Design, (radio)synthesis and applications of radiolabelled matrix metalloproteinase inhibitors for PET
Matusiak, Nathalie

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 14-10-2017
Development and preclinical comparison of two non-peptidomimetic MMP/ADAM inhibitors for PET

Nathalie Matusiak,1 Aren van Waarde,1 Rosalina Wisastra,2 Frank J. Dekker,2 Laurette M. Prély3, Rainer Bischoff,3 Rudi A.J.O Dierckx,1 Philip H. Elsinga1

1 Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2 Department of Pharmaceutical Gene Modulation, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands
3 Department of Analytical Biochemistry, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands

This chapter was submitted for publication
Abstract

Background: Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) are zinc-dependent endopeptidases involved in the degradation of specific protein components of the extracellular matrix (ECM). Several endogenous inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), regulate MMP/ADAM activity. The upregulation of MMP/ADAM activity is an important aspect of the pathophysiology of several diseases. Therefore, the ability to visualize the net balance of MMPs/ADAMs not occupied by TIMPs, would be clinically relevant as it might indicate the proteolytic activity of MMPs and ADAMs in the ECM. Here, we report the design, (radio)synthesis and evaluation of two members of a novel class of hydroxamate-based MMP/ADAM inhibitors: 1-((4-[18F]fluorophenyl)sulfonyl)-N-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide ([18F]-1A) and 4-([1,1'-biphenyl]-4-carbonyl)-1-((4-[18F]fluorophenyl)sulfonyl)-N-hydroxypiperazine-2-carboxamide ([18F]-2) as PET probes for active MMPs/ADAMs.

Materials and methods: Two broad-spectrum piperazine-based MMP/ADAM inhibitors were designed by molecular modeling. Fluoro containing inhibitors 1A and 2 and their nitro analogues were prepared from the dl-piperazine carboxylic acid using multi-step organic synthesis. The inhibitory potencies of 1A and 2 were examined in fluorogenic in vitro inhibition assays for MMP-9, -12 and ADAM-17. The non-peptidomimetic radiotracers [18F]-1A and [18F]-2 were prepared by aromatic nucleophilic substitution of their nitro-precursors and were evaluated in a HT1080 xenograft mouse model.

Results: Molecular modeling indicated a good binding pose of 1A and 2 to the MMP-9 active site. 1A and 2 and their corresponding nitro analogues were obtained with overall yields of 24, 25, 22 and 26%, respectively. The IC50 values for MMP-9, -12 and ADAM-17 were 14.5 ± 2.57, 19.3 ± 4.96 and 620 ± 89.1 nM (for 1A) and 9.19 ± 2.07, 1.12 ± 1.08 and 10.6 ± 0.91 nM (for 2). After high-performance liquid chromatography (HPLC) purification, the radiochemical yields of [18F]-1A and [18F]-2 ranged 1 to 3% based on 18F-fluorine (corrected for decay). The specific activity was 34-78 GBq/µmol at the end of synthesis for [18F]-1A and 42-86 GBq/µmol for [18F]-2. The log P values of [18F]-1A and [18F]-2 were 0.89 ± 0.03 and 2.92 ± 0.06. [18F]-1A and [18F]-2 exhibited low tumor uptake in microPET. Their SUVmean values were 0.14 ± 0.04 (n = 6) and 0.15 ± 0.03 (n = 6) under baseline conditions and were 0.11 ± 0.04 (n = 6) and 0.12 ± 0.02 (n = 6) after co-injection of 2.5 mg/kg.
of the corresponding non-radioactive reference compound. Injected $[^{18}\text{F}]$-1A and $[^{18}\text{F}]$-2 were rapidly excreted through the kidneys and the liver.

**Conclusion:** Molecular modeling resulted in good candidates for MMP-9 targeting greatly differing in lipophilicity. The multi-step organic synthesis of the fluoro-containing inhibitors and the nitro-precursors resulted in good overall yields. 1A and 2 showed good affinities *in vitro* against MMP-9, -12 and ADAM-17. The synthesis of $[^{18}\text{F}]$-1A and $[^{18}\text{F}]$-2 led to low and variable radiochemical yields. These novel PET tracers exhibited low target-to-non-target ratios in a HT1080 xenograft mouse model, which may be due to rapid tracer elimination or low expression levels of active MMPs/ADAMs.

**Keywords**
Hydroxamate, MMP/ADAM inhibitors, PET, $S_{\text{N}}\text{Ar}$
1. Introduction

Proteases are enzymes which are essential in tumor formation and metastatic spread. They are subdivided into four classes: cysteine-, aspartyl-, serine- and metallo-proteinases [1]. About 35% of the proteinases identified so far in the sequenced human genome correspond to metalloproteinases [2]. Among the metalloproteinases, the zinc metalloproteinases, particularly the matrix metalloproteinases (MMPs) and the a disintegrin and metalloproteinases (ADAMs), attracted much consideration as significant targets for a large range of therapeutic applications in cancer [3, 4]. Therefore, synthetic inhibitors were developed to study their biological functions [5, 6].

Matrix metalloproteinase inhibitors (MMPIs) are generally classified into two groups: peptidomimetic inhibitors and non-peptidomimetic inhibitors [7]. The latter demonstrated greater specificity due to their structure-based design, which is characterized by the three-dimensional structure of the MMP active site. These inhibitors bind in a non-covalent mode and they all contain a sulfonyl group which affords hydrogen bonding with the enzymes [8]. We designed two imaging agents based on a novel class of compounds whereby their synthetic approach allowed easy tuning of lipophilicity. These novel imaging agents were the piperazine-based MMP/ADAM inhibitors (1-((4-fluorophenyl)sulfonyl)-N-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide), 1A and (4-((1,1'-biphenyl)-4-carbonyl)-1-((4-fluorophenyl)sulfonyl)-N-hydroxypiperazine-2-carboxamide), 2 [Fig 1] which target particularly MMP-9. These inhibitors have a hydroxamic acid as a zinc binding group, a fluorophenylsulphonamide group to fill the S1’ pocket of the enzymes and either a methanesulfonamide for 1A or a biphenylamide for 2 at the 4-N-position of the piperazine ring to optimize potency and vary the lipophilicity. Each of these

![Figure 1: Structure of 1-((4-fluorophenyl)sulfonyl)-N-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide 1A and 4-((1,1'-biphenyl)-4-carbonyl)-1-((4-fluorophenyl)sulfonyl)-N-hydroxypiperazine-2-carboxamide 2](image-url)
inhibitors was synthesized in a multi-step organic synthesis from the \( dl \)-piperazine carboxylic acid and the inhibitory potencies of 1A and 2 were examined in fluoro-meric in vitro inhibition assays for recombinant human MMP-9, -12 and ADAM-17.

We prepared the \([18F]\)fluoro analogues of 1A and 2 by homoaromatic nucleophilic substitution (\([18F]-S_{N}Ar\)) of their corresponding nitro-precursors. Quality control and stability tests in saline and plasma of the obtained PET-tracers were carried out. Finally, \([18F]-1A\) and \([18F]-2\) were evaluated in vivo in mice bearing HT1080 tumor.

