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Evaluation of $[^{18}F]$BR420 and $[^{18}F]$BR351 as radiotracers for MMP-9 imaging in colorectal cancer

Abbreviated title: Evaluation of $^{18}$F-labeled MMP-9 radiotracers in a colorectal cancer model

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Abstract:

Introduction: MMP-9 is a zinc dependent endopeptidase which is involved in the proteolytic degradation of the extracellular matrix and plays an important role in cancer migration, invasion and metastasis. The aim of this study was to evaluate the potential of MMP-tracers [18F]BR420 and [18F]BR351 for MMP-9 imaging in a colorectal cancer xenograft model.

Methods: [18F]BR420 and [18F]BR351 were synthesized using an automated synthesis module. For [18F]BR420 a novel and improved radiosynthesis was developed. Plasma stability and MMP-9 targeting capacity of both radiotracers was compared in the Colo205 colorectal cancer model. MMP-9 and MPP-2 expression in the tumors was evaluated by immunohistochemistry and in situ zymography.

Results: µPET imaging as well as ex vivo biodistribution revealed a higher tumor uptake for [18F]BR420 (3.15 ± 0.03%ID/g versus 0.94 ± 0.18%ID/g for [18F]BR351 at 2 h p.i.) but slower blood clearance compared to [18F]BR351. [18F]BR351 was quickly metabolized in plasma with 20.28 ± 5.41% of intact tracer remaining at 15 min p.i. [18F]BR420 on the other hand displayed a higher metabolic stability with >86% intact tracer remaining at 2 h p.i. Immunohistochemistry revealed presence of MMP-9 and MMP-2 in the tumor tissue, which was confirmed by in situ zymography. However, autoradiography analysis of tracer distribution in the tumors did not correlate with MMP-9 expression.

Conclusion: [18F]BR420 displayed a higher tumor uptake and higher stability compared to [18F]BR351, yet a low tumor-to-blood ratio and discrepancy between tracer distribution and MMP-9 immunohistochemistry. Therefore both tracers will not be usefulness for MMP-9 imaging in colorectal cancer.
1. Introduction

Matrix metalloproteinases (MMPs) comprise a large family of several structurally related zinc-dependent endopeptidases which contribute to the extracellular matrix (ECM) proteolysis. All MMP family members present a common domain structure composed of at least a predomain, a catalytic domain and a highly conserved active site domain. The catalytic core-domain contains the active, protein-degrading ability of the proteinase, which includes a metal binding site for Zn$^{2+}$. The predomain acts as an internal inhibitor of MMP activity, and activation occurs with cleavage of the predomain leading to a conformational change to the active site. MMPs are synthesized as secreted or membrane-associated (MT-MMPs) inactive zymogens, and must be proteolytically processed to an active state. This processing involves removal of a cysteine residue that interacts with zinc ions from the active site, thereby resulting in MMP activation. Based on their structure and substructure specificity, human MMPs are divided into five groups: collagenases, gelatinases, stromelysins, membrane-type metalloproteinases and the others.

Because of their capacity to degrade the ECM, MMPs are known to play an important role in several diseases. In particular in cancer, excess matrix degradation is one of the hallmarks and an important component in the process of tumor progression. Among the large number of MMPs, gelatinases A (MMP-2) and gelatinases B (MMP-9) are the most frequently investigated proteases for their involvement in cancer migration, invasion and metastasis. Particularly, MMP-9 can show both anti-cancer and tumor-promoting effects. A disturbance of MMP-9 function in animals has been correlated with a decrease in tumor progression while on the other hand overexpression of MMP-9 would induce angiogenesis and increase malignant transformation. Colorectal cancer is the third most common cancer worldwide and the forth most common cause of death. Considerable evidence has implicated MMPs in established colorectal cancer, showing either an increased MMP expression or increased MMP activity in this type of tumor. Overexpression of MMP-9 has been demonstrated in human colorectal cancers, where the degree of overexpression has been shown to correlate with stage of disease and/or prognosis.

Due to their role in cancer, MMPs have gained significant attention as therapeutic target. The search for MMP inhibitors has been largely carried out during the last decades. There are several requirements for the design of an appropriate MMP inhibitor: the presence of a functional group providing a hydrogen bond with the enzyme, side chains able to interact with
the enzyme sub sites and a zinc binding group. The development of MMP inhibitors with a high selectivity towards a particular MMP however remains a big challenge. Most inhibitors are broad spectrum MMP inhibitors although some display favourable binding affinity for a specific MMP type. Recently, a barbiturate-based fluorescent MMP inhibitor (Cy5.5-AF443) with a high affinity toward MMP-2 and MMP-9 has been successfully used for the in vivo evaluation of MMP-2/-9 expression in mouse models of colorectal cancer.\textsuperscript{20, 21} The use of the fluorescent label however requires the application of invasive endoscopic techniques for imaging of MMP activity in the colon. Targeting MMP with Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT) activity-based imaging probes could offer a non-invasive tool to early detect and diagnose MMP-related diseases (e.g. colorectal cancer), to elucidate the exact role of specific MMPs in normal and disease states or to understand the mechanism of action of these enzymes. During the last decades several research groups have been developing PET and SPECT probes for MMP-targeted imaging [see\textsuperscript{22} for a recent review]. Most of them are however broad spectrum MMP inhibitors and currently there are no successful MMP selective radiotracers available. Consequently, in vivo imaging of a particular MMP type activity remains a challenge.

