Development of a radiolabelled hydroxamate-based MMP/ADAM inhibitor, 4-(4-(1-(4-(2-(2-(2-[18F]fluoroethoxy)ethoxy)ethoxy)butyl)-1H-1,2,3-triazol-4-yl)benzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide, for PET

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This chapter was submitted for publication
Abstract

Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) are zinc endopeptidases which are considered attractive targets for the pharmaceutical industry in anti-cancer therapy. Molecular in vivo imaging of locally up-regulated and activated MMPs/ADAMs could be used to understand and detect cancer progression. We developed a novel piperazine-based MMP/ADAM inhibitor 4-(4-(1-(4-(2-(2-(2-[18F]fluoroethoxy)ethoxy)ethoxy)butyl)-1H-1,2,3-triazol-4-yl)benzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide [18F]-1B as a potential PET tracer for imaging MMP/ADAM activity. The non-radioactive analogue was tested in fluorogenic in vitro inhibition assays for MMP-2, MMP-9 and ADAM-17 affinity and the obtained IC50 values were 4.67 ± 0.85, 3.67 ± 0.49 and 43.4 ± 7.74 nM, respectively. The PET probe was prepared with a radiochemical yield of 25-27% and a specific activity of 45-59 GBq/μmol at the end of synthesis. [18F]-1B was evaluated in a HT1080 tumor bearing mouse model. HT1080 tumor was visualized in the microPET images. Retention of [18F]-1B was shown to be MMP/ADAM-mediated. Further evaluation in other disease models is required to validate this radiopharmaceutical as a potential biomarker for imaging MMP/ADAM activity. [18F]-1B provides a novel lead structure that should be further optimized in order to obtain a suitable PET-tracer.

Keywords
Click chemistry, hydroxamate, microPET, MMP/ADAM inhibitor, PEG
1. Introduction

Matrix metalloproteinases (MMPs) are a multi-domain zinc(II)-dependent enzyme family which comprises 23 members [1]. In addition to MMPs, this family also includes, A Disintegrin And Metalloproteinases (ADAMs), with 22 members [2]. MMPs and ADAMs are secreted as pro-enzymes (also called zymogens) and require enzymatic release of their pro-domains to render them proteolytically active. These enzymes are capable of digesting various structural components of the extracellular matrix (ECM) under physiological conditions. MMP/ADAM activity is involved at several stages of tumor development: tumor establishment, growth, angiogenesis, intravasation/extravasation and metastasis [3]. As a result, pharmacologic inhibition of MMP/ADAM activity could be used to halt the spreading of cancer cells [4, 5].

Clinical trials of matrix metalloproteinase inhibitors in cancer were disappointing and the results were not fully understood [6-8]. To overcome these limitations and to improve the results of clinical trials as well as potentially tailor MMP/ADAM inhibitor treatments to the individual patient, markers of tumor progression should be developed and validated with the goal to improve early detection, response to treatment by imaging MMP/ADAM inhibition and to understand how MMP/ADAM activities are regulated. Positron emission tomography (PET) is a non-invasive imaging technology, which is sensitive, has a high spatial resolution and allows quantitative measurements [9]. By molecular modeling, we designed a library of five piperazine-based MMP/ADAM inhibitors [10]. These nonpeptidic inhibitors comprise a hydroxamate as a zinc binding group, a methoxyphenylsulfonamide group to fill the “selective” S1’ pocket of the proteinases and various substituents at the 4-\(N\)-position of the piperazine ring which is solvent-exposed. All residues at the 4-\(N\)-position of the piperazine contain a 1,4-triazole to allow (radio)synthesis of the inhibitors by click chemistry. We applied the click chemistry reaction because this reaction is selective, reliable and fast under mild aqueous Cu(I)-promoted reaction conditions.

In this chapter, we report the synthesis, radiosynthesis and quality control of 4-\(N\)-(4-(4-(1-(4-(2-(2-(2-\([^{18}\text{F}]\text{fluoroethoxy})\text{ethoxy})\text{ethoxy})\text{butyl})-1\text{H}-1,2,3-triazol-4-yl})benzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide \([^{18}\text{F}]\)-1B [Fig 1], a new synthetic MMP/ADAM inhibitor selected from the library of the five modeled inhibitors. The \textit{in vitro} fluorogenic inhibition assays of 1B for re-
combinant human MMP-2, MMP-9 and ADAM-17 affinity are reported. Furthermore, $[^{18}F]-1B$ was evaluated in vivo in a HT1080 xenograft mouse model.

2. Results and discussion

2.1 Design of piperazine-based MMP/ADAM inhibitors by click chemistry

Our strategy for designing new MMP/ADAM inhibitors was based on the inhibitor 4-((1,1’-biphenyl)-4-carbonyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide 2 [Fig 2] developed originally by Cheng et al. [11]. 2 is a broad-spectrum inhibitor with nanomolar affinities for a number of metalloproteinases, like MMP-9 ($IC_{50} = 0.9$ nM) for instance. Modifications of this inhibitor in order to optimize potency, tune the lipophilicity and allow a straightforward radiolabelling method were ideally achieved by changing the substituent at the 4-$N$-position of the piperazine ring which is solvent-exposed [12, 13]. The radiolabelling approach which was adopted was the reliable click chemistry between an azide and an alkyne. A phenyl-triazole linker was supposed to mimic the biphenyl group of 2. Therefore, the biphenyl moiety of 2 was modified in order to incorporate an alkyne group. Several fluorine containing groups with varying size and lipophilicity were attached to the triazole moiety. The binding characteristics to MMP-9 of five different inhibitors were investigated by molecular modeling.
2.2 Molecular modeling of a library of piperazine-based MMP inhibitors

The modeling of the interaction of different inhibitors with MMP-9 was performed by using Molegro Molecular Viewer in order to design a library of five piperazine-based MMP inhibitors targeting specifically MMP-9. Cycloaddition of the alkyne \( \text{3} \) was performed with five different azides and is reported in Table 1. The azido prosthetic groups considered were respectively: 1-azido-4-(fluoromethyl)benzene, 1-azido-3-fluoropropane, 1-azido-4-fluorobutane, 1-azido-3-(2-(2-(2-fluoroethoxy)ethoxy)ethoxy)propane and 1-azido-4-(2-(2-(2-fluoroethoxy)ethoxy)ethoxy)butane to give the corresponding MMP/ADAM inhibitors \( \text{16, 17, 18, 19 and 1B} \). The calculated log P (clog P) and the MolDock Score are shown in Table 1. For comparison, the MMP inhibitor \( \text{2} \) is also reported.

