Selective Imaging of Matrix Metalloproteinase-13 to Detect Extracellular Matrix Remodeling in Atherosclerotic Lesions

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Abstract

Purpose: Overexpression and activation of matrix metalloproteinase-13 (MMP-13) within atheroma increases susceptibility to plaque rupture, a major cause of severe cardiovascular complications. In comparison to pan-MMP targeting [^{18}F]BR-351, we evaluated the potential for [^{18}F]FMBP, a selective PET radiotracer for MMP-13, to detect extracellular matrix (ECM) remodeling in vascular plaques possessing markers of inflammation.

Procedures: [^{18}F]FMBP and [^{18}F]BR-351 were initially assessed *in vitro* by incubation with *en face* aortae from 8 month-old atherogenic *ApoE*$^{-/-}$ mice. *Ex vivo* biodistributions, plasma metabolite analyses, and *ex vivo* autoradiography were analogously performed 30 minutes after intravenous radiotracer administration in age-matched C57Bl/6 and *ApoE*$^{-/-}$ mice under baseline or homologous blocking conditions. *En face* aortae were subsequently stained with Oil Red O (ORO), sectioned, and subject to immunofluorescence staining for Mac-2 and MMP-13.

Results: High-resolution autoradiographic image analysis demonstrated target specificity and regional concordance to lipid-rich lesions. Biodistribution studies revealed hepatobiliary excretion, low accumulation of radioactivity in non-excretory organs, and few differences between strains and conditions in non-target organs. Plasma metabolite analyses uncovered that [^{18}F]FMBP exhibited excellent *in vivo* stability (≥74% intact) while [^{18}F]BR-351 was extensively metabolized (≤37% intact). *Ex vivo* autoradiography and histology of *en face* aortae revealed that [^{18}F]FMBP, relative to [^{18}F]BR-351, exhibited 2.9-fold greater lesion uptake, substantial specific binding (68%), and improved sensitivity to atherosclerotic tissue (2.9-fold vs 2.1-fold). Immunofluorescent staining of aortic *en face* cross-sections demonstrated elevated Mac-2 and MMP-13 positive areas within atherosclerotic lesions identified by [^{18}F]FMBP *ex vivo* autoradiography.

Conclusions: While both radiotracers successfully identified atherosclerotic plaques, [^{18}F]FMBP showed superior specificity and sensitivity for lesions possessing features of destructive plaque

remodeling. The detection of ECM remodeling by selective targeting of MMP-13 may enable characterization of high-risk atherosclerosis featuring elevated collagenase activity.

Keywords: matrix metalloproteinases, atherosclerosis, vulnerable plaques, remodeling, inflammation, autoradiography, positron emission tomography, radiotracer

Introduction

The extracellular matrix (ECM) is a highly dynamic network responsible for maintaining tissue integrity and modulating cellular functions related to proliferation, adhesion and migration.[1] Matrix metalloproteinases (MMPs) are a class of calcium-dependent, zinc-chelating endopeptidases which play an important physiological role in ECM remodeling. Typically, the catalytic activity of MMPs is tightly controlled through transcriptional alterations, tissue-specific MMP release and activation, or inhibition by endogenous tissue inhibitors of metalloproteinases.[2] Contrarily, dysregulation of activated MMPs is associated with several pathologies including metastatic cancer, arthritis, and multiple sclerosis through inflammation-induced tissue remodeling and degradation.[3,4] Of particular interest, uncontrolled ECM remodeling in the vasculature is a hallmark of atherosclerosis.[5]

Atherosclerotic lesions constitute a distinctive feature of progressive disease and risk of acute coronary events is largely associated with molecular markers of remodeling in these plaques.[6] Nevertheless, clinical imaging investigations focus nearly exclusively on anatomical or functional imaging modalities including angiography, computed tomography, and magnetic resonance imaging to identify the extent of coronary occlusion.[7,8] On a molecular level, inflammatory mediators stimulate MMP recruitment and augment atheroma formation by

facilitating mononuclear cell infiltration of the vessel wall.[9,10] Upon differentiation, macrophages mediate MMP secretion and induce proteolytic activity in vascular smooth muscle cells (SMC), contributing directly to plaque destabilization through degradation of the fibrous cap.[9,11,12] Given that thrombosis underlies most adverse clinical outcomes including myocardial infarction and stroke, MMPs represent compelling biomarkers of plaque vulnerability for imaging by positron emission tomography (PET).[13,14]

