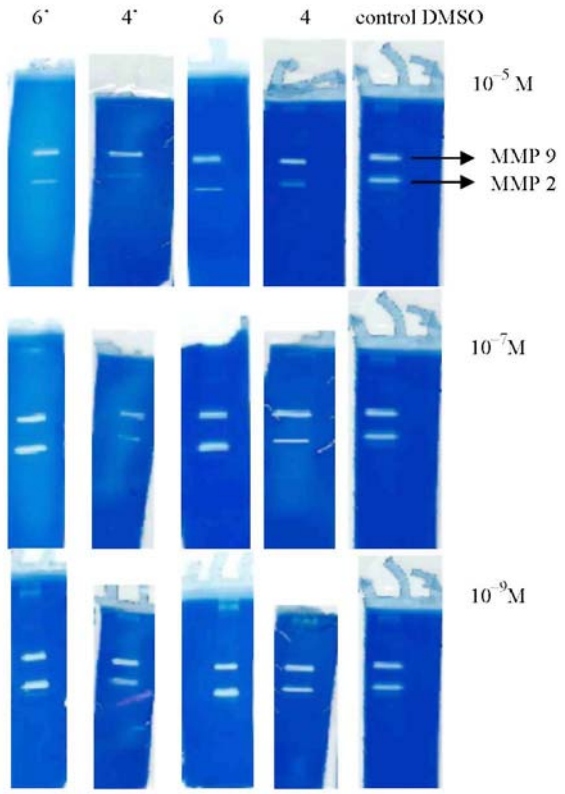
Fig. 1: *Zymogram of compounds (4, 6, 4', 6') in different concentrations (10^{-5}M; 10^{-7}M; 10^{-9}M) next to a control without inhibitor. The control was run to exclude the impact of DMSO on inhibition of gelatinases.*



In vitro enzyme assays using quenched fluorescent peptide substrates were used to determine IC_{50} values of the different compounds for MMP-2, MMP-9, cMT1 and cMT3. The results show for both hydroxamic acid and carboxylic acid compounds (4, 4', 6, 6') a good inhibition activity and a high selectivity for MMP-2 (Table 1).

To further evaluate the pharmacokinetics (dehalo-genation, metabolisation, excretion...) of 2-(4'-[^{123}I] iodo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (9) and 2-(4'-[^{123}I]iodo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (11), biodistributions were performed in NMRI mice. Approximately, 37kBq (1 μCi) of tracer was injected in the tail vein of white mice (NMRI, 20-25 g) and the concentration of radioactivity in various tissues as a function of time was evaluated (Table 2 (9)—Table 3 (11)). Compound 9 showed no long term accumulation in heart, lung and stomach (40min :<3% ID/g). Liver and kidneys showed an uptake of 16.12 ± 2.78 and 2.37 ± 0.81, respectively, at 48 h p.i.. Up to 3.4% ID/g was accumulated in the blood until 48 h p. i. Compound 11 showed a lower uptake in heart, lung, stomach, liver and kidneys (15h:<3% ID/ g). Blood activity was 2.92 ± 0.75 at 6h p.i. No dehalogenation was observed for both compounds.

Table 1: *IC_{50} values of (4, 4', 6, 6') for MMP2, MMP9, cMT1 and cMT3 are expressed in μM and averaged (n = 3), standard deviations are reported*[a]

	MMP 2	MMP 9	cMT1-MMP	CMT3-MMP
Br-COOH (4)	0.075 ± 0.013	1.079 ± 0.069	0.573 ± 0.031	0.450 ± 0.047
I-COOH (4')	0.023 ± 0.003	0.429 ± 0.036	0.180 ± 0.045	0.232 ± 0.029
Br-CONHOH (6)	0.026 ± 0.003	0.445 ± 0.036	1.242 ± 0.16	0.330 ± 0.063
I-CONHOH (6')	0.048 ± 0.002	0.740 ± 0.062	2.509 ± 0.342	0.973 ± 0.150

[a]Kinetic measurements were performed in Tris/HCl 50 mM pH 7.5, $CaCl_2$ 10 mM, Brij 35 0.05%. Details are described in Section 2.