Recently, \textsuperscript{[18}F\textsuperscript{]}BR420 and \textsuperscript{[18}F\textsuperscript{]}BR351 (Figure 1) have been described to be brain penetrant broad spectrum MMP PET radiotracers with favourable affinities for activated MMP-2 and MMP-9 (\textsuperscript{[18}F\textsuperscript{]}BR420, IC\textsubscript{50} = 7 ± 2 and 23 ± 9 nM; and \textsuperscript{[18}F\textsuperscript{]}BR351, IC\textsubscript{50} = 50 ± 27 and 4 ± 3 nM, for MMP-9 and MMP-2, respectively).\textsuperscript{23, 24} Both compounds belong to different families of MMP ligands with BR420 being a barbiturate-based MMP inhibitor and BR351, a hydroxamate-based MMP inhibitor. The in vivo evaluation of \textsuperscript{[18}F\textsuperscript{]}BR420 has so far been limited to an in vivo biodistribution study up to 2 h p.i. in adult C57/BL6 mice, indicating no tissue specific accumulation of the radiotracer.\textsuperscript{23} \textsuperscript{[18}F\textsuperscript{]}BR351 on the other hand has recently successfully been used to image MMP activity after transient middle cerebral artery occlusion in mice.\textsuperscript{25} \textsuperscript{[18}F\textsuperscript{]}BR420 and \textsuperscript{[18}F\textsuperscript{]}BR351 have not yet been evaluated in an oncology context. Considering the prognostic value of MMP-9 expression in colorectal cancer, the aim of the present study was to evaluate and compare the in vivo behaviour of \textsuperscript{[18}F\textsuperscript{]}BR420 and \textsuperscript{[18}F\textsuperscript{]}BR351 in a mouse model of human colorectal cancer. The tumour targeting properties were evaluated as well as the in vivo stability at several time points post tracer injection. For \textsuperscript{[18}F\textsuperscript{]}BR420, a novel and improved automated radiosynthesis was developed.
2. Experimental

2.1. General procedures and materials

Unless stated otherwise, all chemical reagents and solvents were obtained from commercial sources and used without further purification. Characterization of all compounds was performed by $^1$H NMR, $^{13}$C NMR and mass spectrometry. $^1$H NMR and $^{13}$C NMR spectra were recorded on a 400 MHz Bruker Advance DRX spectrometer and analysed by use of MestReNova analytical chemistry software. Chemical shifts are in ppm and coupling constants are in hertz (Hz). Purities were determined using two different UPLC systems based either on mass detection or on UV detection: a Waters Acquity UPLC system coupled to a Waters TUV detector and a Waters TQD ESI mass spectrometer. On both systems, a Waters Acquity UPLC BEH C18 1.7 µm, 2.1 x 50 mm column was used. Solvent A: water with 0.1 % formic acid; Solvent B: acetonitrile with 0.1 % formic acid. Method I: 0.7 mL/min, 0.15 min 95 % A, 5% B then in 1.85 min from 95 % A, 5 % B to 10 % B, 0% A, then 0.25 min, 100 % B, 0 % A, 0.75 min (0.35 mL/min) 95 % A, 5 % B. Method II (purity method): 0.4 mL/min, 0.15 min 95 % A, 5 % B then in 4.85 min from 95 % A, 5% B to 100 % B, 0% A, then 0.25 min, 100 % B, 0 % A, 0.75 min 95 % A, 5 % B. Where necessary, flash purification was performed with a Biotage ISOLERA One flash system equipped with an internal variable dual-wavelength diode array detector (200-400 nm). Biotage® SNAP cartridges were used for normal phase purification (KP-Sil 10 – 100 g, flow rate: 10 – 100 mL/min). For radiosynthesis, no carrier-added [${}^{18}$F]F$^-$ was produced by the $^{18}$O(p,n)${}^{18}$F nuclear reaction in a Siemens Eclipse HP cyclotron by bombardment of $[{}^{18}$O]$\text{H}_2$O (Rotem Industries, Israel). Radiosynthesis of [${}^{18}$F]BR420 and [${}^{18}$F]BR351 was carried out on an automated synthesis module (Fluorsynthon I, Comecer Netherlands, The Netherlands) specifically adapted for these radiosynthesis. Radiochemical yields were calculated from the theoretical initial amount of [${}^{18}$F]F$^-$ and decay corrected to end of bombardment (EOB). Purification of [${}^{18}$F]BR420 and [${}^{18}$F]BR351 was carried out on an automated synthesis module (Fluorsynthon I, Comecer Netherlands, The Netherlands) specifically adapted for these radiosynthesis. Radiochemical yields were calculated from the theoretical initial amount of [${}^{18}$F]F$^-$ and decay corrected to end of bombardment (EOB). Purification of [${}^{18}$F]BR420 and
[18F]BR351 following radiosynthesis was performed by reverse phase semi-preparative HPLC using a Knauer HPLC pump and a Smartline UV detector (λ = 254 nm) in line with a Hi-Rad 1000-CD-X CdWO4 scintillation detector (Scionix, The Netherlands). Radiochemical and chemical purity was determined by analytical reverse phase HPLC using a Shimadzu LC-20AT HPLC pump equipped with a SPD-20A UV/VIS detector (Shimadzu, Japan) in series with a NaI scintillation detector for radiation detection (Raytest, Germany). The recorded data were processed by the GINA-Star 5 software (Raytest, Germany). Radioactivity in samples from animal studies was measured in a Wizard2 2480 automatic gamma counter (Perkin Elmer, Finland).