Specifically, MMP-13 has been identified as a predominant interstitial collagenase in *ApoE*$^{-/-}$ mice and human atheroma. Elevated expression of activated MMP-13 is associated with an increase in *in vivo* collagenolysis primarily in atheromatous compared with fibrous plaques and reduces SMC accumulation in both early and established lesions.[10,15] Additionally, knockout of MMP-13 in mice abolishes lesion collagenolytic activity, while selective inhibition increases local collagen content, suggesting that MMP-13 provokes plaque instability.[16,17]

Nevertheless, current MMP-targeted nuclear medicine agents including [^{18}F]BR-351, [^{99m}Tc]RP-805, and [^{18}F]marimastat-ArBF$_3$ exhibit broad-spectrum activity through coordination to the active site Zn^{2+} ion, conserved across the MMP family.[18–20] These radiotracers may exhibit lower target tissue contrast due to engagement with non-pathologic MMPs expressed in contiguous tissue.[12] Given that MMP-13 expression is limited in non-infiltrated arteries and myocardium, there is tremendous potential for improved imaging contrast, sensitivity, and especially *in vivo* characterization of atherosclerosis with elevated collagenase activity.[10, 21] Fortunately, selective targeting of MMP-13 has been achieved with several small molecule inhibitors by exploiting the unique structural features of its S1′ and S1′* specificity pockets, adjacent the catalytic site.[22–24] In particular, MMP-13 inhibitors based on the pyrimidine dicarboxamide scaffold have shown no detectable inhibition of MMPs-1, -2, -3, -7, -8, -9, -10, -12, -14, and -16 up to 100 μM.[25]

The objective of this study was to evaluate the specificity and sensitivity of an MMP-13 selective radiotracer in comparison to a non-selective MMP radiotracer for the detection of atherosclerotic plaques possessing markers of inflammation and remodeling in mice. To facilitate this analysis, a radiotracer originally developed by Hugenberg *et al.* with reported 10^3-fold MMP-13 selectivity (IC_{50} = 56 ± 2 nM, Table 1), and herein entitled [^{18}F]FMBP, has been chosen for comparison against [^{18}F]BR-351 originally developed by Wagner *et al.*, which is non-selective (2 ≤ IC_{50} ≤ 50 nM, Table 1) and has been previously investigated for imaging MMPs in glioma, colorectal cancer, and stroke.[18,22,26,27,28]

Materials and Methods

Chemical Synthesis and Radiolabeling

See ESM for chemical synthesis, radiolabeling, and quality control testing (Suppl. Figs. 1 & 2).

Animal Model

Male and female C57Bl/6J (strain no. 000664) and *ApoE*$^{-/-}$ (strain no. 002052) mice were obtained from Jackson Laboratory, acclimated upon arrival for 1 week, housed in groups of 4, and monitored periodically until 8-12 months of age. C57Bl/6 mice were fed with normal chow while *ApoE*$^{-/-}$ mice were fed a western atherogenic diet (TD.88137, Envigo) for 16 weeks and returned to normal chow.[29] Both strains were housed in environmentally enriched cages with free access to food and water. All housing, handling, and experimental procedures were in strict accordance with the guidelines of Canadian Council on Animal Care and with approval of the University of Ottawa Animal Care Committee. (Suppl. Fig. 3)

Aortic *En Face*

Procedures for aortic *en face* preparations were adapted from literature.[30] Mice anesthetized with isoflurane were sacrificed by myocardial perfusion with 1× phosphate buffered saline (PBS, 10 mL), briefly fixed with 10% formalin (10 mL), and immediately flushed with additional 1× PBS (5 mL) via left ventricle cannulation. Perfusate was drained from an incision within the right atrium. The heart and aorta were harvested by severing branching arteries and detaching the descending aorta. Upon removal of the adventitia, the aorta was separated from the heart at the root and opened longitudinally.

In Vitro Autoradiography

En face aortae harvested from $ApoE^{-/-}$ mice (n = 4–7 per group) were sequentially washed with EtOH and tris buffer (50 mM, pH 7.4) prior to incubation with the selected tracer (45 kBq) for 1 hour. In separate experiments, non-specific binding was assessed by incubating these same samples with excess non-radioactive standard (2 µM or 10 µM) for 1 hour. Samples were washed with buffer (2 × 5 min) followed by water (1 × 5 min). Aortae were immediately exposed to a super-resolution Storage Phosphor Screen (BAS-IP SR 2025 E) in an Electrophoresis Systems Autoradiography Cassette (FBXC 810) for 15 hours. The screen was scanned with a Cyclone Plus Storage Phosphor System and images were analyzed using OptiQuant software by drawing aortic arch regions of interest (ROI) which were Oil Red O (ORO) positive.[31] Digital light units (DLU) were converted to activity (Bq) using a set of calibration standards with known activities on the same screen. Activity density (Bq/mm^2) was calculated from dividing the sample activity by the ROI area.