2. Results and discussion

2.1 Design of the piperazine-based MMP/ADAM inhibitors 1A and 2

In order to evaluate the effect of probe lipophilicity on MMP/ADAM imaging, we selected two radiolabelled MMP/ADAM inhibitors with different lipophilicities. Cheng et al. [9] developed a library of piperazine-based MMP inhibitors among which the \( N \)-hydroxy-1-\((4\text{-methoxyphenyl})\text{sulfonyl}\)-4-(methylsulfonyl)piperazine-2-carboxamide 3 and 4-\((1,1'\text{-biphenyl})\text{-4-carbonyl}\)-\( N \)-hydroxy-1-\((4\text{-methoxyphenyl})\) sulfonyl)piperazine-2-carboxamide 4 [Fig 2]. 3 and 4 have nanomolar affinities for many matrix metalloproteinases, particularly MMP-9 (IC\(_{50}\) = 0.6 nM for 3 and 0.9 nM for 4), and exhibit different lipophilicities (clog P = 0.24 for 3 and 3.29 for 4). In the design of a MMPI, one of the requirements is to have a functional group which provides a hydrogen bond interaction with the enzyme backbone. The substitution of the methoxy group with a fluoro atom (fluorine-18) to give the radiolabelled inhibitors \([18F]-1A\) and \([18F]-2\) [Fig 1] could result in increased interaction with active MMPs/ADAMs, as a fluoro atom can be considered as a hydrogen substitute [10]. Moreover, the considered radiolabelling procedure was direct incorporation of fluorine-18 by \([18F]-S_{N}Ar\) rather than the use of radiolabelled prosthetic groups. Finally, due to its low positron energy, \(^{18}\text{F}\) gives images with higher resolution than \(^{11}\text{C}\).

2.2 Molecular modeling of the piperazine-based MMP/ADAM inhibitors 1A, 2, 3 and 4

Modeling interaction of inhibitors 1A, 2, 3 and 4 with MMP-9 was performed by using Molegro Molecular Viewer. The calculated log P (clog P) and the MolDock Score are shown in Table 1.
Chapter 5 - Figure 2

Figure 2: Structures of \(N\)-hydroxy-1-((4-methoxyphenyl)sulfonyl)-4-(methylsulfonyl)piperazine-2-carboxamide 3 and 4-[[1,1'-biphenyl]-4-carbonyl]-\(N\)-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide 4

Chapter 5 – Scheme 1

Scheme 1: Synthesis of (1-((4-fluorophenyl)sulfonyl)-\(N\)-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide (1), (4-[[1,1'-biphenyl]-4-carbonyl]-1-((4-fluorophenyl)sulfonyl)-\(N\)-hydroxy-piperazine-2-carboxamide (2), \(N\)-hydroxy-4-(methylsulfonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide (11) and 4-[[1,1'-biphenyl]-4-carbonyl]-\(N\)-hydroxy-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide (12).

(a) 1.1 eq. (t-Boc)\(_2\)O, 3 eq. NaOH, dioxane-water; (b) 1 eq. p-XPhSO\(_2\)Cl (X = F or NO\(_2\)), Et\(_3\)N/cat. DMAP; (c) SOCl\(_2\), MeOH; (d1) 1.3 eq. MeSO\(_2\)Cl, Et\(_3\)N/cat. DMAP; (d2) 1.3 eq. PhPhCOCl, Et\(_3\)N/cat. DMAP; (e) NH\(_2\)OH in KOH and MeOH; (f) 1.1 eq. EDC, 1.3 eq. HOBT, 6.3 eq. NMM, 2 eq. tert-BuONH\(_2\), DCM; (g) TFA, DCM

Chapter 5 - Figure 3

Figure 3: In vivo \[^{18}\text{F}\]-1A/\[^{18}\text{F}\]-2 (left/right) microPET image of a HT1080 tumor bearing mouse shown in coronal view. The microPET image corresponds to the sum of all the frames from 2 to 90 min p.i. of \[^{18}\text{F}\]-1A (or \[^{18}\text{F}\]-2). The position of the tumor is indicated by the crosshair.

Figure 4: Time-activity curve of a tumor-bearing mouse scanned with \[^{18}\text{F}\]-1A (or \[^{18}\text{F}\]-2) and a tumor-bearing mouse scanned with \[^{18}\text{F}\]-1A (or \[^{18}\text{F}\]-2) and co-injection of 2.5 mg/kg of 1A (or 2), from 2 to 90 min p.i. of \[^{18}\text{F}\]-1A ± 1A (or \[^{18}\text{F}\]-2 ± 2). Note that the shape of the curve is not altered by pretreatment for \[^{18}\text{F}\]-1A
The MolDock Score is expressed as the fitness of pose into the binding site by evaluating the intermolecular interaction energy between the ligand and the enzyme, and the intramolecular interaction energy of the enzyme. The docking results of the reported inhibitors to MMP-9 show alignment to the molecule design concept. The substituent construct on 1A, 2, 3 and 4, which are P1’ and P2’, correspond with MMP-9 pockets S1’ and S2’. The hydroxamic acid group was in the proper position to coordinate the Zn²⁺ atom.

In the case of the inhibitors 1A and 3, substitution of the methoxy group by a fluoro atom does not affect the interaction with MMP-9, as suggested by very similar MolDock scores of the original and substituted compounds. However, for the inhibitors 2 and 4, incorporation of the fluoro atom led to significantly improved molecular docking to MMP-9.

2.3 Synthesis of the non-radioactive MMP/ADAM inhibitors 1A and 2

The MMP/ADAM inhibitors 1A and 2 were prepared as HPLC standards and for in vitro fluorogenic inhibition assays. 1A and 2 [Scheme 1] were prepared in five-steps from the commercially available dl-piperazine carboxylic acid 5, according to the procedure of Cheng et al. [9] First, 5 was monoprotected with 1.1 eq. of di-tert-butyl dicarbonate to give the monoamine 6. Addition of para-fluorophenylsulfonyl chloride to the crude amine 6 led to the sulfonamide 7, in an overall yield of 82%. Treatment with methanolic HCl to 7 produced the intermediate 8, obtained in a yield of 84%. 8 was thereafter acylated either with methanesulfonyl chloride or biphenyl-4-carbonyl chloride to result in the substituted piperazine derivatives 9 and 10, with yields of 74% and 81%, respectively. Finally, the incorporation of the hydroxamic acid was performed with hydroxylamine in KOH-MeOH to give the inhibitors 1A and 2 with yields of 47 and 45%, respectively. The overall yields of the synthesis of 1A and 2 were 24 and 25%.

<table>
<thead>
<tr>
<th>MMP/ADAM inhibitor</th>
<th>Mol Dock Score</th>
<th>clog P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-64.0</td>
<td>0.24</td>
</tr>
<tr>
<td>1A</td>
<td>-63.2</td>
<td>0.22</td>
</tr>
<tr>
<td>4</td>
<td>-52.1</td>
<td>3.29</td>
</tr>
<tr>
<td>2</td>
<td>-119.3</td>
<td>3.26</td>
</tr>
</tbody>
</table>

Table 1: Mol Dock Scores and clog P of the MMP/ADAM inhibitors 3, 4, 1A and 2
2.4 Synthesis of the nitro precursors 11 and 12

The synthesis of the nitro precursors N-hydroxy-4-(methylsulfonyl)-1-((4-nitropheno)yl)sulfonyl)piperazine-2-carboxamide 11 and 4-((1,1’-biphenyl)-4-carbony1)-N-hydroxy-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide 12 for the \(^{18}\text{F}\)-radiolabellings was prepared as their fluoro analogues for the first four steps [Scheme 1]. Monoprotection of dl-piperazine carboxylic acid 5 with 1.1 eq. of di-tert-butyl dicarbonate produced the monoamine 6. Addition of paranitrophenyloxon chloride to the crude amine 6 led to the sulfonamide 13, in an overall yield of 73%. Thereafter, 13 was treated with methanolic HCl to give the intermediate 14, obtained in a yield of 86%. 14 was subsequently coupled either with methanesulfonyl chloride or biphenyl-4-carbonyl chloride to afford the substituted piperazine derivatives 15 and 16, with yields of 61% and 66%, respectively.