The MolDock Score is described 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 into MMP-9 show alignment to the molecule design concept. The substituent construct on \( \text{2, 16, 17, 18, 19, and 1B} \), which are P1’ and P2’, correspond with MMP-9 pockets S1’ and S2’. The hydroxamic acid group was in position to form binding coordination with Zn\(^ {2+} \).

As two thirds of MMPs are soluble and to minimize non-specific binding, it seemed more logical for us to develop a hydrophilic MMPI. As a result, the inhibitor \( \text{16} \) was discarded as a potential candidate for radiolabelling. Then, we discriminated the MMPIs according to their affinity for MMP-9 and selected the compounds \( \text{17, 19 and 1B} \) displaying the highest docking scores. Since the PEG-chains are not significantly
The methoxy benzene group is solvent exposed. Due to the rigidity of the piperazine moiety, the hydroxamic acid group had a tendency to move from the zinc binding site.

The inhibitor was forced to bend to opposite direction, most likely attributable to the rigidity and the bulky structure of the benzene-triazole-fluoromethyl benzene. The benzene-triazole-fluoromethyl benzene appeared too long to be fitted into the cavity.

The hydroxamic acid group is shifted from the zinc binding site. The MolDock Score is increased most likely due to the hydrophobic interaction of leucine and arginine inside the cavity, the hydrogen bonding between the triazole and leucine and the aromatic interaction with tyrosine.

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<th>Structure, MolDock Score and clog P of modeled MMP inhibitors</th>
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Table 1: Structure, MolDock Score and clog P of modeled MMP inhibitors
An additional carbon in the alkyl chain made the benzene-triazole did not fit anymore to the cavity, compared to 17. Therefore, the benzene-triazole moiety became solvent exposed and made the inhibitor bent the other way around (similar to 16).

The hydroxamic acid group is moved away from the zinc binding site due to the force of the very long PEG chain. However, as the hydroxamic acid group could form three hydrogen bonds with glutamic acid and histidine residues, an increase in MolDok Score is obtained.

Table 1: Structure, MolDock Score and clog P of modeled MMP inhibitors (continued)
defluorinated \textit{in vivo} and are stable towards metabolism [14-16], we focused our work on the fluoropegylated triazoles 19 and 1B rather than the fluoroalkylated triazole 17. Since 1B demonstrated slightly better affinity and lower lipophilicity, it was selected as the most potent candidate to image active MMPs with PET.

2.3 Synthesis of the terminal alkyne 3

The alkyne 3 [Scheme 2], 4-(4-ethynylbenzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide, was prepared from the commercially available \textit{dl}-piperazine carboxylic acid 4. The three first steps of the synthesis were performed according to the procedure of Cheng et al. [11];[Scheme 1]. Monoprotection of 4 with 1.1 eq. of di-\textit{tert}-butyl dicarbonate led to the monoamine 5. Para-methoxyphenylsulfonyl chloride was added to the crude amine 5 and produced the sulphonamide 6, obtained in an overall yield of 86%. After addition of methanolic HCl to 6, the intermediate 7 was obtained in a yield of 82%. The protected alkyne 4-((trimethylsilyl)ethynyl)benzoic acid 8 was transformed to acid chloride 9 by treatment with thionyl chloride [Scheme 2]. The intermediate 7 was acylated with 4-((trimethylsilyl)ethynyl)benzoyl chloride 9 in order to get the desired substituted piperazine derivative 10. Then the trimethylsilyl group was deprotected with tetrabutylammonium fluoride to produce the free alkyne 11. The hydroxamic acid was

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*Table 1: Structure, MolDock Score and clog P of modeled MMP inhibitors (continued)*
finally incorporated with hydroxylamine in KOH-MeOH to yield the intermediate 3 as precursor for 1,3-dipolar cycloaddition with $^{18}$F-azido prosthetic groups to form 1,4-disubstituted triazoles. The overall yield of the synthesis of 3 was 29%.

### 2.4 Synthesis of the azides Tos-PEG-N$_3$ 17 and F-PEG-N$_3$ 18

The synthesis of the tosyl-precursor 17 and the non-radioactive azide 18 are reported in Scheme 3 and were conducted as previously described [17]. In brief,

**Scheme 1:** Synthesis of methyl 1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxylate hydrochloride  
(a) 1.1 eq. (t-Boc)$_2$O, 3 eq. NaOH, dioxane-water; (b) 1 eq. p-MeOPhSO$_2$Cl, Et$_3$N/cat. DMAP; (c) SOCl$_2$, MeOH

**Scheme 2:** Synthesis of 4-(4-ethynylbenzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl) piperazine-2-carboxamide  
(d) 5 eq. SOCl$_2$, CHCl$_3$, Δ; (e) 1.3 eq. 9, Et$_3$N/cat. DMAP; (f) Bu$_4$NF; (g) NH$_2$OH in KOH and MeOH
commercially available triethylene glycol 12 was monoprotected with trityl chloride to produce the intermediate 13 in an 80% yield. Thereafter, a Williamson synthesis was performed on the primary alcohol of 13 with 1,4-dibromo butane to lead to the mono-bromide 14 in 35-38% yield. In the presence of an excess of sodium azide, the bromo-group was quantitatively substituted by the azido group to form compound 15. The trityl-ether protecting group was then cleaved with TFA and triethylsilane as cation scavenger in a 55% yield. The obtained primary azido-alcohol 16 was reacted with tosyl chloride in a yield of 46% and resulted in the precursor for radiofluorination 17. The primary alcohol of 16 was fluorinated with DAST (diethylamino sulfur trifluoride) to give the fluoropegylated ligand 18. The overall yield of the synthesis of 18 was around 8%.