***In Vivo* Studies**

Dynamic PET imaging was performed on *ApoE*$^{-/-}$ mice over 1 hour (Suppl. Figs. 3 & 4, see ESM). For biodistributions, mice (38 ± 2 g, *n* = 4 per group) were anesthetized with 3% isoflurane and administered non-radioactive standard (5 mg/kg, ip, 50/50 DMSO/water) or equivalently dosed vehicle control 30 minutes prior to intravenous injection of the selected tracer (15 MBq) through a lateral tail vein catheter. After 30 minutes, mice were sacrificed by myocardial perfusion, organs of interest were excised, dipped in water, weighed, and counted for radioactivity using a Hidex Automatic Gamma Counter (energy window: 350–650 keV). Counts per minute (cpm) were converted to activity (Bq) using a set of calibration standards with known activities. Percentage injected dose (%ID) was calculated from dividing the organ activity by the injected dose (decay-corrected) and further normalized by sample mass to obtain the percentage injected dose per gram tissue (%ID/g).

Aortae were also harvested and *en face* specimens were imaged and quantified as described for the *in vitro* autoradiography section. Briefly, percentage injected dose (%ID) was calculated by dividing the lesion activity by the injected dose (decay-corrected) and further normalized by area to obtain activity density (%ID/m^2).[31]

Plasma Metabolite Analysis

Blood samples were further processed and analyzed for plasma metabolites as previously described.[18] Plasma samples spiked with nonradioactive standard were analyzed using the analytical HPLC conditions as described in the quality control protocol (see ESM). Fractions were collected every 2 minutes and counted for radioactivity using a gamma counter. Fractions collected before and after the parent fraction were considered as polar and non-polar metabolites,

respectively. Extraction efficiency was defined as the percentage recovery of radioactivity in protein-free plasma following precipitation with acetonitrile.

Histology

Procedures for ORO staining were adapted from literature.[30] *En face* prepared samples were incubated with the freshly prepared ORO solution for 1.5 hours and washed with water (2 × 5 min). Bright-field images were taken using a Krüss Stereomicroscope (MSL4000-series) adapted with a smartphone camera. Aortic arches, defined by the boundary between the aortic root and the ascending aorta to the same level on the descending aorta, were cropped using Adobe Photoshop CS5. Lesion areas were expressed relative to the total aortic arch surface area (% positive area). Quantification was completed by 2 independent observers using ImageJ.

Immunofluorescence

Following *ex vivo* autoradiography and staining with ORO, *en face* aortic arches were embedded in paraffin wax (TissuePrep), sliced into 5 μm sections, and deparaffinized. Regions which were positive during *ex vivo* [^{18}F]FMBP autoradiography were selected for sectioning (n = 2 per aorta). Antigen retrieval was performed in citrate buffer (pH 6.0) at 100 °C for 150 seconds. Sections were covered in 10% normal goat serum (Vector Laboratories) for 10 minutes and incubated with 1:100 MMP-13 or MMP-2 primary antibody (ab39012 or ab97779, Abcam) and 1:500 Mac-2 primary antibody (CL8942AP, Cedarlane) for 16 hours at 4 °C. Samples were then incubated with secondary antibodies at 1:500 dilution for 30 minutes (MMP-13 & MMP-2: A-11037, Mac-2: A-11006, Invitrogen). Nuclei were counterstained with Hoeschst 33258 (10 mg/mL in PBS) for 8 minutes. Slides were coated with fluorescent protective mounting media (Dako), dried, and covered until imaging by fluorescent microscopy (Zeiss Axio Imager A2). Isotype control antibodies (rabbit IgG ab171870, Abcam and rat IgG CLCR2A00, Cedarlane) were also

utilized to assess non-specific fluorescence at equivalent concentrations to the primary antibodies. Images were acquired with Aperio ImageScope (10× magnification), stitched using Microsoft Image Composite Editor, cropped using Adobe Photoshop CS5 and quantified by 2 independent observers using ImageJ.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Data are presented as mean ± standard error. Differences between 2 groups were tested using a 2-tailed unpaired Student's t-test. Multiple groups were compared using 1-way ANOVA or 2-way ANOVA with Tukey's multiple comparison test. Data are normally distributed. Significance was set at the 0.05 level.