The incorporation of the hydroxamic acid with hydroxylamine in KOH-MeOH was not successful and another approach was adopted. The nitro moiety appeared to be unstable under basic conditions, as was reported by Fei et al. [11]. Therefore, an alternative approach by inserting the hydroxamic acid in acidic conditions was at-

---

**Scheme 1:** Synthesis of (1-((4-fluorophenyl)sulfonyl)-N-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide) (1A), (4-((1,1’-biphenyl)-4-carbonyl)-1-((4-fluorophenyl)sulfonyl)-N-hydroxypiperazine-2-carboxamide) (2), N-hydroxy-4-(methylsulfonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide (11) and 4-((1,1’-biphenyl)-4-carbonyl)-N-hydroxy-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide (12)

(a) 1.1 eq. (t-Boc)_2O, 3 eq. NaOH, dioxane-water; (b) 1 eq. p-XPhSO_2Cl (X = F or NO_2), Et_3N/cat. DMAP; (c) SOCl_2, MeOH; (d1) 1.3 eq. MeSO_2Cl, Et_3N/cat. DMAP; (d2) 1.3 eq. PhPhCOCl, Et_3N/cat. DMAP; (e) NH_2OH in KOH and MeOH; (f) 1.1 eq. EDC, 1.3 eq. HOBT, 6.3 eq. NMM, 2 eq. tert-BuONH, DCM; (g) TFA, DCM
tempted. The methyl esters 15 and 16 were hydrolyzed to their corresponding carboxylic acids, followed by coupling with 0-tert-butylhydroxylamine hydrochloride in the presence of N-[(dimethylamino)propyl]-N-ethylcarbobimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBT) and N-methylmorphorine (NMM). The protected hydroxamic acids 17 and 18 were obtained with yields of 64 and 67%, respectively. Finally, the hydroxamic acids were obtained by deprotection with trifluoroacetic acid in dichloromethane, with yields of 89 and 82%. The overall yields of the synthesis of 11 and 12 were 22 and 26%.

2.5 In vitro evaluation of 1A and 2 in a fluorogenic inhibition assay

The affinities of 1A and 2 were determined in fluorogenic inhibition assays against three selected recombinant human metzincins: MMP-9 (gelatinase B, 92 kDa type IV collagenase), MMP-12 (macrophage metalloelastase) and ADAM-17 (TNF-alpha converting enzyme). The broad-spectrum inhibitor 1A exhibited affinities in the low nanomolar range for both MMP-9 and MMP-12; a higher IC<sub>50</sub> value was determined for ADAM-17. The IC<sub>50</sub> values were 14.5 ± 2.6, 19.3 ± 5.0 and 620 ± 89 nM, respectively. Inhibitor 2 showed higher affinities for MMP-9, -12 and ADAM-17, compared to 1A. The IC<sub>50</sub> values obtained for 2 were respectively 9.2 ± 2.1, 1.1 ± 1.1 and 10.6 ± 0.9 nM. It can be noted that the introduction of a lipophilic moiety at the 4-<i>N</i>-position of the piperazine ring, induces a considerable increase of affinity for ADAM-17. This was already indicated by the molecular modeling.

2.6 Radiosynthesis of [18F]-1A and [18F]-2

The radiosynthesis of [18F]-1A and [18F]-2 was performed in one-step by [18F]-SNAr of the corresponding nitro-precursors 11 and 12. The radiochemical yields (RCYs) of HPLC-purified [18F]-1A and [18F]-2 ranged from 1 to 3 % based on 18F-fluorine (corrected for decay). The specific activity was 34-78 GBq/μmol at the end of synthesis for [18F]-1A and 42-86 GBq/μmol for [18F]-2. The radiosynthesis of [18F]-1A and [18F]-2 led to very low RCYs and not reliable radiosynthesis. Using either microfluidics or microwave synthesis approaches did not improve RCYs. The low RCYs obtained may be attributed to the weak para sulfonyl activating group.

The radiochemical purity of [18F]-1A and [18F]-2 was always > 95% (measured values). Total synthesis times of [18F]-1A and [18F]-2 were about 90 min from end of bombardment.
2.7 In vitro stability of [18F]-1A and [18F]-2 in saline and human plasma

After 1 h and 3 h of incubation, 95% of the radioactivity still corresponded to the intact tracer either in saline or human plasma. This indicates that [18F]-1A and [18F]-2 are highly stable in vitro.

2.8 Octanol/water partition coefficient

The log P of [18F]-1A was 0.89 ± 0.03, indicating moderate lipophilicity of this tracer. The log P of [18F]-2 was 2.92 ± 0.06, indicating that this probe is more lipophilic than [18F]-1A.

2.9 Preclinical investigation of [18F]-1A and [18F]-2 in a HT1080 xenograft mouse model

[18F]-1A and [18F]-2 were evaluated in a HT1080 xenograft mouse model. This animal model of cancer is overexpressing many matrix metalloproteinases and was already used for testing various biomarkers for imaging of MMP/ADAM proteolytic activity [12-15].

After administration of [18F]-1A and [18F]-2, the microPET images [Fig 3] demonstrated low levels of radioactivity in the tumors. For both tracers, the uptake throughout the tumor volume was homogeneous, which indicates that tracer binding was not only to membrane-bound ADAMs but also to extracellular MMPs. In addition, for both radioligands, a high liver and kidney uptake was observed. The small and large intestines retained also much radioactivity. The time-activity curves of [18F]-1A and [18F]-2, in one control mouse and one mouse pretreated with blocking agent showed a slow wash-out of radioactivity from the tumor of both animals [Fig 4]. Based on these time courses it was determined that biodistribution at 90 min post injection was a good time point.

Biodistribution data (SUV mean as mean ± SD) for [18F]-1A are reported in [Fig 5]. Uptake of the radioligand in the tumor was not significantly decreased after co-injection of non-radioactive 1A (from a SUV mean of 0.14 ± 0.04 to 0.11 ± 0.04 at 90 min p.i.). A low uptake in bone was observed which indicates low defluorination of the tracer over the duration of the scan. High uptake of radioactivity in the kidneys and to a lesser extent in the liver at 90 min p.i. was observed. This is most likely due to excretion of the radiotracer and radiometabolites. The amount of activity (i.e. tracer and metabolites) excreted by the liver in the small and large intestines was very high, considering the enormous uptake obtained in the small and large
intestines. No significant differences in the SUV<sub>mean</sub> were noted between control and pretreated mice in other organs than tumor.

Biodistribution data for [18F]-2 are reported in [Fig 5]. Although the lipophilicity was much higher for [18F]-2 than for [18F]-1A, biodistribution data were quite similar. In the past decades, several synthetic MMPIs have been developed and evaluated [7]. Most of these probes exhibited excellent in vitro data but disappointing in vivo results. Only the radiolabelled peptidic MMPIs Marimastat-ArB[18F]F<sub>3</sub> [16] and [18F]FB-ML5 [17] have shown specific accumulation in tumors. Our newly developed [18F]-1A and [18F]-2 - belonging to a novel class of piperazine-based imaging agents – were also not successful in imaging active MMP in a HT1080 xenograft.

Since specific tracer accumulation is dependent on the ratio of target density (B<sub>max</sub>) and affinity of the probe for its target (K<sub>d</sub>), the affinity of [18F]-1A and [18F]-2 may have been not sufficient in consideration of the B<sub>max</sub> for MMP/ADAM imaging. Expression levels of active MMPs may have been too low for PET-labelled MMP-inhibitors with affinities in the 10<sup>-8</sup> M range to act as suitable imaging agents. The increased lipophilicity of [18F]-2 as compared to [18F]-1A did not result in increased tumor uptake. Greater lipophilicity could have resulted in better penetration of the probe into tumors. The specific radioactivities of [18F]-1A and [18F]-2 may have

![Figure 5: Ex vivo biodistribution data of tumor-bearing mice injected with [18F]-1A ([18F]-2) and tumor-bearing mice injected with [18F]-1A (or [18F]-2) and co-injection of 2.5 mg/kg of 1A (or 2), at 90 min p.i. of [18F]-1A ± 1A (or [18F]-2 ± 2).
Bars represent average and error bars SD, n= 6 for each group](image-url)
been too low for MMP/ADAM imaging as natural TIMPs bind to the same domain as MMPIs with a very high affinity in the picomolar range, and in an irreversible manner [18]. Thus, the competition of TIMPs with radioactive probes for MMP/ADAM binding will probably be quite severe. The use of radiolabelled substrates (as opposed to radiolabeled inhibitors) could lead to better results because substrates can be expected to show signal amplification.