2.5 Synthesis of the fluorinated non-radioactive reference compound 1B

The hydroxamate-based MMPI 1B was prepared as HPLC standard and for in vitro fluorogenic inhibition assays. 1B was prepared by cycloaddition of 3 and 18. First of all, it was shown on model compounds that an excess of alkyne improves overall rate enhancement of the click reaction [18], so a slight excess of alkyne (1.2 eq.) was employed. The active copper(I) species was obtained by using the copper(II) salts

![Scheme 3: Synthesis of 1-azido-4-(2-(2-fluoroethoxy)ethoxy)butane 18](image-url)

(h) TrtCl, Py, 0°C to RT; (i) 1,4-dibromobutane (7 eq.), NaH, DMF, 0°C to RT; (j) NaN₃, DMSO, Δ; (k) DCM, Et₃SiH, TFA, 0°C, evaporation and direct chromatography; (l) TsCl, pyridine; (m) DAST

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CuSO$_4$•5H$_2$O in combination with an excess of the reducing agent sodium ascorbate. By employing an excess of reducing agent, the formation of the oxidative coupling products (most commonly alkyne homo-coupling products or bis-triazoles) was avoided [19]. The solvent tested for this reaction is a mixture of water and a polar solvent, either DMF ($N,N$-dimethylformamide), DMSO (dimethylsulfoxide) or $t$BuOH (tert-butanol). The use of water allows acceleration of the reaction and prevents the need of a base [20]. Reaction of 3 and 18 with 1 mol % of CuSO$_4$•5H$_2$O (0.01 eq.) and 5 mol % of sodium ascorbate in a $t$BuOH/H$_2$O mixture (1/3) led to the formation of the triazole products (as judged by LC-MS analysis of the reaction mixture), albeit at a slow rate not suitable for radiosynthesis with fluorine-18. Addition of 1.1 mol % of the ligand tris-(benzyltriazolylmethyl)amine (TBTA) to the reaction mixture again showed poor conversion (ca. 10%) after one hour, which increased to around 50% conversion after 24 h. To obviate the diminished reactivity of the alkyne 3 (electron-poor alkyne), a very large excess of copper salt and reducing agent was employed. In analogy to the report of Hugenberg et al. [21], the cycloaddition of 3 with the fluoropegylated triazole 18 under optimized conditions proceeded at an acceptable rate when employing 50 mol % of CuSO$_4$•5H$_2$O and 60 mol % of sodium ascorbate in a DMF/H$_2$O mixture (4/1) for 24 h. 1B was formed in around 90% yield (complete conversion observed after 6 h).

Compound 1B was prepared by using a convergent synthesis, with an overall yield of 2.3%. The alkyne 3 was obtained with a satisfactory overall yield of 29%. On the other hand, the multistep organic synthesis of the azide 18 was achieved in a moderate yield of about 8%, partly due to the highly hydrophilic nature of all the intermediates. Given that only small amounts of 1B were needed for HPLC standard and IC$_{50}$ measurements, improvement of the synthesis of 1B was not pursued.

### 2.6 In vitro evaluation of 1B in a fluorogenic inhibition assay

The affinity of 1B was determined in a fluorogenic inhibition assay against three selected recombinant human metzincins: MMP-2 (gelatinase A, 72 kDa type IV collagenase), MMP-9 (gelatinase B, 92 kDa type IV collagenase) and ADAM-17 (TNF alpha converting enzyme). The broad-spectrum inhibitor 1B exhibited affinities in the low nanomolar range for both MMP-2 and MMP-9; a higher IC$_{50}$ value was determined for ADAM-17, the IC$_{50}$ values were 4.67 ± 0.85, 3.67 ± 0.49 and 43.4 ± 7.74 nM, respectively. The introduction of a triazole moiety is an asset for gaining affinity. Indeed, Hugenberg et al. [21] (radio)synthesized a library of triazole-based...
inhibitors with high affinity for a range of MMPs. The gain in affinity was attributed to attractive interactions between the hydrophilic triazole nitrogen atoms and the Zn$^{2+}$ ion in the catalytic domain or other functional substituents in the enzyme.

2.7 Strategy for radiolabelling $[^{18}\text{F}]-\text{1B}$

Two different approaches were considered for the radiosynthesis of the triazole-based MMPI $[^{18}\text{F}]-\text{1B}$.

**First approach:** in two steps by radiofluorination of the Tos-PEG chain 17 followed by an intermediate purification either by SPE purification to remove the free fluorine with an Alumina N Sep-Pak light cartridge or by semi-preparative HPLC (or a combination of SPE and HPLC) to remove the non-reacted fluorine and the unlabelled precursor. Then, the second step would consist of the click chemistry of the resulting $[^{18}\text{F}]-\text{18}$ with the cycloaddition mixture with a final HPLC purification and formulation.

**Second approach:** a one-pot two step reaction [Scheme 4] by radiofluorination of 17 directly followed by the cycloaddition with 3. A final semi-preparative HPLC purification and formulation would be performed.

**Scheme 4.** One-pot two step radiosynthesis of $[^{18}\text{F}]-\text{1B}$

(n) $[^{18}\text{F}]\text{KF/K222, DMSO, 110°C then CuSO}_4\cdot\text{5H}_2\text{O (500 mol%), Na Ascorbate (750 mol%), DMSO/H}_2\text{O 4/1, 60°C, 15 min}$
2.8 Radiosynthesis of $^{18}\text{F}$-1B

**First approach:** the radiofluorination of 17 was performed at 120°C during 10 min. $^{18}\text{F}$-18 was purified by SPE with an Alumina N cartridge followed by semi-preparative HPLC and was obtained with an average RCY of 21 ± 2% (corrected for decay) after purification. Considering the moderate RCY and the time-consuming isolation of the intermediate $^{18}\text{F}$-18, also a one-pot two-step procedure was considered.