Results

Radiosyntheses of [^{18}F]FMBP and [^{18}F]BR-351

With minor modifications to previously reported conditions, automated radiosyntheses were established to produce the target radiotracers.[22,26] [^{18}F]FMBP and [^{18}F]BR-351 were obtained in decay-corrected radiochemical yields of 28 ± 1% (n = 10) and 27 ± 6% (n = 3), 60 and 80 minutes after bombardment, with radiochemical purities >99% (Fig. 1). Molar activities were determined to be 92 ± 11 GBq/µmol and 90 ± 53 GBq/µmol at the end of synthesis for [^{18}F]FMBP and [^{18}F]BR-351, respectively (Fig. 1).

***In Vitro* Autoradiographic Validation**

Aortic arch autoradiographic uptake patterns of [^{18}F]FMBP and [^{18}F]BR-351 displayed colocalization with ORO-positive lipid-rich atherosclerotic lesions (Fig. 2a). At baseline, [^{18}F]FMBP exhibited an aortic lesion activity density of 61.45 ± 2.08 Bq/mm^2, reducible by 23% to 47.57 ± 2.04 Bq/mm^2 (P = 0.0363) and 45% to 33.79 ± 4.18 Bq/mm^2 (P = 0.0001) upon co-

incubation with 2 μM and 10 μM non-radioactive FMBP, respectively (Fig. 2b). At baseline, [^{18}F]BR-351 exhibited aortic lesion activity density of 37.62 ± 5.00 Bq/mm^2, reducible by 55% to 17.05 ± 3.61 Bq/mm^2 upon co-incubation with 2 μM non-radioactive BR-351 (Fig. 2b, $P = 0.0046$). Similarly, incubation with 10 μM non-radioactive BR-351 reduced aortic lesion activity density by 56% to 16.54 ± 1.94 Bq/mm^2 (Fig. 2b, $P = 0.0017$).

PET Imaging and Biodistribution

Under baseline conditions in *ApoE* $^{-/-}$ mice, [^{18}F]FMBP predominantly exhibited hepatic (17.22 ± 2.56 %ID/g), intestinal (4.73 ± 0.78 %ID/g), myocardial (4.66 ± 0.11 %ID/g), and renal (4.04 ± 0.36 %ID/g) retention (Fig. 3a). Similar to previous reports for C57Bl/6 mice and consistent with *ex vivo* findings, blood time-activity curves revealed rapid washout of radioactivity (T_{max}: 0.75 min) while myocardial uptake stabilized at 4.82 ± 0.31 %ID/cc with slow clearance 10 minutes after injection (Suppl. Fig. 4).[22] [^{18}F]BR-351 exhibited uptake within these same organs with greater accumulation in the intestine (Fig. 3b, intestine: 5.79 ± 0.89 %ID/g, kidney: 2.88 ± 0.67 %ID/g, and liver: 2.72 ± 0.41 %ID/g). Low levels of radioactivity (<2 %ID/g) were observed in all other measured organs and few differences were observed between strains and conditions (Fig. 3). Particularly, [^{18}F]FMBP and [^{18}F]BR-351 exhibited statistically significant differences in the liver ($P < 0.0007$) and intestine ($P < 0.0011$), respectively (Fig. 3).

Plasma Metabolite Analysis

Following measurement of whole-blood radioactivity, samples were immediately pooled for plasma metabolite analysis using fractional radio-HPLC and gamma counting (Table 2 & Suppl. Fig. 5). In *ApoE*$^{-/-}$ mice, 79% and 74% of plasma radioactivity corresponded to intact [^{18}F]FMBP under baseline and blocking conditions, comparable to levels observed in C57Bl/6 mice (Table 2). Conversely, 37% of plasma radioactivity corresponded to intact [^{18}F]BR-351 in *ApoE*$^{-/-}$ mice, with

a reduction to 16% under blocking conditions and 9% in C57Bl/6 mice (Table 2). In both analyses, remaining radioactivity was mainly attributed to polar radio-metabolites. The observed extraction efficiencies (≥75%) suggest representative sampling of plasma radioactivity.