In addition, in order to design a more suitable probe for MMP/ADAM imaging, the use of alternative zinc binding group may be attempted to overcome the rapid elimination problem. Finally, in order to increase the subtype specificity of an imaging probe, investigators may try to target a specific MMP by using antibody based recombinant protein.

3. Conclusion and future perspectives

Two novel MMP/ADAM inhibitors 1A and 2 with different lipophilicities were prepared with satisfactory overall yields. 1A and 2 exhibited good affinities against MMP-9, -12 and ADAM-17 in vitro. [18F]-1A and [18F]-2 were radiolabelled by aromatic nucleophilic substitution of their corresponding nitro-precursors with very low RCYs and poor reliability. These novel [18F]-PET tracers exhibited low uptake in a HT1080 xenograft mouse model which was not reduced after pretreatment with cold compound. Thus, they cannot be considered as potential probes for imaging MMP/ADAM activity.

4. Materials and methods

4.1 General.

All chemicals, reagents, and solvents for the (radio)synthesis of the compounds were analytical grade, purchased from commercial suppliers (Aldrich, Fluka, Sigma and Merck) and were used without further purification, unless otherwise specified. Solid phase extraction cartridges were obtained from Waters Chromatography Division, Millipore Corporation. Flash chromatography was performed on silica gel 60 (0.040-0.063, Merck). All reactions were monitored by thin layer chromatography on Merck F-254 silica gel plates using solvent mixture of ethyl acetate (EtOAc) and methanol (MeOH). Detection of the compounds on the TLC plates was performed with UV light (254 nm). 1H-NMR spectra were recorded on a Varian AMX400 spectrometer (400 MHz). Chemical shifts were determined relative to the signal of the solvent, converted to the TMS (tetramethylsilane) scale, and expressed in δ.
units (ppm) downfield from TMS (for DMSO-d$_6$: $\delta$ 2.504 for $^1$H). Data are reported as follows: chemical shifts, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, dd=doublet of doublets, dt=doublet of triplets, td=triplet of doublets, m=multiplet, br=broad), coupling constants (Hz), and integration. Mass spectrometry was recorded on an AEI-MS-902 mass spectrometer by EI (70 eV) measurements. Radioactivity measurements for log P determination, saline/plasma stability, biodistribution and metabolite were performed using an automated gammacounter (LKB Wallac, Turku, Finland).

4.2 Statistical analysis

Calculations were performed using Excel 2007 (Microsoft) and GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, USA). Results are expressed as mean ± SD. Comparisons between different experimental groups were made using unpaired two-sided student t-test. Data were considered statistically significant when p values were smaller than 0.05.

4.3 Molecular modeling of the piperazine-based MMP/ADAM inhibitors 1A, 2, 3 and 4

The crystal structure of MMP-9 was downloaded from the Protein Data Bank (PDB) (PDB code 2OW1). All molecules were drawn using Chemaxon MarvinSketch (www.chemaxon.com) and prepared (structure recognition and protonation) using SPORES (www.tcd.uni-konstanz.de/research/spores.php). Molecular docking simulations were performed using PLANTS v1.6.140,141. The docking site center was determined by applying a constraint for the hydroxamic group to be able to form a coordination with the zinc in the active site. Fifteen poses were generated for each compound and the docking results were analyzed using Molegro Virtual Docker (www.molegro.com). Docking solutions were selected based on the MOLDOCKSCORE and the docking solutions were evaluated manually, followed by energy minimization of the ligand.

4.4 Synthesis of 1A, 2, 11 and 12

4-(Tert-butoxycarbonyl)-1-((4-fluorophenyl)sulfonyl)piperazine-2-carboxylic acid (7)

An aqueous solution of sodium hydroxide (4.6 mL, 50% w/w, 19.4 N, 88.6 mmol) was slowly added to a solution of dl-piperazine carboxylic acid dihydrochloride 5
(5.99 g, 29.5 mmol) in 60 mL of p-dioxane and 30 mL of water at 0°C. Addition of di-tert-butyl dicarbonate (7.09 g, 32.5 mmol) was followed and the mixture was stirred overnight at room temperature yielding 6. Freshly distilled triethylamine (8.23 mL, 59.0 mmol), 4-dimethylaminopyridine (721 mg, 5.90 mmol) and 4-fluorophenylsulfonyl chloride (5.74 g, 29.5 mmol) were added to the mixture at 0°C and the reaction was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and 1 N HCl. The EtOAc layer (2 x 100 mL) was washed with brine, dried over Na₂SO₄, filtered, and concentrated in vacuo to give the title compound 7 (9.40 g, 82%):

1H NMR (400 MHz, DMSO-d₆) δ 12.07 (s, 1H), 7.80 – 7.71 (m, 2H), 7.21 – 7.11 (m, 2H), 4.09 – 3.95 (m, 2H), 3.87 (dt, J = 12.5, 4.9 Hz, 1H), 3.53 (dt, J = 12.2, 4.8 Hz, 1H), 3.38 – 3.22 (m, 2H), 3.17 (dt, J = 12.2, 4.8 Hz, 1H), 1.37 (s, 9H).

HRMS-ESI: calc for C₁₆H₂₂FN₂O₆S ([M + H]+), 389.4, found 389.1.

Methyl 1-((4-fluorophenyl)sulfonyl)piperazine-2-carboxylate hydrochloride (8)

Thionyl chloride (7.98 mL, 110 mmol) was added dropwise to a solution of 4-(tert-butoxycarbonyl)-1-((4-fluorophenyl)sulfonyl)piperazine-2-carboxylic acid 7 (8.50 g, 21.9 mmol) in 50 mL of methanol at 0°C. The mixture was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure to a solid residue, which was triturated with 5% methanol/hexane to give the title compound 8 (6.23 g, 84%):

1H NMR (400 MHz, DMSO-d₆) δ 7.82 – 7.71 (m, 2H), 7.21 – 7.12 (m, 2H), 3.79 (t, J = 7.1 Hz, 1H), 3.42 (dt, J = 12.2, 4.9 Hz, 1H), 3.14 – 2.94 (m, 2H), 2.95 – 2.82 (m, 2H), 2.71 (dt, J = 12.5, 5.0 Hz, 1H), 1.87 (s, 1H).

HRMS-ESI: calc for C₁₂H₁₆FN₂O₄S ([M + H]+ without HCl), 303.3, found 303.2.

Methyl 1-((4-fluorophenyl)sulfonyl)-4-(methylsulfonyl)piperazine-2-carboxylate (9)

To a solution of amine hydrochloride 8 (2.50 g, 7.38 mmol) in 4 mL of water and 16 mL of p-dioxane at 0°C was added subsequently freshly distilled triethylamine (3.19 mL, 22.9 mmol), DMAP (91.0 mg, 0.74 mmol) and dropwise methanesulfonyl chloride (742 µL, 9.59 mmol). The reaction was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and water. The organic layer was washed with 1 N HCl,
water, and brine, dried over Na$_2$SO$_4$, and concentrated under reduced pressure to give the title compound 9 (2.08 g, 74%):

$^1$H NMR (400 MHz, DMSO-\textit{d}$_6$) δ 7.81 – 7.71 (m, 2H), 7.21 – 7.11 (m, 2H), 3.80 (t, $J$ = 6.7 Hz, 1H), 3.58 (dt, $J$ = 12.5, 5.0 Hz, 1H), 3.45 (dd, $J$ = 12.4, 6.8 Hz, 1H), 3.19 (dt, $J$ = 12.2, 5.0 Hz, 1H), 3.08 (dt, $J$ = 12.2, 4.9 Hz, 1H), 3.01 – 2.82 (m, 5H).