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Table 2: *Tissue concentrations of radioactivity at various time points post i.v. administration of [^{123}I] 2-(4'-iodo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (9)*[a]

	Time (min)										Time (h)							
	0.33	0.66	1	1.5	2	3	5	10	20	40	1	2	3	6	9	15	24	48
Blood	60.45±25.30	39.62±8.95	35.73±14.02	25.87±10.21	15.49±7.90	12.77±6.40	9.06±2.07	7.40±0.51	6.13±2.82	5.31±1.00	5.32±0.62	4.84±1.25	6.39±1.38	6.77±3.32	5.52±2.16	5.62±2.11	4.67±0.70	3.40±0.11
Brain	1.29±0.31	0.82±0.37	0.83±0.34	0.64±0.31	0.36±0.17	1.69±2.56	0.22±0.09	0.20±0.03	0.17±0.10	1.62±2.60	0.18±0.06	0.14±0.02	0.14±0.03	0.50±0.60	0.19±0.08	0.19±0.06	0.39±0.48	0.12±0.06
Heart	11.36±3.89	9.45±3.44	10.65±7.16	8.35±3.40	5.95±2.91	4.80±2.55	3.81±1.04	2.77±0.11	2.64±1.38	2.46±0.97	2.52±0.23	2.05±0.67	2.98±0.57	2.64±1.27	2.49±1.07	2.54±1.21	1.98±0.05	1.52±0.27
Lungs	21.92±7.33	15.69±6.08	12.51±1.72	12.74±6.78	6.33±3.50	5.30±2.28	3.88±0.74	3.71±0.33	2.98±0.61	2.65±0.86	2.55±0.36	1.56±1.35	3.11±0.46	2.72±1.66	2.64±1.01	2.58±1.20	2.61±0.86	1.62±0.00
Stomach	0.70±0.18	0.94±0.04	0.80±0.22	0.72±0.47	1.16±1.27	0.77±0.26	0.61±0.32	0.43±0.34	0.65±0.37	0.84±0.25	0.92±0.28	1.31±0.81	2.01±1.25	2.18±1.20	2.36±0.65	0.68±0.72	1.36±1.13	0.81±0.26
Spleen	1.20±0.15	2.70±1.11	3.62±1.48	3.69±1.06	3.17±1.29	2.46±1.38	1.68±0.31	1.28±0.09	1.18±0.26	1.41±0.75	1.00±0.09	0.90±0.21	1.12±0.22	1.03±0.57	0.93±0.36	1.03±0.43	1.03±0.51	0.59±0.08
Liver	9.32±1.42	14.63±2.86	27.36±12.22	29.47±4.27	41.73±15.92	39.84±22.35	46.07±16.59	40.66±3.25	50.59±17.39	43.78±8.32	54.68±9.77	42.56±14.19	66.09±11.48	61.21±28.00	61.43±23.35	32.74±15.56	35.06±9.39	16.12±2.78
Kidneys	13.70±3.47	13.68±1.88	16.82±7.43	15.56±5.63	11.11±1.07	6.42±3.40	5.97±1.71	4.60±2.04	3.39±0.57	3.86±1.48	6.12±4.33	2.22±1.97	4.54±1.11	5.50±0.31	3.42±1.45	3.42±0.83	3.14±0.95	2.37±0.81
Small intestine	1.19±0.25	1.30±0.36	1.47±0.80	1.72±0.86	1.31±0.39	1.27±0.51	1.13±0.35	1.21±0.29	1.30±0.51	1.75±0.21	1.98±0.26	3.06±2.46	3.01±1.17	3.02±1.15	3.00±1.42	2.12±1.11	2.82±1.30	1.11±0.23
Large intestine	0.47±0.16	0.69±0.26	0.62±0.34	0.82±0.29	0.58±0.33	0.72±0.41	0.59±0.15	0.52±0.03	0.56±0.16	0.90±0.42	0.77±0.15	0.52±0.45	1.99±0.47	2.63±1.00	3.75±2.82	1.81±0.77	3.73±2.60	1.27±0.19
Bladder	1.85±0.89	2.17±0.59	2.14±0.94	2.68±1.10	2.48±1.24	13.33±21.14	2.23±0.92	2.45±0.67	2.19±0.91	2.06±0.89	2.28±1.15	1.15±0.25	1.76±0.47	2.42±1.64	2.04±1.18	1.93±1.07	2.41±1.43	1.40±0.20
Fat	2.65±0.47	1.89±0.18	4.21±2.20	2.31±1.59	3.48±2.03	4.47±3.25	1.69±0.40	1.83±0.82	1.96±0.89	4.27±5.34	2.90±2.80	0.92±0.89	3.98±3.15	1.91±1.19	1.76±0.92	2.08±1.07	1.96±1.52	0.96±0.37
Pancreas	3.84±1.52	3.02±0.45	3.85±1.88	4.14±1.80	3.52±1.22	2.96±1.23	4.89±4.27	3.39±2.65	2.02±0.40	2.02±1.24	1.19±0.44	1.14±0.99	1.83±1.63	1.92±0.77	1.59±0.08	1.65±0.89	2.34±1.72	0.70±0.28