Ex Vivo Autoradiography and Oil Red O Quantification

Following intravenous administration, aortic arch uptake of [^{18}F]FMBP and [^{18}F]BR-351 was visualized by *en face* autoradiography and displayed high regional concordance with ORO-positive lipid-rich atherosclerotic lesions (Figs. 4a & 4b). At baseline, [^{18}F]FMBP exhibited significantly higher lesion activity density in the *ApoE*$^{-/-}$ cohort (244 ± 37 %ID/m^2) relative to C57Bl/6 controls (Fig. 4c, 86 ± 22 %ID/m^2, $P = 0.0011$). Pre-treatment with non-radioactive FMBP significantly decreased [^{18}F]FMBP aortic lesion uptake by 68% to 78 ± 24 %ID/m^2 (Fig. 4c, $P = 0.0007$), equivalent to levels observed in control mice, absent of vascular lesions. [^{18}F]BR-351 also exhibited elevated lesion activity density in the *ApoE*$^{-/-}$ cohort (84 ± 6 %ID/m^2) relative to controls (40 ± 11 %ID/m^2), however, observed differences were not statistically significant (Fig. 4c, $P = 0.6251$). No significant change in [^{18}F]BR-351 uptake was observed following pre-administration with BR-351 (Fig. 4c, $P = 0.9935$). Quantification of ORO revealed that positive areas were significantly elevated within *ApoE*$^{-/-}$ aortae (25.51 ± 3.74%) and limited in C57Bl/6 controls (0.16 ± 0.07%, Fig. 4d, $P = 0.0002$).

Markers of Inflammation and Remodeling

Percentage positive Mac-2 (7.16 ± 1.15%), MMP-13 (5.52 ± 1.54%), and MMP-2 (9.44 ± 2.14%) areas were distinctly increased within atherosclerotic lesions of *ApoE*$^{-/-}$ mice, as detected by immunofluorescence and predicted by [^{18}F]FMBP *ex vivo* autoradiography (Figs. 5a–l & Suppl. Fig. 6). Additionally, MMP-13 and Mac-2 positive areas colocalized (Figs. 5i & 5j). Isotype control

experiments revealed a moderate extent of non-specific binding within atherosclerotic lesions, but none in C57Bl/6 controls (Suppl. Fig. 7).

Discussion

Few MMP-targeted radiotracers have been evaluated in mouse models of atherosclerosis, namely [^{99m}Tc]RP805, [^{111}In]RP782, [^{123}I]I-HO-CGS 27023A and [^{68}Ga]Ga-DOTA-TCTP-1.[32–38] Investigations with these radiotracers have primarily focused on colocalizing and in some cases correlating *in vivo* and *ex vivo* uptake in atherosclerosis with markers such as macrophages and MMPs, determined by immunostaining or mRNA expression. Nevertheless, direct evidence toward the differentiation of stable from vulnerable plaques remains elusive. Limited availability of mouse models recapitulating atherosclerotic plaque rupture contribute to this shortcoming,[39] though it has also been acknowledged that quantification of broad metalloproteinase activity may not be appropriate.[32,38] The target density of a non-selective radiotracer is subject to dynamic MMP expression patterns in various tissues and diseases. While many MMPs have been shown to be upregulated in human carotid plaques, these enzymes possess varying pathophysiological roles in plaque progression.[5,12,40,41] Therefore, imaging specific subtypes which have been shown to directly contribute to atherosclerotic plaque rupture may be advantageous. The present study sought to evaluate an approach to selective targeting of MMP-13, a predominant collagenase implicated in plaque vulnerability, for sensitivity and characterization of ECM remodeling in inflamed plaques, in comparison to a broad-spectrum MMP radiotracer.[10,15-17]

To facilitate this comparative analysis, automated syntheses were adapted to reliably obtain [^{18}F]FMBP and [^{18}F]BR-351 with slightly improved radiochemical yield and molar activity (>90 GBq/µmol) suitable for high contrast imaging (Fig. 1 & Suppl. Fig 1). Initial validations were performed by high resolution *in vitro* autoradiography in *en face* aortae to visualize and quantify

radioligand binding in atherosclerotic tissue without potential confounds related to target-tissue delivery and metabolism. Given the unavailability of heterologous MMP-selective inhibitors, and the aforementioned differences in target engagement, radiotracer binding specificity was assessed with the corresponding non-radioactive standard. [^{18}F]FMBP and [^{18}F]BR-351 demonstrated moderate levels of specific binding (45% and 56% displaceable, respectively) and evident colocalization to ORO-positive areas, representing lipid-laden atheromatous plaques (Fig. 2). [^{18}F]FMBP demonstrated dose dependent blocking and lesion activity density was 1.6-fold greater than [^{18}F]BR-351 at baseline, while [^{18}F]BR-351 specific uptake was fully saturated at 2 μM (Fig. 2). Taken together, though levels of specificity were suboptimal during *in vitro* evaluation, these findings provided justification for more comprehensive investigations.