**Second approach:** the tosylate PEG azide 17 was radiofluorinated in DMSO at 120°C during 10 min. After 10 min of cooling down, the alkyne 3 dissolved in DMSO and an aqueous mixture of CuSO$_4$•5H$_2$O and sodium ascorbate were added to the crude $^{18}\text{F}$-18. The mixture was heated at 60°C during 15 min. This approach successfully provided $^{18}\text{F}$-1B in a total synthesis time of about 105 min. The radiochemical yield of HPLC-purified $^{18}\text{F}$-1B was 25-27% based on “dried” $^{18}$F-fluorine (corrected for decay), the specific activity was 45-59 GBq/μmol at the end of synthesis and the radiochemical purity > 95%.

Different amounts of catalysts and temperatures were tested. Initially, the cycloaddition with 50 mol % of CuSO$_4$•5H$_2$O and 75 mol % of sodium ascorbate in a DMSO/H$_2$O mixture (4/1) at room temperature for 30 min was attempted but no cycloaddition product was detected. So increase of the temperature to 40°C and then 60°C were performed but it did not afford the formation of $^{18}\text{F}$-1B after 30 min. Therefore, increase of the amount of copper salt and reducing agent 10 times more was carried out. By employing 500 mol % of CuSO$_4$•5H$_2$O and 750 mol % of sodium ascorbate at 60°C, $^{18}\text{F}$-1B was obtained with a satisfactory overall RCY of around 25-27%.

The influence of the reaction time on the radiochemical yield of the cycloaddition process was also tested. The cycloaddition was quite fast and the reaction was complete within about 15 min.

Click chemistry allowed the successful and reliable synthesis of $^{18}\text{F}$-1B in a satisfactory overall RCY and radiosynthesis time.

2.9 Octanol/water partition coefficient

The measured log P of $^{18}\text{F}$-1B was 0.54 ± 0.1, showing the rather hydrophilic nature of the tracer. The relative hydrophilicity of $^{18}\text{F}$-1B is very suitable for imaging MMP activity as most MMPs are water-soluble enzymes.
2.10 \textit{In vitro} stability of $[^{18}\text{F}]-1\text{B}$ in saline, rat plasma and human plasma

After 1 h and 3 h of incubation, 99% of the radioactivity still corresponded to the intact tracer either in saline, rat plasma or human plasma. This indicates that $[^{18}\text{F}]-1\text{B}$ is highly stable \textit{in vitro}. Indeed, the 1,2,3-triazole bond is robust and is known to be stable toward oxidation and reduction.

2.11 Preclinical evaluation of $[^{18}\text{F}]-1\text{B}$ in a HT1080 tumor bearing mouse model

$[^{18}\text{F}]-1\text{B}$ was evaluated in a HT1080 tumor bearing mouse model. This animal model, overexpressing a high number of MMPs/ADAMs, was already employed for the assessment of various biomarkers for the non-invasive imaging of the proteolytic activity [22-25].

Plasma, urine, tumor, kidney and liver samples at 90 min post injection (p.i.) were analysed for parent and metabolite levels by HPLC and radioTLC. In the control group, metabolite assays showed that parent tracer represented $55 \pm 11\%$, $4.5 \pm 2.5\%$, $39 \pm 11\%$, $18 \pm 9\%$ and $25 \pm 23\%$ of total radioactivity in plasma, urine, tumor, kidney and liver samples, respectively. In the blocking group, metabolite assays demonstrated that parent tracer represented $56 \pm 19\%$, $5.8 \pm 2.2\%$, $52 \pm 18\%$, $20 \pm 6\%$ and $18 \pm 7\%$ of total radioactivity, respectively. The metabolism of $[^{18}\text{F}]-1\text{B}$ was relatively moderate in plasma to reach more than half of parent compound present at 90 min p.i. According to HPLC and TLC, only more polar radio-metabolite(s) was detected, which indicates that the radiometabolite(s) is structurally different from $[^{18}\text{F}]-1\text{B}$ and, as a result, probably unlikely to have affinity for active MMPs/ADAMs. The metabolism rate of $[^{18}\text{F}]-1\text{B}$ was very similar under both tracer and co-injection of 1B, therefore the effect of the co-injection of the non-radioactive inhibitor did not influence the metabolism of $[^{18}\text{F}]-1\text{B}$.

The radioactivity uptake in the selected tissues (SUV$_{\text{mean}}$ data presented in mean ± SD) is reported in Figure 3. The uptake in the tumor was low and significantly decreased from an average SUV$_{\text{mean}}$ of $0.40 \pm 0.06$ for the control mice to $0.27 \pm 0.12$ for the blocked animals ($p = 0.0454$) at 90 min p.i. A low uptake in the bone was observed which indicates neglectible 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 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 into the small intestine was very high, considering the enormous uptake.
obtained in the small intestine. The rest of the dissected tissues did not show high accumulation of $[^{18}F]-1B$. Except for the tumor, no significant different retention in any tissue between the control and the homologous block groups was obtained.

Following administration of $[^{18}F]-1B$, the microPET images [Fig 4] exhibited an homogeneous uptake throughout the tumor volume with a relatively low signal to noise ratio. This suggests that tracer binding was not only to membrane-bound ADAMs but also to extracellular MMPs. Indeed, soluble enzymes diffuse around their source. A high kidney uptake was also observed in the microPET images.

The average time-activity curves in the tumor of the control and blocked animals are depicted in Figure 5. $[^{18}F]-1B$ demonstrated a slow wash out in the tumor. PET-SUV$_{mean}$ showed a significant reduction of the tracer accumulation in the tumor at 90 min p.i. between both groups of mice: $0.37 \pm 0.07$ vs $0.25 \pm 0.10$ (p = 0.0380).