[a]Animals were injected intravenously with 37kBq [^{123}I] 2-(4'-iodo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (9) and sacrificed at designated times. Units are expressed as % injected dose/g of tissue *(n* = 3) corrected for background radiation and averaged. Below are reported the standard deviations.

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Table 3: *Tissue concentrations of radioactivity at various time points post i.v. administration of [^{123}I] 2-(4'-iodo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (11)*[a]

	Time (min)										Time (h)							
	0.33	0.66	1	1.5	2	3	5	10	20	40	1	2	3	6	9	15	24	48
Blood	27.80±2.22	14.72±2.05	7.50±2.49	6.14±0.42	4.84±0.66	4.58±0.61	4.81±0.47	4.91± 0.42	5.02±0.26	4.92±0.28	5.19±0.60	4.57±0.27	5.17±1.79	2.92±0.75	1.94±0.74	0.86±0.51	0.73±0.36	0.80±0.79
Brain	1.00±0.17	3.09±4.36	0.42±0.28	0.33±0.02	0.51± 0.42	0.29±0.06	0.73±0.65	0.56±0.07	0.99±0.12	1.18±0.05	1.96±0.67	1.24±0.08	1.37±0.45	0.76±0.12	0.53±0.18	0.18±0.10	0.11±0.06	0.23±0.33
Heart	19.68±0.77	16.17±1.73	10.72±1.45	14.01±4.07	12.18±2.62	11.09±1.78	11.51±2.23	8.82±1.65	6.16±1.11	3.47±0.38	3.31±0.51	2.33±0.32	2.67± 1.12	1.94±0.55	1.80±0.53	0.99±0.58	0.81±0.66	0.36±0.10
Lungs	43.89±2.24	30.03±6.37	18.83±5.69	19.45±2.52	16.00±2.29	12.70±1.26	9.93±0.22	7.70±1.41	5.32±0.79	3.50±0.38	3.75±0.74	2.82±0.28	3.07±0.71	2.40±0.69	2.29±0.44	1.12±0.64	0.88±0.70	0.36±0.10
Stomach	0.65±0.37	2.10±0.55	2.08±0.04	2.14±0.54	3.07±1.43	2.40±0.28	4.32± 1.88	2.39±0.71	4.24±1.46	3.56±0.96	1.51±0.56	3.27±1.54	3.59±1.39	2.41± 0.92	2.40±1.56	0.59±0.36	0.75±0.53	0.18±0.08
Spleen	0.74±0.72	7.23±4.63	5.95±0.52	7.49±0.72	5.17±0.79	4.45±0.81	4.38± 1.39	5.14±0.76	3.96±1.70	2.66±0.50	3.73±0.63	2.26±0.14	2.62±1.02	1.33±0.20	1.05±0.09	0.68±0.37	0.49±0.38	0.45±0.43
Liver	3.49±2.37	13.39±0.90	17.58±2.40	23.25±3.50	24.62±7.49	21.77±2.32	22.86±2.41	16.69±1.61	13.57±1.62	10.71±0.91	8.10±1.61	8.22±0.52	11.92±5.92	8.54±2.73	7.14±3.33	2.62±1.01	2.77±1.35	1.30±0.78
Kidneys	10.55±8.21	24.27±6.67	26.78±5.57	28.44±2.72	23.32±2.22	21.87±2.38	16.47±1.20	12.72±1.47	9.80±1.31	9.20±2.21	6.81±0.98	7.80±2.68	11.67±6.64	7.42±2.06	5.72±2.11	1.89±0.71	1.39±0.80	0.52±0.17