To further establish radioligand localization profiles, *ex vivo* biodistribution was performed in C57Bl/6 and *ApoE*$^{-/-}$ mice. *In vivo* PET imaging was conducted in *ApoE*$^{-/-}$ mice to select an optimal timepoint for *ex vivo* sampling (Suppl. Fig. 4). Subsequent experiments were conducted at a 30-minute timepoint following stabilization of [^{18}F]FMBP myocardial and blood time-activity curves to sufficiently allow for radiotracer circulation. Both [^{18}F]FMBP and [^{18}F]BR-351 predominantly undergo renal clearance and hepatobiliary excretion with low accumulation in non-excretory organs (Fig. 3), as previously reported in mice.[22,26] While the observed differences in hepatic [^{18}F]FMBP uptake are not fully understood, constitutive expression of MMP-13 and western-diet induced fibrosis suggest potential explanations.[42,43] Observed differences in intestinal [^{18}F]BR-351 uptake are suspected to be related to metabolic variations (Table 2). Low bone uptake indicated that radiodefluorination did not occur, offering the potential for non-invasive aortic imaging of calcific vascular lesions in close proximity to the spine in larger species using PET/CT. Most notably, distribution patterns were similar among mouse strains and few differences

were observed under blocking conditions, emphasizing that target expression and specific binding are low in organs unassociated with atherosclerosis (Fig. 3).

A metabolic study of [^{18}F]FMBP and [^{18}F]BR-351 was conducted to assess *in vivo* stability (Table 2 & Suppl. Fig. 5). Analysis of blood plasma revealed high metabolic stability of [^{18}F]FMBP, with the parent fraction representing the major detectable species under all testing conditions at a 30-minute timepoint. Although metabolites were not identified, the remaining polar radio-metabolites likely correspond to O-dealkylation or amide hydrolysis products.[22] In contrast, analysis of blood plasma revealed low metabolic stability of [^{18}F]BR-351, with polar radio-metabolites representing the major detectable species under all testing conditions at an equivalent timepoint. Differences in the extent of [^{18}F]BR-351 metabolism are consistent with the observed variability in *ex vivo* intestinal uptake (Fig. 3b). Similarly, polar radio-metabolites likely correspond to O-dealkylation with the sulfonamide and hydroxamate moieties representing other labile positions.[18,44]

Ex vivo autoradiography on *en face* aortae served to reinforce *in vitro* findings in atherosclerotic tissue (Figs. 4a & 4b). [^{18}F]FMBP possessed 2.9-fold greater uptake in *ApoE*$^{-/-}$ models compared to C57Bl/6 animals and exhibited regional concordance with lipid-laden ORO-positive areas. Gratifyingly, pharmacological dosing of non-radioactive FMBP completely blocked aortic lesion tracer uptake such that it was indistinguishable from controls (Fig. 4c). Although [^{18}F]BR-351 seemingly possessed 2.1-fold sensitivity to atherosclerotic tissue, this difference along with the slight reduction observed by pre-treatment with non-radioactive BR-351 were not statistically significant in the sample groups (Fig. 4c). The discrepancy between *in vitro* and *ex vivo* findings is likely attributed to the poor metabolic stability of [^{18}F]BR-351 resulting in limited MMP engagement (Table 2). Relative to [^{18}F]BR-351, [^{18}F]FMBP further exhibited 2.9-fold greater

aortic lesion uptake (Fig. 4c). Quantification of ORO-positive areas highlighted that aortic lipid accumulation was elevated in *ApoE*$^{-/-}$ mice and negligible in C57Bl/6 controls (Fig. 4d).

En face aortae obtained during *ex vivo* analyses were subsequently embedded in paraffin, sectioned, and stained for immunofluorescent detection of Mac-2 and MMP-13 as markers of inflammation and remodeling, alongside nuclear counterstaining with Hoescht (Figs. 5a-j). As expected, a negative result was obtained for C57Bl/6 samples lacking aortic lesions (Figs. 5a-j). In contrast, atherosclerotic lesions which were ORO-positive and detected by [^{18}F]FMBP *ex vivo* autoradiography possessed elevated Mac-2 and MMP-13 positive areas, suggesting that radiotracer uptake is sensitive to the presence of these biomarkers (Figs. 5a-i). Notably, MMP-13 density appears to be markedly increased within the outer curvature of the atherosclerotic lesion, consistent with localization of this enzyme within the fibrous cap, which is a major determinant of plaque destabilization (Figs. 5i & 5j).[10,15] Quantification recapitulated that Mac-2 and MMP-13 are uniquely expressed in response to atherosclerosis, consistent with macrophage mediated secretion and activation of this collagenase (Figs. 5k & 5l).