In the past two decades, several synthetic MMPIs have been prepared and evaluated [26]. Most of these compounds are nonpeptidomimetic, which were designed on the three-dimensional structure of the MMP active site. They bind in a non-covalent mode and all contain a sulfonyl group which affords hydrogen bonding with the enzymes. Most of them are also hydroxamic acid-based MMPIs. Up to now,

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Figure 3: Ex vivo biodistribution data of tumor bearing mice scanned with $[^{18}F]-1B$ and tumor bearing mice scanned with $[^{18}F]-1B$ and co-injection of 2.5 mg/kg of 1B, at 90 min p.i. of $[^{18}F]-1B \pm 1B$. Bars represent average and error bars SD, n= 6 for each group, * p < 0.05
none of this subtype of radiolabelled MMPIs was evaluated in an animal model of cancer. 

$^{[18F]}$-1B represents the first in vivo evaluation with such model of disease.

Figure 4: In vivo $^{[18F]}$-1B microPET images of a HT1080 tumor bearing mouse shown in transaxial (left) and coronal (right) views. The microPET images correspond to the sum of all the frames from 2 to 90 min p.i. of $^{[18F]}$-1B. The arrows indicate the position of the tumor.

Figure 5: Tumor time-activity curves of tumor-bearing mice scanned with $^{[18F]}$-1B and tumor-bearing mice scanned with $^{[18F]}$-1B and co-injection of 2.5 mg/kg of 1B, from 2 to 90 min p.i. of $^{[18F]}$-1B ± 1B. Points represent average and error bars SD, n= 6 for each group.
In regard of the obtained results, further evaluation should be performed in order to know if $[^{18}\text{F}]-1\text{B}$ may be considered as a suitable radioligand for imaging/quantification of the proteolytic activity. $[^{18}\text{F}]-1\text{B}$ provides a novel lead structure that should be further optimized in order to obtain a suitable PET-tracer. The affinity of $[^{18}\text{F}]-1\text{B}$ may have been too low in relation to the $B_{\text{max}}$ for MMP/ADAM imaging. Further information is also required on possible species differences with regard to MMP/ADAM $B_{\text{max}}$ to understand if there is scope for clinical translation of $[^{18}\text{F}]-1\text{B}$.

Previous work performed on radiolabelled MMPIs exhibited some accumulation in tissues with no MMP overexpression like the liver [27]. MMP levels in plasma and urine were reported to be elevated, for instance, in breast cancer [28]. $[^{18}\text{F}]-1\text{B}$ and most radiolabelled MMP-targeting probes are broad-spectrum inhibitors. As a result, MMP probes may bind or be activated directly in the bloodstream, which was suggested to lead to poor target/non-target contrast when imaging tissues. This finding can explain our small significant reduction obtained for the SUV$_{\text{mean}}$ in the tumor. In addition, Digilio et al. [29] demonstrated that high affinity albumin binding represents a limitation for the specificity reachable by MMPIs. Indeed, albumin accumulates aspecifically in tumors, which decreases the binding potential of arylsulfone-based MMPIs.

On the other hand, a ‘more’ specific inhibitor targeting a specific MMP could be of great interest and led to high signal to noise ratio. However, the preparation of such a probe is very challenging, as for instance, MMP-2, -3, -7, -8 and -9 possess homologous active site features [26]. Therefore, antibody based recombinant proteins might be more suited for the purpose of subtype selectivity.

3. Conclusion

$[^{18}\text{F}]-1\text{B}$ was successfully radiolabelled by click chemistry with a good overall RCY. This relatively hydrophilic tracer showed high in vitro stability in saline and rat/human plasma. The hydroxamate-based MMPI 1B exhibited nanomolar affinities in vitro. HT1080 tumor was detected in the microPET image and uptake of $[^{18}\text{F}]-1\text{B}$ was shown to be MMP-mediated. Further analysis regarding the $B_{\text{max}}$ for MMP/ADAM imaging should be performed in order to validate $[^{18}\text{F}]-1\text{B}$ as a potential radiopharmaceutical for the non-invasive imaging of the proteolytic activity. $[^{18}\text{F}]-1\text{B}$ provides a novel lead structure that should be further optimized in order to obtain an appropriate PET-tracer.
4. 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 performed using unpaired two-sided student t-test. Data were considered statistically significant when p values were smaller than 0.05.

5. Experimental section

5.1 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- and ¹³C-NMR spectra were recorded on a Varian AMX400 spectrometer (400 and 100 MHz respectively). 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-d₆: δ 2.504 for ¹H and for MeOH: δ 3.312, 4.867 for ¹H). 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).

5.2 Molecular modeling of a library of piperazine-based MMP inhibitors

The crystal structure of MMP-9 was downloaded from the Protein Data Bank (PDB) (PDB code 2OW1). All molecules were drawn using ChemaxonMarvinSketch (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.

5.3 4-((Tert-butoxycarbonyl)-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxylic acid (6)

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 4 (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 5. Freshly distilled triethylamine (8.23 mL, 59.0 mmol), 4-dimethylaminopyridine (721 mg, 5.90 mmol) and 4-methoxyphenylsulfonyl chloride (6.09 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 6 (10.2 g, 86%):

1H NMR (400 MHz, DMSO-d6) δ 12.97 (s, 1H), 7.83 – 7.74 (m, 2H), 7.10 – 7.01 (m, 2H), 4.32 (t, J = 7.3 Hz, 1H), 4.06 (dd, J = 12.4, 7.3 Hz, 1H), 3.83 – 3.67 (m, 5H), 3.56 – 3.40 (m, 2H), 3.32 (dd, J = 12.5, 7.3 Hz, 1H), 1.39 (s, 9H).

HRMS-ESI: calc for C17H25N2O7S ([M + H]+), 401.5, found 401.2; calc for C17H28N3O7S ([M + NH4]+), 418.5, found 418.3; calc for C34H49N4O14S2 ([2M + H]+), 801.9, found 801.5.