2.2. Chemistry

2.2.1. 5-[4-(2-chloroethyl)piperazin-1-yl]-5-(4-phenoxyphenyl)pyrimidine-2,4,6-trione (I)

N-(2-Chloroethyl) piperazine (42%) was prepared as the trifluoroacetic acid salt 3 by the reaction of N-Boc piperazine 2 (97%) with bromo-chloroethane. Barbiturate scaffold 4 (67%) was prepared as previously described.26 To a cooled solution of 4 (0.186 g, 0.628 mmol) in DMF (2 mL) at 0 °C, a solution of NBS (0.112 g, 0.628 mmol) in DMF (2 mL) was dropwise added over a period of 15 min. Then, the addition funnel was rinsed with DMF (2 mL) and the rinse was also added to the reaction mixture. After stirring for 20 min, compound 3 (0.165 g, 0.628 mmol) and K2CO3 (0.174 g, 1.256 mmol) were added and the reaction mixture was stirred at 0 °C for 60 min and then allowed to reach room temperature and stirred for additional 20 h. The reaction mixture was then diluted in EtOAc and an aqueous solution of citric acid (0.628 mmol, 0.1 M) was added. The organic phase was separated and washed with water and brine, dried over Na2SO4, filtered and concentrated under reduced pressure. The crude product was purified by manual chromatography (Hexane/EtOAc, 1/2, V/V) to yield compound 1 (0.054 g, 20%). 1H NMR (400MHz, CDCl3): δ = 9.18 (bs, 2H), 7.43-7.39 (m, 4H), 7.21-7.17 (m, 1H), 7.08-7.02 (m, 4H), 4.25-4.22 (m, 2H), 3.80-3.78 (m, 2H), 3.40-3.34 (m, 4H), 2.60-2.56 (m, 4H) ppm. 13C NMR (100 MHz, CDCl3): δ 30.72, 42.94, 64.79, 74.30, 78.61, 78.98, 79.26, 118.19, 119.37, 124.19, 128.82, 129.23, 129.56, 130.19, 149.35, 153.55, 153.97, 155.70, 157.60, 169.91 ppm. MS (ESI) m/z = 487.2 [M + H + CH3CN]+.

2.3. Radiochemistry

[18F]F− was transferred from the cyclotron to the synthesis module and trapped on an activated anion exchange cartridge (QMA Sep-Pak Light, Waters). [18F]F− was eluted with 1 mL of a
solution of Kryptofix 222 (220 mg) and K$_2$CO$_3$ (40 mg) in CH$_3$CN/H$_2$O (83/17, V/V) and collected in the reaction vial. The solution was evaporated at 130°C with a stream of helium (1 bar) and under vacuum for 8 min. To ensure dryness, azeotropic distillation of 1 mL of acetonitrile was performed twice for 6 min at 140 °C with a stream of helium (1 bar). The reaction was then cooled down to 90 °C with compressed air and a solution of the precursor 1 (2 mg in 1 mL of DMSO) was added to the reaction vial. The reaction mixture was heated at 120°C for 10 min, cooled down to 80 °C and diluted with 1.9 mL of a solution of 0.05 M NaOAc, pH 5.5 / 96% EtOH (50/50, V/V). This solution was passed through an Alumina N light cartridge (Waters) and injected into the HPLC loop. The crude reaction mixture was purified on a Phenomenex Luna C18, 10 µm, 10 x 250 mm column and eluted with a solution of 0.05 M NaOAc, pH 5.5 / 96 % EtOH (50/50, V/V) at 4 mL/min. $[^{18}\text{F}]$BR420 was collected ($R_t = 12-13$ min), filtered through a 0.22 µm sterile filter (PES syringe filter, 25 mm, 0.2 µm) and diluted with 0.9% sodium chloride solution to reduce the EtOH concentration to < 10 %.

Chemical and radiochemical purity of the tracer was measured by analytical HPLC (Phenomenex Luna C18, 5 µm, 4.6 x 150 mm) eluted with 0.1 % TFA in H$_2$O / 0.1 % TFA in CH$_3$CN (55/45, V/V) at a flowrate of 1 mL/min. The specific activity was determined using a UV calibration curve ($\lambda = 272$ nm).

$[^{18}\text{F}]$BR351 was reproduced according to the literature with slight modifications. A solution of the precursor 5 (2 mg in 1 mL of CH$_3$CN) was added to the azeotropically dried $[^{18}\text{F}]$K$_{222}$F and the reaction mixture was heated at 90 °C for 20 min, cooled down to 80 °C and diluted with 1.9 mL of a solution of 0.05 M NaOAc, pH 5.5 / 96 % EtOH (45/55, V/V). This solution was passed through an Alumina N light cartridge (Waters) and injected into the HPLC loop. The crude reaction mixture was purified on a Phenomenex Luna C18, 10 µm, 10 x 250 mm column and eluted with a solution of 0.05 M NaOAc, pH 5.5 / 96 % EtOH (45/55, V/V) at 3 mL/min. $[^{18}\text{F}]$BR351 eluted with a retention time of 10-11 min, was collected, filtered through a 0.22 µm sterile filter (PES syringe filter, 25 mm, 0.2 µm) and diluted with 0.9% sodium chloride solution to reduce the EtOH concentration to < 10 %. Chemical and radiochemical purity of the tracer was measured by analytical HPLC (Phenomenex Luna C18, 5 µm, 4.6 x 150 mm) eluted with 0.1 % TFA in H$_2$O / 0.1 % TFA in CH$_3$CN (52/48, V/V) at a flowrate of 1 mL/min. The specific activity was determined using a UV calibration curve ($\lambda = 254$ nm).