Considering that [^{18}F]BR-351 effectively binds to MMP-2, -8, -9 and -13, the contributions of each target to radiotracer uptake must be clearly identified to facilitate interpretation. While, the percentage positive area of MMP-2 was also found to be elevated in atherosclerotic plaques as detected by immunofluorescence (Suppl. Fig. 6), literature on MMP-2 deficient atherogenic mice suggests that this gelatinase imparts stability to atherosclerotic lesions by accumulating SMCs into the fibrous cap.[45] Likewise for MMP-9, inconsistent findings necessitate further elucidation of its diverse effects on cellular plaque composition.[45,46] Regarding the interstitial collagenases, MMP-13 predominates over MMP-8 in degrading intraplaque collagen, and reducing SMC accumulation in atherogenic mice.[15] Most significantly, MMP-13 colocalizes with MMP-1 in

inflamed human atheromatous plaques, demonstrating that selective imaging of MMP-13 remains a promising strategy for pre-clinical evaluations.[10]

Limitations

BR-351 and FMBP bind active site zinc ions or specificity pockets adjacent the MMP active site. Although the possibility for engagement with inactive enzymes was not assessed in this study, latent MMP active sites are inaccessible to both substrate and aqueous medium prior to cleavage of the pro-peptide domain.[25,47] Therefore, it is reasonable to infer radiotracer specific binding reflects active MMP concentrations. Due to the unavailability of well-characterized selective MMP-13 inhibitors, heterologous blocking studies were not possible in the current study and target engagement of MMP-13 could not be directly evaluated. Biological variance of MMP-13 expression was insufficient to establish meaningful correlations using *ApoE*$^{-/-}$ mice, though may be possible with additional model strains.[32]

From an imaging perspective, while PET radiotracers inherently hold several advantages over SPECT analogues, accurate *in vivo* localization and quantification of small lesions in mice remains challenging due to limited spatial resolution and partial volume effects. *In vivo* PET imaging was conducted in this study to observe dynamic distribution among organs, but could not be used to localize aortic uptake due to the small physical size of the vessel and significant liver uptake. It is also important to acknowledge that the employed atherogenic mouse model may lack the underlying biochemical and physiological processes necessary to truly develop rupture-prone atherosclerotic plaques.[48] As such, the present study sought to co-localize radiotracer uptake with biomarkers of inflammation and remodelling consistent with disrupted human plaques. Future studies on larger mammals with advanced human-like atherosclerosis are planned to not only localize but differentiate stable atherosclerotic plaques from those which are susceptible to rupture.

Given the promising results of this approach, second generation PET radiotracers derived from alternative inhibitor scaffolds with improved potency and selectivity are under parallel development.

Conclusions

The feasibility of imaging extracellular matrix remodeling *ex vivo* in mouse models of atherosclerosis with MMP-13 targeted PET radiotracers has been established. While both [^{18}F]BR351 and [^{18}F]FMBP successfully localized atherosclerotic lesions, MMP-13-targeted imaging with [^{18}F]FMBP showed improved binding specificity and sensitivity to atherosclerotic tissue possessing markers of destructive plaque remodeling relative to pan-selective [^{18}F]BR-351. Altogether, [^{18}F]FMBP has proven useful for the *ex vivo* detection of extracellular matrix remodeling in inflamed atherosclerotic lesions, suggesting that selective imaging represents a promising approach towards the characterization of high-risk atherosclerosis. Selective MMP-13-targeted radiotracers with superior specificity and pharmacokinetics may enable *in vivo* localization of collagenase activity in atherosclerotic plaques.

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Author Contributions

Project design – A.B. and B.H.R.

Method development – A.B., M.M., G.F., X.Z., and R.A-H.

Data acquisition and analysis – A.B., M.M., G.F., and E.F.

Writing and revision of the manuscript - A.B., M.M., G.F., and B.H.R.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Bonnans C, Chou J, Werb Z (2014) Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol 15:786–801.

2. Löffek S, Schilling O, Franzke C-W (2011) Biological role of matrix metalloproteinases: a critical balance. Eur Respir J 38:191–208.

3. Li H, Wang D, Yuan Y, Min J (2017) New insights on the MMP-13 regulatory network in the pathogenesis of early osteoarthritis. Arthritis Res Ther 19:248.

4. Hu J, Van den Steen PE, Sang Q-XA, Opdenakker G (2007) Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. Nat Rev Drug Discov 6:480–498.

5. Johnson JL (2017) Metalloproteinases in Atherosclerosis. Eur J Pharmacol 816:93-106.

6. Giannakou S, Angelidis G, Tsougos I, et al (2020) Pet tracers for vulnerable plaque imaging. Ann Nucl Med. 34:305–313.