5.4 Methyl 1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxylate hydrochloride (7)

Thionyl chloride (8.72 mL, 120.0 mmol) was added dropwise to a solution of 4-((tert-butoxycarbonyl)-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxylic acid 6 (9.60 g, 24.0 mmol) in 50 mL of methanol at 0°C. The mixture was stirred overnight at room temperature. The reaction mixture was concentrated under re-
duced pressure to a solid residue, which was triturated with 5% methanol/hexane to give the title compound 7 (6.90 g, 82%):

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.83 – 7.75 (m, 2H), 7.08 – 7.02 (m, 2H), 3.87 (t, $J = 8.1$ Hz, 1H), 3.65 (s, 3H), 3.41 – 3.13 (m, 3H), 2.95 – 2.71 (m, 3H), 1.91 (s, 1H).

HRMS-ESI: calc for $C_{13}H_{19}N_2O_5S$ ([M + H]$^+$ without HCl), 315.4, found 315.2; calc for $C_{26}H_{37}N_4O_{10}S_2$ ([2M + H]$^+$ without HCl), 629.7, found 629.3.

5.5 4-((Trimethylsilyl)ethynyl)benzoyl chloride (9)

Thionyl chloride (6.25 mL, 86.0 mmol) was added dropwise to a solution of 4-((trimethylsilyl)ethynyl)benzoic acid 8 (4.69 g, 21.5 mmol) in 50 mL of chloroform at 0°C. The mixture was kept under reflux for 2 h. The reaction mixture was concentrated under reduced pressure and coevaporated three times with chloroform to give the corresponding acyl chloride 9, which was immediately used without further purification.

5.6 Methyl 1-((4-methoxyphenyl)sulfonyl)-4-(4-((trimethylsilyl)ethynyl)benzoyl)piperazine-2-carboxylate (10)

To a solution of amine hydrochloride 7 (5.02 g, 14.3 mmol) in 10 mL of water and 20 mL of p-dioxane at 0°C was added subsequently freshly distilled triethylamine (6.18 mL, 44.3 mmol), 4-dimethylaminopyridine (175 mg, 1.43 mmol) and dropwise 4-((trimethylsilyl)ethynyl)benzoyl chloride 9 freshly prepared as described above. 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 (6.11 g, 83%):

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.15 – 8.07 (m, 2H), 7.92 – 7.84 (m, 2H), 7.78 – 7.70 (m, 2H), 7.09 – 7.02 (m, 2H), 4.63 (t, $J = 6.7$ Hz, 1H), 3.79 (s, 3H), 3.59 – 3.37 (m, 3H), 3.32 (dd, $J = 12.5$, 6.6 Hz, 1H), 3.25 – 3.13 (m, 2H), 0.08 (s, 9H).

HRMS-ESI: calc for $C_{25}H_{31}N_2O_6Si$ ([M + H]$^+$), 515.7, found 515.3; calc for $C_{25}H_{34}N_3O_6Si$ ([M + NH$_4$]$^+$), 532.7, found 532.3; calc for $C_{25}H_{30}N_2O_6SSiNa$ ([M + Na]$^+$), 537.7, found 537.3; calc for $C_{50}H_{61}N_4O_{12}Si_2$ ([2M + H]$^+$), 1030.3, found 1029.6.
5.7 Methyl 4-(4-ethylnylbenzoyl)-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxylate (11)

To a solution of methyl 1-((4-methoxyphenyl)sulfonyl)-4-(4-((trimethylsilyl)ethylnyl)benzoyl)piperazine-2-carboxylate 10 (5.82 g, 11.3 mmol) in 60 mL of THF at 0°C was added subsequently acetic acid (403 μL) and acetic anhydride (403 μL). Dropwise addition of tetra-n-butylammonium fluoride (7.10 g, 22.5 mmol) in 60 mL of THF was followed and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and partitioned between EtOAc and H₂O. The EtOAc layer (2 x 100 mL) was washed with brine, dried over Na₂SO₄, filtered and concentrated in vacuo to give the title compound 11 (4.75 g, 95%): ¹H NMR (400 MHz, DMSO-d₆) δ 8.08 – 8.00 (m, 2H), 7.80 – 7.69 (m, 4H), 7.09 – 7.03 (m, 2H), 4.33 (t, J = 6.5 Hz, 1H), 3.66 – 3.56 (m, 4H), 3.54 – 3.34 (m, 3H), 3.25 – 3.13 (m, 2H), 3.04 (s, 1H).

HRMS-ESI: calc for C₂₂H₂₃N₂O₆S ([M + H]+), 443.5, found 443.2; calc for C₂₂H₂₆N₃O₆S ([M + NH₄]+), 460.5, found 460.3; calc for C₂₂H₂₂N₂O₆Na ([M + Na]+), 465.5, found 465.3; calc for C₄₄H₄₅N₄O₁₂S₂ ([2M + H]+), 906.0, found 905.5; calc for C₄₄H₄₈N₅O₁₂S₂ ([2M + NH₄]+), 903.0, found 902.5; calc for C₄₄H₄₄N₄O₁₂S₂Na ([2M + Na]+), 908.0, found 907.5.

5.8 4-(4-Ethylnylbenzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide (3)