HRMS-ESI: calc for C$_{13}$H$_{18}$FN$_2$O$_6$S$_2$ ([M + H]$^+$), 381.4, found 381.7.

1-((4-Fluorophenyl)sulfonyl)-N-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide (1A)

Preparation of NH$_2$OK/NH$_2$OH solution: NH$_2$OH.HCl (2.74 g, 39.4 mmol) was dissolved in MeOH (15 mL) by heating to reflux overnight. The solution was cooled at 0°C, and a solution of KOH (3.32 g, 59.1 mmol) in MeOH (10 mL) was added in one portion. The resulting suspension was used without prior removal of precipitated material. A solution of methyl ester 9 (1.50 g, 3.94 mmol) in NH$_2$OK/NH$_2$OH solution (as described above) was stirred at room temperature for 2 days. The reaction mixture was taken up in dilute aqueous HCl (pH = 3, 50 mL), extracted with EtOAc (3 x 50 mL), dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (0-5% MeOH in EtOAc) to afford hydroxamic acid 1A (706 mg, 47%) as a white solid.

$^1$H NMR (400 MHz, DMSO-\textit{d}$_6$) δ 10.67 (s, 1H), 8.86 (s, 1H), 7.82 – 7.71 (m, 2H), 7.20 – 7.11 (m, 2H), 3.62 (dt, $J$ = 12.2, 4.9 Hz, 1H), 3.26 – 3.05 (m, 3H), 2.98 – 2.83 (m, 5H).

HRMS-ESI: calc for C$_{13}$H$_{18}$FN$_2$O$_6$S$_2$ ([M + H]$^+$), 382.4, found 382.6.

Methyl 4-[(1,1’-biphenyl]-4-carbonyl)-1-((4-fluorophenyl)sulfonyl) piperazine-2-carboxylate (10)

To a solution of amine hydrochloride 8 (2.50 g, 7.38 mmol) in 5 mL of water and 10 mL of p-dioxane at 0°C was added subsequently freshly distilled triethylamine (3.19 mL, 22.9 mmol), DMAP (91.0 mg, 0.74 mmol) and slowly biphenyl-4-carbonyl chloride (2.08 g, 9.59 mmol). The reaction was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and water. The organic layer was washed with 1 N HCl, aqueous NaHCO$_3$, water, and brine, dried over Na$_2$SO$_4$, and concentrated under reduced pressure to give the title compound 10 (2.88 g, 81%):
1H NMR (400 MHz, DMSO-\textit{d}_6) \delta 7.81 – 7.62 (m, 8H), 7.53 – 7.36 (m, 3H), 7.21 – 7.10 (m, 2H), 4.29 (t, \textit{J} = 6.7 Hz, 1H), 3.76 (dt, \textit{J} = 12.0, 4.8 Hz, 1H), 3.70 – 3.60 (m, 4H), 3.57 – 3.36 (m, 3H), 3.14 (dt, \textit{J} = 12.2, 5.0 Hz, 1H).

HRMS-ESI: calc for C\textsubscript{25}H\textsubscript{24}FN\textsubscript{2}O\textsubscript{5}S ([M + H]\textsuperscript{+}), 483.5, found 483.8.

4-[[1,1’-Biphenyl]-4-carbonyl]-1-((4-fluorophenyl)sulfonyl)-N-hydroxypiperazine-2-carboxamide (2)

Preparation of NH\textsubscript{2}OK/NH\textsubscript{2}OH solution: NH\textsubscript{2}OH.HCl (2.88 g, 41.5 mmol) was dissolved in MeOH (15 mL) by heating to reflux overnight. The solution was cooled at 0°C, and a solution of KOH (3.49 g, 62.2 mmol) in MeOH (10 mL) was added in one portion. The resulting suspension was used without prior removal of precipitated material. A solution of methyl ester 10 (2.00 g, 4.15 mmol) in NH\textsubscript{2}OK/NH\textsubscript{2}OH solution (as described above) was stirred at room temperature for 2 days. The reaction mixture was taken up in dilute aqueous HCl (pH = 3, 50 mL), extracted with EtOAc (3 x 50 mL), dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated in vacuo. The residue was purified by column chromatography (0-5% MeOH in EtOAc) to afford hydroxamic acid 2 (903 mg, 45%) as a white solid.

1H NMR (400 MHz, DMSO-\textit{d}_6) \delta 9.95 (s, 1H), 8.86 (s, 1H), 7.87 – 7.66 (m, 8H), 7.54 – 7.35 (m, 3H), 7.21 – 7.10 (m, 2H), 4.46 (t, \textit{J} = 6.8 Hz, 1H), 3.64 – 3.38 (m, 4H), 3.22 (ddt, \textit{J} = 29.9, 12.0, 4.9 Hz, 2H).

HRMS-ESI: calc for C\textsubscript{24}H\textsubscript{23}FN\textsubscript{3}O\textsubscript{5}S ([M + H]\textsuperscript{+}), 484.5, found 484.7.

4-(Tert-butoxycarbonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxylic acid (13)

An aqueous solution of sodium hydroxide (4.6 mL, 50% w/w, 19.4 N, 88.6 mmol) was slowly added to a solution of \textit{dl}-piperazine carboxylic acid dihydrochloride 5 (5.99 g, 29.5 mmol) in 60 mL of p-dioxane and 30 mL of water at 0°C. Addition of di-\textit{tert}-butyl dicarbonate (7.09 g, 32.5 mmol) was followed and the mixture was stirred overnight at room temperature yielding 6. Freshly distilled triethylamine (8.23 mL, 59.0 mmol), 4-dimethylaminopyridine (721 mg, 5.90 mmol) and 4-nitrophenylsulfonyl chloride (6.54 g, 29.5 mmol) were added to the mixture at 0°C and the reaction was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and 1 N HCl. The EtOAc layer (2 x 100 mL) was washed with brine, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated in vacuo to give the title compound 13 (8.95 g, 73%).
\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 12.13 (s, 1H), 8.42 – 8.33 (m, 2H), 8.07 – 7.97 (m, 2H), 4.22 (t, \(J = 6.9\) Hz, 1H), 3.97 (dd, \(J = 12.3, 7.0\) Hz, 1H), 3.86 (dt, \(J = 12.3, 5.0\) Hz, 1H), 3.64 (dt, \(J = 12.2, 5.0\) Hz, 1H), 3.42 – 3.28 (m, 2H), 3.19 (dt, \(J = 12.2, 5.1\) Hz, 1H), 1.37 (s, 9H).

HRMS-ESI: calc for C\(_{16}\)H\(_{22}\)N\(_3\)O\(_8\)S \([\text{M} + \text{H}]^+\) 416.4, found 416.4.

**Methyl 1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxylate hydrochloride (14)**

Thionyl chloride (7.00 mL, 96.5 mmol) was added dropwise to a solution of 4-(tert-butoxycarbonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxylic acid \(13\) (8.00 g, 19.3 mmol) in 50 mL of methanol at 0°C. The mixture was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure to a solid residue, which was triturated with 5% methanol/hexane to give the title compound \(14\) (6.07 g, 86%):

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.43 – 8.34 (m, 2H), 8.15 – 8.08 (m, 2H), 3.72 (t, \(J = 7.1\) Hz, 1H), 3.44 (dt, \(J = 12.4, 5.0\) Hz, 1H), 3.23 – 3.08 (m, 2H), 2.92 (dt, \(J = 12.5, 5.0\) Hz, 1H), 2.86 – 2.72 (m, 2H), 1.91 (s, 1H).

HRMS-ESI: calc for C\(_{12}\)H\(_{16}\)N\(_3\)O\(_6\)S \([\text{M} + \text{H}]^+\) without HCl, 330.3, found 330.1.