2.4. Cell cultures and animal model
The experimental protocol was approved by the Antwerp University Ethical Committee for Animal Experiments (ECD 2015-13), and all applicable institutional and national guidelines for the care and use of animals were followed. Human colorectal cancer cell line Colo205 was used for induction of xenografts because of its known MMP-9 expression. \(^{27}\) Low-passage Colo205-luc2 cells (PerkinElmer, Maltham, MA, USA) were cultured in RPMI1640 medium enriched with 10 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 % penicillin-streptomycin (Invitrogen, Merelbeke, Belgium) at 37 °C and 5 % CO\(_2\) in a humidified incubator. For inoculation, cells were harvested by trypsinization with trypsin-EDTA (0.05 %; Invitrogen) and counted with the Muse™ Cell Count and Viability Assay (Merck Millipore, Overijse, Belgium). Colo205 cells were then resuspended in sterile phosphate buffered saline (PBS) at a concentration of 2 × 10\(^6\) viable cells per 100 µL for inoculation. Female CD1-/- athymic nude mice aged 6-8 weeks (Charles Rivers, Calco, Italy) were subcutaneously inoculated in both hind legs with 100 µL of cell suspension. Tumor growth was evaluated three times a week with digital caliper measurements from the moment tumors became palpable. Tumor volume was calculated with the formula \(V = 0.5 \times (\text{length} \times \text{width}^2)\).

2.5. In vivo µPET imaging

Fully body PET/CT imaging was performed on an Inveon µPET/CT scanner (Siemens Preclinical Solutions Inc, Knoxville, TN, USA). CD1-/- nude female mice bearing Colo205 xenografts were injected intravenously (i.v.) via a catheter in the lateral tail vein with \[^{18}\text{F}]\text{BR420}\) (37.8 MBq, \(n = 1\)) or \[^{18}\text{F}]\text{BR351}\) (5.6 ± 3.4 MBq, \(n = 2\)) and underwent a 60 min dynamic µPET scan followed by static scans at 2 h p.i. (20 min) and 4 h p.i. (46 min). For the scan, mice were anesthetized with isoflurane (5% for induction, 2% for maintenance) supplemented with oxygen. PET data was recorded in list-mode format and the 60 min scan was divided in a total of 33 frames: 12 x 10 s, 3 x 20 s, 3 x 30 s, 3 x 60 s, 3 x 150 s and 9 x 300 s. The PET images were reconstructed using 2 iterations with 16 subsets of the three-dimensional ordered subset expectation maximization (OSEM) algorithm\(^{28}\) followed by 18 maximum a posteriori (MAP) iterations. Normalization, dead time, random, CT-based attenuation and scatter corrections were applied. CT imaging was done using a 220 degrees rotation with 120 rotation steps. Voltage and amperage were set to 80 keV and 500 µA, respectively.

Volume-of-interests (VOIs) for the whole tumors, heart (a measure of the blood pool), muscle, liver and kidneys were drawn manually on the co-registered CT images using PMOD
v3.3 (PMOD Technologies, Switzerland). Using this VOI the tracer uptake was then obtained and defined as the percent injected dose per cubic centimeter (%ID/cc) = 100 x activity concentration in the VOI (kBq/cc) / the injected dose (kBq).

2.6. Ex vivo biodistribution and blocking study

CD1-/- nude female mice bearing Colo205 xenografts (n = 4 for $[^{18}F]$BR420, n = 3 for $[^{18}F]$BR351), were i.v. injected with 18.76 ± 1.02 MBq $[^{18}F]$BR420 or 5.92 ± 1.23 MBq $[^{18}F]$BR351 via the lateral tail vein under isoflurane anesthesia. At 2 h post injection (p.i.), animals were sacrificed by cervical dislocation to perform ex vivo biodistribution. Blood was collected via cardiac puncture and thereafter tumors and main organs were rapidly removed, rinsed in PBS, blotted dry and measured. The uptake of radioactivity in blood and organs was expressed as percentage of the injected dose per gram of tissue plus or minus the standard deviation (%ID/g ± SD).

A blocking study was performed for $[^{18}F]$BR420 (n = 4), using a different and more potent MMP-9 ligand (IC$_{50}$ = 0.2 nM for MMP-9; 20 nM for MMP-3; 30 nM for MMP-2) (Figure 2). The blocking agent was injected 30 min before the radiotracer at a dose of 1 mg/kg. Two hours p.i. (6.66 ± 1.68 MBq), the animals were sacrificed. Blood, tumors and main organs were collected, weighed and counted as described above.