Preparation of NH₂OK/NH₂OH solution: NH₂OH.HCl (6.74 g, 97.0 mmol) was dissolved in MeOH (35 mL) by heating to reflux overnight. The solution was cooled at 0°C, and a solution of KOH (8.16 g, 145.5 mmol) in MeOH (20 mL) was added in one portion. The resulting suspension was used without prior removal of precipitated material. A solution of methyl ester 11 (4.29 g, 9.70 mmol) in NH₂OK/NH₂OH solution (as described above) was stirred at room temperature for 2 days. The reaction mixture was taken up in dilute aqueous HCl (pH = 3, 100 mL), extracted with EtOAc (2 x 100 mL), dried over Na₂SO₄, filtered and concentrated in vacuo. The residue was purified by column chromatography (0-5% MeOH in EtOAc) to afford hydroxamic acid 3 (2.24 g, 52%) as a white solid: ¹H NMR (400 MHz, DMSO-d₆) δ 10.44 (s, 1H), 8.87 (s, 1H), 8.08 – 8.00 (m, 2H), 7.81 – 7.68 (m, 4H), 7.10 – 7.02 (m, 2H), 4.45 (t, J = 6.8 Hz, 1H), 3.64 (dd, J = 12.3, 6.7 Hz, 1H), 3.55 – 3.37 (m, 3H), 3.26 – 3.15 (m, 2H), 3.04 (s, 1H).
HRMS-ESI: calc for C_{21}H_{22}N_{3}O_{6}S ([M + H]^+), 444.5, found 444.5; calc for C_{21}H_{25}N_{4}O_{6}S ([M + NH_4]^+), 461.5, found 461.4; calc for C_{21}H_{21}N_{3}O_{6}SNa ([M + Na]^+), 466.5, found 466.5; calc for C_{42}H_{43}N_{6}O_{12}S_{2} ([2M + H]^+), 888.0, found 887.5; calc for C_{42}H_{46}N_{7}O_{12}S_{2} ([2M + NH_4]^+), 905.0, found 904.5; calc for C_{42}H_{42}N_{6}O_{12}S_{2}Na ([2M + Na]^+), 909.9, found 910.0.

5.9 4-(4-(1-(4-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)butyl)-1H-1,2,3-triazol-4-yl)benzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide (1B)

1H-NMR (400 MHz, CD_{3}OD): 8.41 (s, 1H), 7.88 (d, 2H, J = 8.4 Hz), 7.78 (d, 2H, J = 8.4 Hz), 7.43 (d, 2H, J = 8.4 Hz), 7.08 (d, 2H, J = 8.8 Hz), 4.54 (dd, 1H, J = 4.0, 5.2 Hz), 4.52 (t, 2H, J = 7.2 Hz), 4.42 (m, 1H), 3.89 (s, 3H), 3.74 (dd, 1H, J = 2.8, 4.0 Hz), 3.66-3.56 (m, 11H), 3.53 (t, 2H, J = 6.4 Hz), 2.05 (m, 2H), 1.61 (m, 2H).

HSQC-NMR (100 MHz, CD_{3}OD): 130.8, 128.5, 126.4, 122.5, 115.4, 84.8, 83.1, 71.6, 71.6, 70.9, 70.6, 56.0, 50.9, 48.8, 43.2, 28.0, 27.2.

HRMS-ESI: calc for C_{31}H_{42}FN_{6}O_{9}S ([M + H]^+), 693.8, found 693.6.

5.10 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_{2}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 0.5 mL anhydrous acetonitrile at 130°C.

5.11 1-Azido-4-(2-(2-(2-[18F]fluoroethoxy)ethoxy)ethoxy)butane ([18F]-18) (two-step reaction (first approach))

A solution of 17 (1.0 mg, 2.49 μmol) in anhydrous DMSO (500 μL) was added to the dry [18F]KF / kryptofix[2.2.2] complex and was heated for 10 min at 120°C. The mixture was allowed to cool down for 10 min and was then passed through an
Alumina N Sep-Pak light cartridge. The reaction vial was rinsed with 700 μL H₂O which was passed through the Alumina N cartridge. The cartridge was then dried by airflow. To remove unreacted [¹⁸F]fluorine and unreacted 17, [¹⁸F]-18 was purified by semi-preparative reverse phase HPLC using a Symmetry Prep C18 column (7.8 mm x 300 mm, 7 μm) from Waters, equipped with a 20 x 4.6 mm² precolumn. The mobile phase used was a mixture of 0.05 M monosodium phosphate buffer NaH₂PO₄/MeOH/THF 550/220/180 at a flow rate of 2.5 mL.min⁻¹. The column effluent was monitored using an Elite Lachrom VWR Hitachi L-2400 UV detector (λ = 254 nm, AUFS = 0.5) and a Bicron frisk-tech radioactivity detector. Sample injection was carried out using an injector block with a loop of 1 mL. The retention time of [¹⁸F]-18 was 9.4-13.6 min (the retention time of 17 was 14.2 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 700 μL of DMSO to yield purified [¹⁸F]-18.

5.12 4-((1-(4-(1H-1,2,3-triazol-4-yl)benzoyl)-N-hydroxy-1-((4-methoxyphenyl)sulfonyl)piperazine-2-carboxamide ([¹⁸F]-18) (one pot two-step reaction (second approach))

The copper(I) species was prepared in situ by using copper(II) sulfate / sodium ascorbate 10 min before addition to the crude [¹⁸F]-18. To a solution of copper(II) sulfate pentahydrate (0.4 M, 31.1 μL, 12.4 μmol) was added sodium ascorbate (0.6 M, 31.1 μL, 18.7 μmol) and 62.8 μL H₂O. The mixture was stirred for 10 min.

A solution of 17 (1.0 mg, 2.49 μmol) in anhydrous DMSO (400 μL) was added to the dry [¹⁸F]KF / kryptofix[2.2.2] complex and heated for 10 min at 120°C. The mixture was allowed to cool down for 10 min. A solution of 3 (1.66 mg, 3.74 μmol) in anhydrous DMSO (100 μL) and a solution of copper(II) sulfate pentahydrate / sodium ascorbate (as prepared above) were added. The mixture was heated at 60°C during 15 min. The mixture was allowed to cool down for 5 min then it was diluted with a solution of 0.1 M Na₂EDTA (15 mL) and passed through a Sep-Pak Plus C18 cartridge (preconditioned with 10 mL of ethanol and 10 mL of water). The cartridge was washed with 10 mL of water for injection, followed by elution of crude [¹⁸F]-1B with 2 mL of absolute ethanol. The solvent was evaporated under reduced pressure and a stream of argon at 60°C within 10-15 min. The dried crude product was re-suspended in water. Separation of the radiosynthesized and unlabelled compounds was performed by semi-preparative reverse phase HPLC using a Symmetry Prep
C18 column (7.8 mm x 300 mm, 7 μm) from Waters, equipped with a 20 x 4.6 mm² precolumn. The mobile phase used was a mixture of 0.05 M monosodium phosphate buffer NaH2PO4/MeOH/THF 700/70/180 at a flow rate of 3.5 mL.min⁻¹. The column effluent was monitored using an Elite Lachrom VWR Hitachi L-2400 UV detector (λ = 254 nm, AUFS = 0.5) and a Bicron frisk-tech radioactivity detector. Sample injection was carried out using an injector block with a loop of 1 mL. The retention time of [18F]-1B was 14.8-16.6 min (the retention time of 3 was 36.4 min). The HPLC-collected fraction was diluted with about 100 mL of water for injection and passed through a Sep-Pak Light C18 cartridge preceded of an Alumina N 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 redissolved in 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 X-Terra RP 18 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 30/70 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]-1B was 13.0 min.