Small intestine	0.76±0.71	2.05±0.56	2.24±0.55	3.15±0.14	2.59±0.25	2.90±0.54	3.69±0.81	6.24±2.72	10.66±4.67	17.36±3.21	24.79±6.32	14.55±1.35	11.64±3.03	4.05±0.39	2.58±0.89	1.19±0.69	0.98±0.73	0.29±0.08
Large intestine	0.29±0.32	1.34±0.63	0.69±0.22	1.09±0.15	1.07±0.07	1.11±0.31	1.35±0.33	1.47±0.53	1.88±0.65	1.65±0.21	2.13±0.72	13.57±4.17	42.93±24.59	15.43±3.54	6.98±3.40	2.07±1.71	1.57±1.08	0.63±0.46
Bladder	1.86±1.13	11.87±17.42	1.44±0.37	3.32±2.58	2.44±1.74	2.25±0.96	4.34±3.72	2.74± 1.05	3.35±0.78	3.10±0.28	4.04±1.78	6.77± 1.88	9.97±12.72	6.03±5.68	7.63±9.27	1.18±1.03	0.88±0.28	0.79±1.11
Fat	2.29±1.80	2.71±1.94	3.45±2.32	3.13±1.66	2.61±1.48	3.11±1.59	5.06±2.84	3.45±0.23	3.46±1.81	2.20±0.21	2.72±1.44	1.90±0.41	2.38±0.83	1.55±0.31	1.65±0.45	0.78±0.40	0.52±0.28	0.50±0.86
Pancreas	2.48±2.31	6.32±3.75	3.69±0.69	6.75±0.71	5.57±0.94	6.66±1.79	7.33±0.48	8.39±1.31	7.11±1.14	3.86±0.24	3.04±0.50	2.28±0.43	2.46±1.04	1.84±0.64	1.93±0.58	0.97±0.47	0.89±0.70	0.60±0.44

[a]Animals were injected intravenously with 37kBq [^{123}I] 2-(4'-iodo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (11) and sacrificed at designated times. Units are expressed as % injected dose/g of tissue *(n* = 3) corrected for background radiation and averaged. Below are reported the standard deviations.

4. CONCLUSION

Radioiodinated carboxylic and hydroxamic MMP inhibitors 2-(4'-[^{123}I] iodo-biphenyl-4-sulphonylamino)-3-methyl-butyric acid (9) and 2-(4'-[^{123}I] iodo-biphenyl-4-sulphonylamino)-3-methyl-butyramide (11), their unlabeled standards and precursors were synthesized in good yield. In vitro zymography and enzyme assays showed for both hydroxamic acid and carboxylic acid compounds a good inhibition activity and a high selectivity for MMP-2. In vivo biodistribution showed no long-term accumulation in organs and blood activities, which give the possibility to accumulate in the tumor.

These data suggest that these may be potential useful agents for non-invasive monitoring of enhanced tumor MMP levels in vivo and thus diagnosis of primary and secondary tumors and tumor response to MMP inhibitor therapy using SPECT. These results also warrant further evaluation of tumor uptake in tumor-bearing athymic mice and metabolite studies of the radioiodinated MMP inhibitors.

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