![Figure 2: Structure of the MMP-9 blocking agent.](image)

2.7. Plasma metabolite analysis

Plasma radiometabolite analysis was performed at 2 h p.i. for $[^{18}F]$BR420 (n = 4, 5.72 ± 0.24 MBq) and at 5 min, 15 min, 30 min and 2 h p.i. for $[^{18}F]$BR351 (n = 3 per time point, 14.41 ± 5.72 MBq). At the indicated time points, mice were sacrificed and blood (500-800 µL, withdrawn by cardiac puncture) was centrifuged at 4°C for 7 min at 4000 rpm. The plasma was removed (200-300 µL) and the same amount of ice-cold CH$_3$CN was added followed by addition of 10 µL of a solution of the cold standard (BR420 or BR351, 1 mg/mL). The sample
was counted in the automatic gamma-counter and vortexed for 30 s. Following centrifugation for 4 min at 4000 rpm, the supernatant was separated from the pellet. Subsequently, both fractions were counted in the gamma-counter to calculate the extraction efficiency (% recovery of radioactivity in CH$_3$CN). Hundred µL of the supernatant was analyzed by reverse phase HPLC using a Phenomex Luna (C18, 5 µm, 4.6 mm x 150 mm) column eluted with 0.1 %TFA in H$_2$O/0.1 % TFA in CH$_3$CN (55/45, V/V for $[^{18}$F]BR420; 52/48, V/V for $[^{18}$F]BR351) at a flow rate of 1 mL/min. The HPLC column was coupled to a Phenomenex guard column. Fractions were automatically collected every 30 s and measured with the gamma-counter. A validation study for metabolite analysis was also performed to exclude degradation of the radiotracer during work-up. For this, an aliquot of the radiotracer was added to blood in vitro and the samples were processed as described for the metabolite analysis and analyzed by HPLC.

2.8. Ex vivo autoradiography, immunohistochemistry and in situ zymography

Immediately after gamma counting, the tumors were snap-frozen in cooled isopentane (-35°C) and sectioned. Serial cryosections were collected in triplicate for ex vivo autoradiography (20 µm), immunohistochemistry (20 µm) and in situ zymography (10 µm).

For ex vivo autoradiography, 20 µm fresh-frozen sections were exposed overnight to high-sensitivity phosphor imaging plates and subsequently scanned using the Fujifilm image reader FLA-7000.

Immunohistochemical staining of MMP-9 and MMP-2 was performed on 20 µm fresh-frozen sections using respectively a rabbit polyclonal anti-MMP-9 antibody (ab38898, Abcam, UK) and a rabbit polyclonal anti-MMP-2 antibody (ab37150, Abcam, UK). Tumor sections were fixed using 4% paraformaldehyde and washed with PBS. Non-specific binding was blocked using 3% hydrogen peroxide and 3% Normal Goat Serum (Jackson ImmunoResearch, UK). 3% Bovine Serum Albumin (Sigma-Aldrich, Belgium) was included in the blocking step for immunohistochemical staining of MMP-2. Sections were incubated overnight with the primary antibody, diluted 1:1000 (anti-MMP-9) or 1:500 (anti-MMP-2) in antibody diluent containing 0.1% Bovine Serum Albumin, 0.2% Triton X-100 (Sigma-Aldrich, Belgium) and 2% Normal Goat Serum. The following morning, sections were washed and incubated for 1 h with horse radish peroxidase-conjugated secondary antibody (goat anti-rabbit; 1:500; Jackson ImmunoResearch, UK). Finally, sections were exposed for 10 min to the colorimetric substrate 3,3’-diaminobenzidine (Dako, Denmark). The tumor sections were counterstained
with cresyl violet, dehydrated and coverslipped. Images were obtained using an Olympus CX31 light microscope (Olympus Corporation, Japan) equipped with an AxioCam Erc 5s camera (Zeiss, Germany). ZEN 2012 software (Zeiss, Germany) was used for image acquisition.

*In situ* zymography was performed to assess gelatinase activity (MMP-9 and MMP-2) in the same tumor tissue. 10 µm fresh-frozen sections were pre-incubated in water at 37°C for 100 min and subsequently covered with a fluorogenic substrate DQ gelatin (DQ gelation from pig skin, fluorescein conjugate, Life Technologies/Molecular Probes, USA), which was diluted 1:100 in the buffer supplied by the manufacturer, at 37°C for 45 min. After washing, sections were mounted using VECTASHIELD mounting medium with DAPI (Vector Laboratories, USA) and coverslipped. To examine the specificity of the reaction, 10 mM 1,10-phenanthroline (Life Technologies/Molecular Probes, USA), a chelator and general MMP inhibitor, was added to the pre-incubation and reaction steps in sections adjacent to those used for the normal *in situ* zymography. Images were acquired at room temperature using an Olympus BX51 fluorescence microscope equipped with PlanC-N 4x/0.10 and UplanFL-N 20x/0.5 objective lenses and an Olympus DP71 digital camera. Olympus cellSens Dimension software was used for image acquisition and processing.

### 3. Results and discussion

#### 3.1. Chemistry

Cold standard BR420 was synthesized in a moderate yield (38%) following the published methodology. Since the two-step radiosynthesis of \(^{18}\text{F}\)BR420 according to the reported method failed in our hands, alternative one-step radiolabeling procedures using different precursors were explored. First the synthesis of a tosylate derivative was evaluated. The synthesis of the labelling precursor however failed most likely because tosylation/mesitylation of the primary alcohol results in a highly unstable aziridinium salt which undergoes a ring-opening reaction to yield the starting alcohol. We therefore decided to prepare chloro-derivative 1 as alternative precursor for the one-step radiosynthesis of \(^{18}\text{F}\)BR420 following the synthetic pathway described in Scheme 1.
**Scheme 1**: Synthesis of precursor 1. Reagents and conditions: (a) i) Cs$_2$CO$_3$, DMF, rt, 3d; ii) TFA, DCM, 0°C-rt; (b) NBS, K$_2$CO$_3$, DMF, 5°C-rt.

Cold standard BR351 and the corresponding precursor 5 were reproduced as previously described in the literature$^{24}$ in moderate yields of 38% and 54%, respectively.