The cycloaddition of the alkyne 3 with [18F]-1B was monitored by silica gel TLC analysis using EtOAc/MeOH 9/1 as mobile phase. The reaction was followed from 0 to 30 min and reaction mixture samples (2.5 μL of reaction mixture diluted in 1 mL EtOAc) were taken every 5 min.

5.13 In vitro evaluation of 1B in a fluorogenic inhibition assay

Recombinant ADAM-17 (ectodomain) was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant catalytic domain (CD) of human MMP-2 was from Biomol International (Butler Pike, PA, USA). Recombinant human MMP-9 CD without fibronectin type II insert (expressed in E. Coli as described [30,31]) was 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 1B. For MMP-2 and -9, 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-2 or MMP-9 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). For MMP-2 and MMP-9, nine-point inhibition curves (0, 0.5, 1, 2, 6, 12.5, 25, 50 and 100 nM) were plotted in GraphPad Prism. For ADAM-17, nine-point inhibition curves (0, 20, 50, 100, 125, 250, 500, 750 and 1000 nM) were plotted. IC₅₀ values were determined by sigmoidal fitting. Each experiment was performed in triplicate.

5.14 In vitro stability of [¹⁸F]-1B in saline, rat plasma and human plasma

5.14.1 Saline stability
The stability of [¹⁸F]-1B was evaluated in vitro in saline. Formulated [¹⁸F]-1B 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 X-Terra RP 18 column (4.6 mm x 250 mm, 5 µm) at a flow rate of 1.5 mL.min⁻¹ with ACN/H₂O 30/70 as mobile phase. One-minute fractions of the eluate were collected and radioactivity in the fractions was counted with a gamma counter.

5.14.2 Rat plasma stability
The stability of [¹⁸F]-1B was evaluated in vitro in rat plasma. Blood from rats was centrifuged at 3000 rpm for 5 min, subsequently the supernatant was taken. 100 µL of formulated [¹⁸F]-1B was dissolved in 1 mL rat plasma and incubated at 37°C for 3 h. After 1 h and 3 h of incubation, aliquots of 250 µL were taken. 750 µL of ACN were added in order to deproteinise the plasma; then it was centrifuged 3 min at 3000 rpm. The supernatant was passed through a Millex Filter (0.22 µm) and
subsequently diluted with 500 µL ACN and 500 µL H₂O and analysed by analytical HPLC as described previously.

5.14.3 Human plasma stability
The stability of \(^{18}\text{F}\)-1B was evaluated \textit{in vitro} in human plasma. Full 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 \(^{18}\text{F}\)-1B 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.

5.15 Octanol/water partition coefficient
About 5 kBq of formulated \(^{18}\text{F}\)-1B 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 layers was counted in a gammacounter. The experiment was performed in triplicate. The partition coefficient (log P) was calculated as: \( \log P = \log_{10} \left( \frac{\text{cpm}_{\text{octanol layer}}}{\text{cpm}_{\text{aqueous layer}}} \right) \)

5.16 HT1080 fibrosarcoma xenograft mouse model

5.16.1 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).

5.16.2 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 T₂₅ culture flask. Cells were grown in a humidified
atmosphere containing 5% CO₂ and were passaged twice per week. (2-2.5) x 10⁶ 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).

5.17 MicroPET studies
Animals were scanned when the tumors 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 scanned with [¹⁸F]-1B and with or without coinjection of 2.5 mg/kg of 1B. [¹⁸F]-1B (16.0 ± 5.1 MBq, 0.31 ± 0.11 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. 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.

5.18 Ex vivo biodistribution
After the microPET scan, ex vivo biodistribution studies were performed on the sacrificed mice. 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_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_mean), defined as [tissue activity concentration (MBq/g)/(injected activity (MBq)/mouse body weight (g))].
5.19 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\(_{\text{mean}}\) using the following formula: \[\text{tissue activity concentration (MBq/cc)/}(\text{injected activity (MBq)/mouse body weight (g)})\].

5.20 Metabolite analysis (plasma, urine, tumor, kidney and liver) of \([^{18}\text{F}]-1\text{B}\) in a HT1080 xenograft mouse model

Metabolite analysis was performed on plasma and urine after the \textit{ex vivo} biodistribution. Protein precipitation was carried out: 750 µL of ACN were added to an aliquot of 250 µL of plasma which was then centrifuged for 3 min at 3000 rpm. The supernatant was passed through a Millex Filter (0.22 µm) and subsequently diluted with 500 µL ACN and 500 µL H\(_2\)O and analysed by analytical HPLC using a X-Terra RP 18 column (4.6 mm x 250 mm, 5 µm) at a flow rate of 1.5 mL.min\(^{-1}\) with ACN/H\(_2\)O 30/70 as mobile phase. One minute fractions of the eluate were collected and radioactivity in the fractions was determined with a gamma counter. Urine samples were treated in the same way as plasma. Tumor, liver and kidney were dissolved in 1 mL PBS and were triturated using an Ultra-Turrax mixer. Tissue homogenates were diluted with 10 mL H\(_2\)O and passed through a normal C18 cartridge. Then, 0.5 mL ACN was eluted through the cartridge for radio-TLC and radio-HPLC analysis.

Funding Sources

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