**Methyl 4-(methylsulfonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxylate (15)**

To a solution of amine hydrochloride \(14\) (2.50 g, 6.84 mmol) in 4 mL of water and 16 mL of p-dioxane at 0°C was added subsequently freshly distilled triethylamine (2.96 mL, 21.2 mmol), DMAP (83.1 mg, 0.68 mmol) and dropwise methanesulfonyl chloride (687 µL, 8.89 mmol). The reaction was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and water. The organic layer was washed with 1 N HCl, water, and brine, dried over Na\(_2\)SO\(_4\), and concentrated under reduced pressure to give the title compound \(15\) (1.70 g, 61%):

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 8.43 – 8.34 (m, 2H), 7.97 – 7.89 (m, 2H), 4.19 (t, \(J = 6.5\) Hz, 1H), 3.52 (dt, \(J = 12.5, 5.0\) Hz, 1H), 3.38 – 3.23 (m, 2H), 3.21 – 3.03 (m, 2H), 2.97 – 2.83 (m, 4H).

HRMS-ESI: calc for C\(_{13}\)H\(_{18}\)N\(_3\)O\(_8\)S\(_2\) \([\text{M} + \text{H}]^+\), 408.4, found 408.5.
Methyl 4-[[1,1’-biphenyl]-4-carbonyl]-1-[(4-nitrophenyl)sulfonyl]piperazine-2-carboxylate (16)

To a solution of amine hydrochloride 14 (2.50 g, 6.84 mmol) in 5 mL of water and 10 mL of p-dioxane at 0°C was added subsequently freshly distilled triethylamine (2.96 mL, 21.2 mmol), DMAP (83.1 mg, 0.68 mmol) and slowly biphenyl-4-carbonyl chloride (1.93 g, 8.89 mmol). The reaction was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and water. The organic layer was washed with 1 N HCl, aqueous NaHCO₃, water, and brine, dried over Na₂SO₄, and concentrated under reduced pressure to give the title compound 16 (2.3 g, 66%):

\[1H \text{ NMR (400 MHz, DMSO-}d_6\) \delta 8.42 – 8.34 (m, 2H), 8.09 – 8.01 (m, 2H), 7.88 – 7.80 (m, 2H), 7.78 – 7.66 (m, 4H), 7.54 – 7.36 (m, 3H), 3.98 – 3.81 (m, 2H), 3.64 – 3.48 (m, 2H), 3.31 – 3.18 (m, 2H). \]

HRMS-ESI: calc for C₂₅H₂₄N₃O₇S ([M + H]+), 510.5, found 510.7.

N-([Tert-butoxy]-4-(methylsulfonyl)-1-[(4-nitrophenyl)sulfonyl]piperazine-2-carboxamide (17)

A solution of methyl ester 15 (1.20 g, 2.95 mmol) in 1 N HCl (60 mL) was refluxed overnight. The solution was cooled at room temperature and the solvent was removed under reduced pressure. The obtained residue was dissolved in CH₂Cl₂ (20 mL). The solution was cooled at 0°C, and N-[(dimethylamino)propyl]-N-ethyl-carbodiimide hydrochloride (505 mg, 3.25 mmol), 1-hydroxybenzotriazole (519 mg, 3.84 mmol) and N-methylmorphorine (2.05 mL, 18.6 mmol) were added. After stirring 1 h at room temperature, O-tert-butyldihydroxylamine hydrochloride (741 mg, 5.90 mmol) was added. The reaction was stirred at room temperature for 2 days. The reaction mixture was concentrated in vacuo. The residue was dissolved in EtOAc, washed with brine, dried over Na₂SO₄, and concentrated under reduced pressure. The residue was purified by column chromatography (0-5% MeOH in EtOAc) to afford protected hydroxamic acid 17 (877 mg, 64%).

\[1H \text{ NMR (400 MHz, DMSO-}d_6\) \delta 11.25 (s, 1H), 8.42 – 8.34 (m, 2H), 8.09 – 8.01 (m, 2H), 7.88 – 7.77 (m, 2H), 4.38 (t, J = 6.5 Hz, 1H), 3.59 (dd, J = 12.3, 6.5 Hz, 1H), 3.51 – 3.32 (m, 2H), 3.26 – 3.05 (m, 2H), 2.98 (dd, J = 12.4, 6.6 Hz, 1H), 2.85 (s, 3H), 1.14 (s, 9H). \]

4-[(1,1’-Biphenyl]-4-carbonyl)-N-(tert-butoxy)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide (18)

A solution of methyl ester 16 (1.50 g, 2.94 mmol) in 1 N HCl (60 mL) was refluxed overnight. The solution was cooled at room temperature and the solvent was removed under reduced pressure. The obtained residue was dissolved in CH$_2$Cl$_2$ (20 mL). The solution was cooled at 0°C, and N-[[(dimethylamino)propyl]-N-ethyl-carbodiimide hydrochloride (501 mg, 3.23 mmol), 1-hydroxybenzotrizole (516 mg, 3.82 mmol) and N-methylmorphorine (2.05 mL, 18.6 mmol) were added. After stirring 1 h at room temperature, 0-tert-butyldihydroxylamine hydrochloride (739 mg, 5.88 mmol) was added. The reaction was stirred at room temperature for 2 days. The reaction mixture was concentrated in vacuo. The residue was diluted in EtOAc, washed with brine, dried over Na$_2$SO$_4$, and concentrated under reduced pressure. The residue was purified by column chromatography (0-5% MeOH in EtOAc) to afford protected hydroxamic acid 18 (1.12 g, 67%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.82 (s, 1H), 8.42 – 8.34 (m, 2H), 8.07 – 7.99 (m, 2H), 7.85 – 7.66 (m, 6H), 7.54 – 7.36 (m, 3H), 4.52 (t, $J$ = 7.7 Hz, 1H), 3.93 – 3.75 (m, 2H), 3.59 – 3.48 (m, 1H), 3.37 (dd, $J$ = 12.5, 7.5 Hz, 1H), 3.18 – 3.06 (m, 1H), 1.19 (s, 9H).

HRMS-ESI: calc for C$_{28}$H$_{31}$N$_4$O$_7$S $([M + H]^+)$, 567.6, found 567.7.

N-Hydroxy-4-(methylsulfonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide (11)

Trifluoroacetic acid (10 mL) was added dropwise to a stirred solution of protected hydroxamic acid 17 (500 mg, 1.08 mmol) in CH$_2$Cl$_2$ (20 mL) at 0°C under argon. After stirring overnight at room temperature, the reaction mixture was concentrated in vacuo. The residue was treated with saturated aqueous NaHCO$_3$ (100 mL) and extracted with CH$_2$Cl$_2$ (3 x 60 mL). The combined organic fractions were dried over Na$_2$SO$_4$, and concentrated under reduced pressure to give the hydroxamic acid 11 (393 mg, 89%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 10.49 (s, 1H), 8.87 (s, 1H), 8.42 – 8.34 (m, 2H), 8.06 – 7.98 (m, 2H), 4.49 (t, $J$ = 6.9 Hz, 1H), 3.81 – 3.67 (m, 2H), 3.59 – 3.48 (m, 1H), 3.41 (dd, $J$ = 12.3, 6.9 Hz, 1H), 3.11 – 2.97 (m, 2H), 2.85 (s, 3H).