3.2. **Radiochemistry**

While evaluating the two-steps radiosynthesis of $^{[18\text{F}]}$BR420 described in literature$^{23}$ radio-HPLC analysis of the second step (reaction of the 1-$^{[18\text{F}]}$fluoro-2-tosyloxyethane with the piperazine-containing barbiturate scaffold) at different time points indicated formation of several byproducts while $^{[18\text{F}]}$BR420 could not be obtained. By implementing a one-step approach using direct displacement of the chloro derivative 1 by $^{[18\text{F}]}$fluoride (Scheme 2), $^{[18\text{F}]}$BR420 could be successfully obtained in a radiochemical yield of 16.22 ± 3.12% (n = 10, decay-corrected to EOB). By using a biocompatible mobile phase for HPLC purification of the reaction mixture a simple dilution and sterile filtration step could be used to obtain an injection-ready solution of $^{[18\text{F}]}$BR420, thereby reducing the total synthesis to 60 min (EOB), including formulation. Chemical and radiochemical purity were > 98% and the determined specific activity was 65.07 ± 27.63 GBq/µmol (EOS, n = 10).
Scheme 2: Fully automated synthesis of $^{[18}F$BR420 and $^{[18}F$BR351. Reagents and conditions: (a) $^{[18}F$Fluoride, K$_2$CO$_3$, Kryptofix-2.2.2, DMSO, 120°C, 10 min; (b) $^{[18}F$Fluoride, K$_2$CO$_3$, Kryptofix-2.2.2, CH$_3$CN, 90°C, 20 min.

Radiosynthesis of $^{[18}F$BR351 was performed as previously reported$^{24}$ by reaction of tosylate precursor 5 dissolved in CH$_3$CN with dried $^{[18}F$F for 20 min at 90°C (Scheme 2). Again, a biocompatible mobile phase was used for the purification, omitting the need for an extra Sep-Pak purification step. Purified $^{[18}F$BR351 could be obtained in a decay corrected radiochemical yield of 22.42 ± 12.79% (n = 5), in a total synthesis time of 70 min (EOB), including formulation, and with a radiochemical purity > 98%. The determined specific activity was 172.0 ± 153.10 GBq/µmol (EOS, n = 5).

3.3. Exploratory in vivo µPET imaging study

An ex vivo biodistribution study at different time points up to 60 min post injection of $^{[18}F$BR351 as well as a µPET imaging study up to 2 h post injection of $^{[18}F$BR420 in normal C57/BL6 mice have previously been published.$^{23,24}$ These studies indicated low uptake for both tracers in heart, brain, lung and muscle with clearance of radioactivity out of excretory organs such as liver and kidneys with time after injection. Therefore in the current study, only a limited number of animals was evaluated by µPET imaging to enable the selection of a time point for a more elaborate ex vivo evaluation of tumor uptake and tracer stability.
Figure 3: Representative µPET/CT images of CD1-/- athymic nude mice intravenously injected with 38 MBq $^{18}$F]BR420 (n = 1) (A) or 6 MBq $^{18}$F]BR351 (n = 2) (B). PET images are presented in sagittal and coronal orientation at 1 min, 30 min and 2h post tracer injection. Tumors are pointed with arrow heads.

Representative µPET/CT images are presented in Figure 3. For $^{18}$F]BR420 tumor uptake increased from 0.45 %ID/cc at 1 min p.i. to a maximum value of 1.94 %ID/cc at 2 h post tracer injection with little decrease in uptake up to 4h post injection (1.78 %ID/cc) (Figure 4).
Figure 4: Time-Activity curve (%ID/cc) for heart, tumor and muscle (A) and kidneys and liver (B) of CD1-/ athymic nude mice injected with $[^{18}\text{F}]$BR420 ($n = 1$) or $[^{18}\text{F}]$BR351 ($n = 2$).

For $[^{18}\text{F}]$BR351, an initial higher tumor uptake of 1.11 %ID/cc was detected at 1 min p.i. which increased to a maximum uptake of 1.23 %ID/cc at 9 min p.i.. Tumor uptake then decreased to 0.42 %ID/cc at 2 h p.i. and remained stable up to 4 h p.i. (0.41 %ID/cc). In contrast to $[^{18}\text{F}]$BR420 for which tumor uptake exceeded uptake in the muscle at 2h p.i. (1.66 %ID/cc in the muscle) and 4h p.i. (1.22 %ID/cc in the muscle), tumor uptake of $[^{18}\text{F}]$BR351 did not exceed uptake in the muscle at any of the investigated time points. $[^{18}\text{F}]$BR420 demonstrated a slow blood clearance with an initial peak uptake of 17.78 %ID/cc in the heart decreasing to 5.53 %ID/cc at 2 min p.i., 3.90 %ID/cc at 30 min p.i. and 3.09% ID/cc at 2h p.i..

For $[^{18}\text{F}]$BR351 an initial higher peak uptake of 20.56 %ID/cc was detected in the heart followed by a faster wash-out compared to $[^{18}\text{F}]$BR420 (5.93 %ID/cc at 2 min p.i., 1.03 %ID/cc at 30 min p.i. and 0.76 %ID/cc at 2 h p.i.). For $[^{18}\text{F}]$BR420 highest uptake could be detected in the liver (26.27 %ID/cc at 4 min p.i. and 15.69 %ID/cc at 2 h p.i.) indicating predominant hepatobiliary clearance of the tracer. For $[^{18}\text{F}]$BR351 lower (13.24 %ID/cc at 4
min p.i. and 1.25 %ID/cc at 2 h p.i.) but also predominant hepatobilliary clearance could be detected. In the previously reported µPET study of [^{18}F]BR420 in C57/BL6 mice an initial high liver uptake was demonstrated but in contrast to our study, the liver uptake decreased with at 2 h p.i. uptake mainly being visible in intestines and bladder. The slower clearance from the liver seen in our study might be related to a mouse strain difference. For [^{18}F]BR351 an ex vivo biodistribution study in C57/BL6 mice indicated predominant hepatobiliary clearance at 60 min post tracer injection which is comparable to the excretion profile witnessed in our study.

3.4. Ex vivo evaluation of tumor uptake

For a more elaborate comparison of tumor uptake and stability of [^{18}F]BR420 and [^{18}F]BR351 the 2 h p.i. time point was selected as the exploratory µPET imaging study indicated clearance of radioactivity from non-target organs at 2 h p.i. while tumor uptake stabilized. The 2 h p.i. time point was also the time point demonstrating the highest tumor uptake which was attained for [^{18}F]BR420. Distribution of {^{18}F} radioactivity in various organs at 2 h p.i. following iv administration of [^{18}F]BR420 (with and without blocking) and [^{18}F]BR351 is presented in Figure 5. As was also visible in the µPET imaging, [^{18}F]BR420 displays a slower blood clearance compared to [^{18}F]BR351 with 3.44 ± 0.93 %ID/g remaining in the blood at 2 h p.i. for [^{18}F]BR420 versus 1.40 ± 0.17 %ID/g for [^{18}F]BR351. For [^{18}F]BR420 the biodistribution study showed a large accumulation of the tracer in the liver (19.33 ± 4.76%ID/g) and large intestines (9.49 ± 3.70 %ID/g), while for [^{18}F]BR351 the highest uptake could be detected in large and small intestines (27.90 ± 0.79 and 14.14 ± 3.22%ID/g, respectively) (Figure 5A). Tumor uptake was significantly higher for [^{18}F]BR420 (3.15 ±0.03%ID/g versus 0.94 ± 0.18%ID/g for [^{18}F]BR351, p < 0.05, Figure 5B). The slow blood clearance of [^{18}F]BR420 resulted in low a tumor-to-blood (T/B) ratio of 0.92 and a tumor-to-muscle (T/M) ratio of 2.03. T/B and T/M ratios for [^{18}F]BR351 were 0.67 and 1.06, respectively.

As the best tumor accumulation was obtained for [^{18}F]BR420, a blocking study was performed to evaluate MMP-9 selectivity of the tumor signal. Pre-treatment of the animals with a more potent MMP-9 inhibitor resulted in a significant decrease in [^{18}F]BR420 uptake in the tumor (p < 0.01) (Figure 5B). Administration of the blocking agent also influenced the tracer uptake in all other organs with a significant reduction of tracer accumulation observed for liver (p < 0.01) and brain (p < 0.05). This might be related to constitutive physiological
expression of MMPs in normal tissue, reflecting the broad spectrum of both $[^{18}\text{F}]\text{BR420}$ and our blocking agent.

**Figure 5:** Ex vivo biodistribution (%ID/g ± SD) in selected organs (A) and tumors (B) at 2 h p.i. of $[^{18}\text{F}]\text{BR420}$ (n = 4 for non blocked and blocked) or $[^{18}\text{F}]\text{BR351}$ (n = 3). Statistical analysis was performed by unpaired two-tailed t-test, *p < 0.05, **p < 0.01, significantly different from non-blocked.

3.5. Plasma metabolite analysis

A possible explanation for the higher tumor uptake of $[^{18}\text{F}]\text{BR420}$ compared to $[^{18}\text{F}]\text{BR351}$ could be its lower binding affinity for MMP-9 ($IC_{50} = 50 ± 27$ nM versus $7 ± 2$ nM for $[^{18}\text{F}]\text{BR420}$), the faster blood clearance of $[^{18}\text{F}]\text{BR351}$, or a lower stability compared to $[^{18}\text{F}]\text{BR420}$ resulting in a reduced tumor accumulation. A plasma metabolite study was therefore performed to analyse the in vivo stability of both tracers. The metabolite validation assay demonstrated high extraction yields (93.06 ± 0.69% for $[^{18}\text{F}]\text{BR420}$ and 94.75 ± 1.48% for $[^{18}\text{F}]\text{BR351}$) indicating limited or reversible binding to plasma proteins. No degradation of the radiotracers was observed during work-up.
The radio-HPLC metabolite analysis for $[^{18}F]$BR420 revealed high stability of the radiotracer in plasma at 2 h p.i following iv injection, where the concentration of unmetabolized $[^{18}F]$BR420 ($t_R = 8.5$ min) represented $86.18 \pm 3.12\%$ of the total radioactivity. Three minor radiometabolite peaks could be detected eluting with retention times of 2 min (M1, $7.02 \pm 2.76\%$), 4 min (M2, $1.96 \pm 0.32\%$) and 10 min (M3, $5.60 \pm 0.68\%$), respectively. Extraction yield for plasma remained high ($91.32 \pm 1.10\%$).