HRMS-ESI: calc for C$_{12}$H$_{17}$N$_4$O$_8$S$_2$ $([M + H]^+)$, 409.4, found 409.6.
4-[[1,1’-Biphenyl]-4-carbonyl]-N-hydroxy-1-((4-nitrophenyl)sulfonyl) piperazine-2-carboxamide (12)

Trifluoroacetic acid (10 mL) was added dropwise to a stirred solution of protected hydroxamic acid 18 (700 mg, 1.24 mmol) in CH₂Cl₂ (20 mL) at 0°C under argon. After stirring overnight at room temperature, the reaction mixture was concentrated in vacuo. The residue was treated with saturated aqueous NaHCO₃ (100 mL) and extracted with CH₂Cl₂ (3 x 60 mL). The combined organic fractions were dried over Na₂SO₄, and concentrated under reduced pressure to give the hydroxamic acid 12 (582 mg, 92%):

\[ \delta \text{H NMR (400 MHz, DMSO-d₆)} \delta 11.11 (s, 1H), 8.87 (s, 1H), 8.42 – 8.34 (m, 2H), 8.09 – 8.01 (m, 2H), 7.80 – 7.66 (m, 6H), 7.54 – 7.36 (m, 3H), 4.26 (t, J = 6.7 Hz, 1H), 3.74 – 3.49 (m, 3H), 3.34 – 3.07 (m, 3H). \]

HRMS-ESI: calc for C₂₄H₂₃N₄O₇S ([M + H]+), 511.5, found 511.4.

4.5 In vitro evaluation of 1A and 2 in a fluorogenic inhibition assay

Recombinant ADAM-17 (ectodomain) was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human MMP-9 CD and recombinant human MMP-12 CD without fibronectin type II insert (expressed in E.coli as described in [19, 20]) were a kind gift from AstraZeneca R&D (Lund &Moelndal, Sweden).

This competitive enzyme activity assay was performed by monitoring the conversion of the fluorogenic substrate Mca-PLAQAV-Dpa-RSSSR-NH₂ (R&D systems) by recombinant ADAM-17 in presence of increasing concentrations of 1A or 2. For MMP-9 and -12, the conversion of the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (Bachem, Bubendorf, Switzerland) was followed. Measurements were performed in Costar White 96-well plates (Corning, Schiphol-Rijk, The Netherlands), where each well contained 10 ng ADAM-17 and a final concentration of 10 µM substrate in a final volume of 100 µL ADAM assay buffer (25 mM Tris pH 9.0, 2.5 µM ZnCl₂, 0.005% w/v Brij-35). Inhibition of MMP proteolytic activity was determined with 10 ng of MMP-9 or MMP-12 per well with a final concentration of 2 µM substrate in 100 µL MMP assay buffer (50 mM Tris pH 7.4, 0.2 M NaCl, 10 mM CaCl₂, 2.5 µM ZnCl₂, 0.05% (v/v) Brij-35). Proteolysis rates were followed by measuring fluorescence (λ_ex/em = 320, 440 nm) increase using a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany), at 20°C for recombinant MMPs and at 37°C for recombinant ADAM-17, for 15 min (conditions of the experiments not in the stationary phase). Seven-point inhibition curves (0, 0.1, 1, 10, 100, 1000
and 10000 nM) were plotted. IC<sub>50</sub> values were determined by sigmoidal fitting. Each experiment was performed in triplicate.

4.6 Radiosynthesis of [18F]-1A and [18F]-2

Production of n.c.a. 18F-fluorine and preparation of dry 18F-fluorine-cryptate complex

Aqueous 18F-fluorine was produced by irradiation of 18O-water with a Scanditronix MC-17 cyclotron via the 18O(p,n) 18F nuclear reaction. The 18F-fluorine solution was trapped on a SepPak Light Accell plus QMA anion exchange cartridge (preconditioned with 5 mL of sodium bicarbonate 1.4% and 100 mL of H<sub>2</sub>O and then dried under a flow of Argon) to recycle the 18O-enriched water. The 18F-fluorine was eluted from the QMA anion exchange cartridge with 1 mg potassium carbonate in 1 mL of water and collected into a vial containing 5 mg kryptofix[2.2.2]. Subsequently, 1 mL of acetonitrile was added and the solvents were removed at 130°C under an argon stream. The [18F]KF / kryptofix[2.2.2] complex was then dried by azeotropic distillation with 3 times addition of 0.5 mL anhydrous acetonitrile at 130°C.

1-((4-[18F]Fluorophenyl)sulfonyl)-N-hydroxy-4-(methylsulfonyl)piperazine-2-carboxamide ([18F]-1A)

A solution of N-hydroxy-4-(methylsulfonyl)-1-((4-nitrophenyl)sulfonyl)piperazine-2-carboxamide 11 (5.0 mg, 12.2 mmol) in anhydrous DMSO (400 μL) was added to the dry [18F]KF / kryptofix[2.2.2] complex and heated for 20 min at 140°C. After cooling, the reaction mixture was diluted with 250 μL acetonitrile and 750 μL H<sub>2</sub>O and was purified by semi-preparative reverse phase HPLC. HPLC was performed with Elite LaChrom Merck Hitachi L-7100 pump system using a Phenomenex reversed-phase Luna C18 column (10 mm x 250 mm, 5µm), preceded of a 20 x 4.6 mm<sup>2</sup> precolumn, equipped with both a UV (Elite LaChrom VWR Hitachi L-2400 UV detector set at 254 nm, AUFS = 0.5) and a Bicron radioactivity monitor. Sample injection was carried out using an injector block with a loop of 1 mL. The mobile phase used was a mixture of acetonitrile/water 25/75 at a flow rate of 2.5 mL.min<sup>-1</sup>. The retention time of [18F]-1A was about 18 min. The HPLC-collected fraction was diluted with about 100 mL of water for injection and passed through a Sep-Pak Light C18 cartridge. The cartridge was washed with 10 mL of water for injection and eluted with 0.7 mL of absolute EtOH. The obtained product was diluted with
saline to decrease the percentage of EtOH to less than 10% for the subsequent animal experiments. Quality control was performed by analytical HPLC, using a Phenomenex Luna C18 column (4.6 mm x 250 mm, 5 μm) from Waters, equipped with a 20 x 4.6 mm² precolumn. The mobile phase employed was ACN / H₂O 70 / 30 at a flow rate of 1.5 mL.min⁻¹. The column effluent was monitored using an Elite Lachrom VWR Hitachi L-2400 UV detector (λ = 254 nm, AUFS = 0.010) and a Bicron frisk-tech radioactivity detector. Sample injection was carried out using an injector block with a loop of 100 μL. The retention time of [¹⁸F]-1A was about 8 min.

4-[[1,1'-Biphenyl]-4-carbonyl]-1-((4-[¹⁸F]fluorophenyl)sulfonyl)-N-hydroxypiperazine-2-carboxamide ([¹⁸F]-2)

A solution of 4-([1,1'-biphenyl]-4-carbonyl)-N-hydroxy-[18F]fluorophenylsulfonyl)piperazine-2-carboxamide 12 (5.0 mg, 9.79 mmol) in anhydrous DMSO (400 μL) was added to the dry [¹⁸F]KF / kryptofix[2.2.2] complex and heated for 20 min at 140°C. After cooling, the reaction mixture was diluted with 450 μL acetonitrile and 550 μL H₂O and was purified by semi-preparative reverse phase HPLC. HPLC was performed with Elite LaChrom Merck Hitachi L-7100 pump system using a Phenomenex reversed-phase Luna C18 column (10 mm x 250 mm, 5µm), preceded of a 20 x 4.6 mm² precolumn, equipped with both UV (Elite LaChrom VWR Hitachi L-2400 UV detector set at 254 nm, AUFS = 0.5) and a Bicron radioactivity monitor. Sample injection was carried out using an injector block with a loop of 1 mL. The mobile phase used was a mixture of acetonitrile/water 45/55 at a flow rate of 2.5 mL.min⁻¹. The retention time of [¹⁸F]-2 was about 23 min. The HPLC-collected fraction was diluted with about 100 mL of water for injection and passed through a Sep-Pak Light C18 cartridge. The cartridge was washed with 10 mL of water for injection and eluted with 0.7 mL of absolute EtOH. The obtained product was diluted with saline to decrease the percentage of EtOH to less than 10% for the subsequent animal experiments. Quality control was performed by analytical HPLC, using a Phenomenex Luna C18 column (4.6 mm x 250 mm, 5 μm) from Waters, equipped with a 20 x 4.6 mm² precolumn. The mobile phase employed was ACN/H₂O 40/60 at a flow rate of 1.5 mL.min⁻¹. The column effluent was monitored using an Elite Lachrom VWR Hitachi L-2400 UV detector (λ = 254 nm, AUFS = 0.010) and a Bicron frisk-tech radioactivity...
detector. Sample injection was carried out using an injector block with a loop of 100 µL. The retention time of $[^{18}\text{F}]-2$ was about 12 min.