For $[^{18}F]$BR351 on the other hand, the radio-HPLC metabolite analysis of plasma indicated only $6.95 \pm 0.95\%$ of intact tracer remaining at 2 h p.i. Additional time points were therefore analyzed indicating a fast metabolism with almost $50\%$ of the tracer being metabolized as early as 5 min p.i. (Table 1). At 15 min p.i. the concentration of unmetabolized $[^{18}F]$BR351 ($t_R = 7$ min) represented only $20.28 \pm 5.41\%$ of the total radioactivity. Two polar radiometabolites were detected in the plasma, eluting at 2 min (M1) and 3.5 min (M2). The presence of metabolite M1 increased with time p.i. and it became the main radiometabolite peak at 2 h p.i. ($80.45 \pm 3.24\%$). M1 was identified as 2-[$^{18}F$]fluoroethanol, a known brain penetrant metabolite, by injection of a solution of 2-fluoroethanol under the same HPLC conditions. Extraction yields for plasma remained relatively constant and high ($85.50 \pm 5.27\%$) at all investigated time points. A plasma metabolite study by Wagner et al. reported $86\%$ of intact $[^{18}F]$BR351 remaining at 2.5 min p.i. and $37\%$ at 20 min p.i. with formation of two polar metabolites.$^{24}$ The much lower plasma stability observed in our study might be mouse strain related or might be attributed to different HPLC conditions used for the analysis. Wagner et al did not identify the major metabolite that was formed, but assumed phase-I metabolism and thus O-dealkylation of the parent compound, resulting in 2-[$^{18}F$]fluoroethanol to be the mechanism of metabolite generation. This was confirmed in the current study by co-elution with 2-fluoroethanol.

3.6. Ex vivo autoradiography, immunohistochemistry and in situ zymography

As both $[^{18}F]$BR351 and $[^{18}F]$BR420 are broad spectrum MMP-inhibitors, yet with favourable affinities for activated MMP-2 and MMP-9, the presence of MMP-9 and MMP-2 in the tumor tissue was assessed by immunohistochemistry. MMP-9 was sparsely present in the stroma of the tumor (Figure 6B-C and F-G). MMP-2 immunostaining on the other hand demonstrated a higher expression and homogeneous distribution of MMP-2 throughout the tumor tissue (Figure 6D and H).
**Figure 6:** Ex vivo autoradiography and immunohistochemical staining of MMP-9 and MMP-2 in the tumor tissue. A-E: Representative ex vivo autoradiographs of respectively [\(^{18}\text{F}\)]BR420 (A) and [\(^{18}\text{F}\)]BR351 (E) uptake in Colo205 tumor tissue at 2 h p.i.. Ex vivo autoradiography reveals a homogeneous distribution of both radiotracers in the tumors. B-C-F-G: MMP-9 immunostaining reveals sparse expression of MMP-9 in the stroma of the tumor. B-F: 4x magnification; C-G: 10x magnification, scale bar = 10\(\mu\)m. D-H: MMP-2 immunostaining reveals a high and homogeneous expression of MMP-2 throughout the tumor.

**In situ** zymography revealed gelatinolytic activity in the tumor tissue (Figure 7A). Gelatinolytic activity could be blocked by adding 1,10-phenanthroline, a zinc chelator (Figure 7B).

**Figure 7:** Gelatinolytic activity in Colo205 tumor tissue. Fluorescence micrographs of in situ zymography with DQ gelatin as fluorogenic substrate (green) in the absence (A) and presence (B) of 10 mM 1,10-phenanthroline, a general MMP inhibitor. Scale bar = 200 \(\mu\)m.
Ex vivo autoradiography of tumor slices revealed a homogeneous distribution of both radiotracers in tumor tissue (Figure 6A and E). Although in the blocking study a significant reduction in [18F]BR420 tumor uptake could be obtained by pre-treatment with a more potent MMP-9 inhibitor, indicating possible selective binding of [18F]BR420 to MMP-9, the discrepancy between radioactivity distribution of [18F]BR420 in the autoradiography analysis and the distribution of MMP-9 in the IHC analysis does not support selective binding of the tracer to MMP-9. As the tracer distribution in the tumor is more correlated with MMP-2 IHC and since the blocking agent also has a relative affinity for MMP-2 (IC50 = 30 nM versus 24 nM for [18F]BR420), the reduction in [18F]BR420 uptake in the tumor might also be related to MMP-2 binding. However, the homogeneous distribution might as well just reflect a broad spectrum binding profile.

4. Conclusion

MMP-9 plays an important role in cancer migration, invasion and metastasis. The availability of a MMP-9 selective radiotracer could aid in drug development, diagnosis and staging of tumors with known MMP-9 expression, including colorectal cancer. Here we present the first evaluation of [18F]BR420 and [18F]BR351 as potential MMP-9 PET radiotracers in a colorectal cancer model expressing MMP-9. Preparation of [18F]BR420 was improved using a novel, faster and fully automated strategy. [18F]BR351 was quickly metabolized and displayed only low tumor uptake. These unpromising results make [18F]BR351 not suited for MMP imaging in mouse models of cancer. [18F]BR420 showed a significantly higher tumor uptake compared to [18F]BR351 and was found to be stable in plasma up to 2 h p.i. Nevertheless, due to a low T/B ratio and discrepancy of tracer distribution in the tumor with MMP-9 immunostaining [18F]BR420 will not be useful for selective in vivo imaging of MMP-9 in colorectal cancer.

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References


Table 1: In vivo plasma metabolite analysis of [18F]BR351 at different time points post tracer injection. Values are expressed as % of total radioactivity, mean ± S.D. (n = 3). ND = not detected.

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<tr>
<th>Time p.i. (min)</th>
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<td>M1 2 min</td>
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<tr>
<td>5 min</td>
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