4.7 In vitro stability of $[^{18}\text{F}]-1\text{A}$ and $[^{18}\text{F}]-2$ in saline and human plasma

Saline stability of $[^{18}\text{F}]-1\text{A}$ and $[^{18}\text{F}]-2$

The stability of $[^{18}\text{F}]-1\text{A}$ (or $[^{18}\text{F}]-2$) was evaluated in vitro in saline. Formulated $[^{18}\text{F}]-1\text{A}$ (or $[^{18}\text{F}]-2$) was dissolved in 1 mL saline and incubated at 37°C for 3 h. After 1 h and 3 h of incubation, aliquots of 250 µL were taken and diluted with 500 µL ACN and 500 µL H₂O and analysed by analytical HPLC using a Phenomenex Luna C18 column (4.6 mm x 250 mm, 5 µm), equipped with a 20 x 4.6 mm² precolumn. The mobile phase employed was ACN/H₂O 30/70 for $[^{18}\text{F}]-1\text{A}$ (or ACN/H₂O 40/60 for $[^{18}\text{F}]-2$) at a flow rate of 1.5 mL min⁻¹. One-minute fractions of the eluate were collected and radioactivity in the fractions was counted with a gamma counter.

Human plasma stability of $[^{18}\text{F}]-1\text{A}$ and $[^{18}\text{F}]-2$

The stability of $[^{18}\text{F}]-1\text{A}$ (or $[^{18}\text{F}]-2$) was evaluated in vitro in human plasma. Blood from a healthy donor, kept at room temperature for 15 min, was centrifuged at 3000 rpm for 5 min, subsequently the supernatant was taken. 100 µL of formulated $[^{18}\text{F}]-1\text{A}$ (or $[^{18}\text{F}]-2$) was dissolved in 1 mL human plasma and incubated at 37°C for 3 h. After 1 h and 3 h of incubation, aliquots were taken and analysed as described previously.

4.8 Octanol/water partition coefficient

About 5 kBq of formulated $[^{18}\text{F}]-1\text{A}$ or $[^{18}\text{F}]-2$ diluted in 5 µL saline was diluted in 495 µL PBS (pH = 7.4) and 500 µL $n$-octanol in an Eppendorf cup. The mixture was vortexed for 5 min and the cup was centrifuged at 3000 rpm for 5 min. A 100 µL aliquot of both water and $n$-octanol layer was counted in a gammacounter. The experiment was performed in triplicate. The partition coefficient (log P) was calculated as: $\log P = \log_{10} \left( \frac{\text{cpm}_{\text{octanol layer}}}{\text{cpm}_{\text{aqueous layer}}} \right)$.

4.9 HT1080 fibrosarcoma xenograft mouse model

Animals

Male BALB/c nu/nu (BALB/cOlaHsd-Foxn1nu) mice (nude mice) were obtained from Harlan (Lelystad, The Netherlands). The mice were housed in IVC cages with paper bedding on a layer of wood shavings in a room with constant temperature
(~ 20°C) and fixed, 12-hour light-dark regime. Food (standard laboratory chow, RMH-B, Hope Farms, The Netherlands) and water were available ad libitum. After arrival, the mice were allowed to acclimatize for at least seven days. The study was approved by the Animal Ethics Committee of the University of Groningen, The Netherlands (DEC 6058B).

**HT1080 inoculation**

Human fibrosarcoma HT1080 cells were obtained from American Type Culture Collection, Manassas, USA. HT1080 cells were maintained in 15 mL Eagle's Minimum Essential Medium (EMEM) (Lonza, Walkersville, USA) supplemented with 10% fetal calf serum (FCS) in a T75 culture flask. Cells were grown in a humidified atmosphere containing 5% CO_2 and were passaged twice per week. (2-2.5) x 10^6 HT1080 cells, in a 1:1 (v/v) mixture of Matrigel (extracellular matrix compound, Becton Dickinson) and EMEM with 10% FCS, were subcutaneously injected into the right shoulder of the BALB/c nude mice (7-8 weeks old).

**4.10 Preclinical studies**

Animals were injected with [18F]-1A or [18F]-2 when the tumors had reached an adequate size (0.3-0.6 mL), after 14 to 21 days of inoculation. The mice were randomly divided into two groups: tumor-bearing mice injected with [18F]-1A (or [18F]-2) and with or without coinjection of 2.5 mg/kg of 1A (or 2). [18F]-1A (1.55 ± 1.38 MBq, 0.031 ± 0.028 nmol) (or [18F]-2 (2.12 ± 3.28 MBq, 0.043 ± 0.080 nmol)), dissolved in saline (maximum volume 200 μL per injection), was intravenously injected into the penile vein of mice anesthetized with 5% isoflurane (Pharmacie BV, The Netherlands) that was mixed with medical air at a flow rate of 2 mL.min⁻¹, after which anesthesia was maintained with 2% isoflurane. For each tracer, 12 mice were used. In each group (either baseline or block), one animal was scanned using PET. Following induction of anesthesia, the mice were then positioned in the bed of the microPET camera (Focus 220, Siemens Medical Solutions USA, Inc.) in transaxial position. The body temperature of mice was maintained by electronically regulated heating pads. Data acquisition of the microPET camera was initiated and continued for a period of 90 min. After the completion of the dynamic emission scan, a 515 sec transmission scan with a Co-57 point source was made for correction of attenuation of 511 keV photons by tissue. After microPET scanning, the mice were terminated by administering a high dose of isoflurane (5%) for about 20 min.
Ex vivo biodistribution

*Ex vivo* biodistribution studies were performed on the sacrificed mice 90 min p.i. of $[^{18}\text{F}]$-1A ($\pm$ 1A) or $[^{18}\text{F}]$-2 ($\pm$ 2). The following organs were taken: bladder, bone, brain, heart, kidney, large intestine, liver, lungs, muscle, pancreas, small intestine, spleen, stomach, tumor and urine. A small drop of infusate was collected for calculation of SUV\textunderscore{mean}. The blood was centrifuged in order to collect plasma and red blood cells. All samples were weighed and levels of radioactivity were determined using a gamma counter. Tracer uptake was expressed as the standardized uptake value (SUV\textunderscore{mean}), defined as $\left[\frac{\text{tissue activity concentration (MBq/g)}}{\text{injected activity (MBq)/mouse body weight (g)}}\right]$.

MicroPET image analysis

Emission sinograms were iteratively reconstructed (OSEM2d) after being normalized, corrected for attenuation, and corrected for decay of radioactivity. The list-mode data of the emission scans were separated into 22 frame sinograms (15 frames of 2 minutes, 3 frames of 5 minutes, 2 frames of 7.5 minutes, 2 frames of 15 minutes; zoom factor, 4). PET image analysis was performed using Inveon Research Workplace (Siemens) software. Regions of interest were drawn around the tumor. The uptake of the tracer in the region of interest was determined in Bq cm$^{-3}$, which was converted to PET-SUV\textunderscore{mean} using the following formula: $\left[\frac{\text{tissue activity concentration (MBq/cc)}}{\text{injected activity (MBq)/mouse body weight (g)}}\right]$.

Acknowledgements

The authors wish to thank the Dutch Technology Foundation (STW) for financial support (project 08008).
References