7. Heo R, Nakazato R, Kalra D, Min JK (2014) Noninvasive imaging in coronary artery disease. Semin Nucl Med 44:398–409.

8. Owen DRJ, Lindsay AC, Choudhury RP, Fayad ZA (2011) Imaging of Atherosclerosis. Annu Rev Med 62:25–40.

9. Libby P (2002) Inflammation in atherosclerosis. Nature 420:868–874.

10. Sukhova GK, Schönbeck U, Rabkin E, et al (1999) Evidence for Increased Collagenolysis by Interstitial Collagenases-1 and -3 in Vulnerable Human Atheromatous Plaques. Circulation 99:2503–2509.

11. Lessner SM, Galis ZS (2004) Matrix metalloproteinases and vascular endothelium-mononuclear cell close encounters. Trends Cardiovasc Med 14:105–111.

12. Newby AC (2012) Matrix metalloproteinase inhibition therapy for vascular diseases. Vascul Pharmacol 56:232–244.

13. Schaar J (2004) Terminology for high-risk and vulnerable coronary artery plaques. Eur Heart J 25:1077–1082.

14. Newby AC (2014) Proteinases and plaque rupture: unblocking the road to translation. Curr Opin Lipidol 25:358–366.

15. Quillard T, Araújo HA, Franck G, et al (2014) Matrix Metalloproteinase-13 Predominates Over Matrix Metalloproteinase-8 as the Functional Interstitial Collagenase in Mouse Atheromata. Arterioscler Thromb Vasc Biol 34:1179–1186.

16. Quillard T, Tesmenitsky Y, Croce K, et al (2011) Selective Inhibition of Matrix Metalloproteinase-13 Increases Collagen Content of Established Mouse Atherosclerosis. Arterioscler Thromb Vasc Biol 31:2464–2472.

17. Deguchi J, Aikawa E, Libby P, et al (2005) Matrix Metalloproteinase-13/Collagenase-3 Deletion Promotes Collagen Accumulation and Organization in Mouse Atherosclerotic Plaques. Circulation 112:2708–2715.

18. Vazquez N, Missault S, Vangestel C, et al (2017) Evaluation of [18F]BR420 and [18F]BR351 as radiotracers for MMP-9 imaging in colorectal cancer. J Label Compd Radiopharm 60:69–79.

19. Ye Y, Toczek J, Gona K, et al (2018) Novel arginine-containing macrocyclic MMP inhibitors: synthesis, ^{99m}Tc-labeling, and evaluation. Sci Rep 8:11647.

20. auf dem Keller U, Bellac CL, Li Y, et al (2010) Novel matrix metalloproteinase inhibitor [18F]marimastat-aryltrifluoroborate as a probe for in vivo positron emission tomography imaging in cancer. Cancer Res 70:7562–7569.

21. Spinale FG, Coker ML, Heung LJ, et al (2000) A Matrix Metalloproteinase Induction/Activation System Exists in the Human Left Ventricular Myocardium and Is Upregulated in Heart Failure. Circulation 102:1944–1949.

22. Hugenberg V, Wagner S, Kopka K, et al (2017) Radiolabeled Selective Matrix Metalloproteinase 13 (MMP-13) Inhibitors: (Radio)Syntheses and in Vitro and First in Vivo Evaluation. J Med Chem 60:307–321.

23. Nara H, Sato K, Naito T, et al (2014) Discovery of Novel, Highly Potent, and Selective Quinazoline-2-carboxamide-Based Matrix Metalloproteinase (MMP)-13 Inhibitors without a Zinc Binding Group Using a Structure-Based Design Approach. J Med Chem 57:8886–8902.

24. Rangasamy L, Di Geronimo B, Ortín I, et al (2019) Molecular Imaging Probes Based on Matrix Metalloproteinase Inhibitors (MMPIs). Molecules 24:2982.

25. Engel CK, Pirard B, Schimanski S, et al (2005) Structural basis for the highly selective inhibition of MMP-13. Chem Biol 12:181-189.

26. Wagner S, Breyholz H-J, Law MP, et al (2007) Novel Fluorinated Derivatives of the Broad-Spectrum MMP Inhibitors *N* -Hydroxy-2(*R*)-[[(4-methoxyphenyl)sulfonyl](benzyl)- and (3-picolyl)-amino]-3-methyl-butanamide as Potential Tools for the Molecular Imaging of Activated MMPs with PET. J Med Chem 50:5752–5764.

27. Zinnhardt B, Pigeon H, Thézé B, et al (2017) Combined PET imaging of the inflammatory tumor microenvironment identifies margins of unique radiotracer uptake. Cancer Res 77: 1831–1841.

28. Zinnhardt B, Viel T, Wachsmuth L, et al (2015) Multimodal imaging reveals temporal and spatial microglia and matrix metalloproteinase activity after experimental stroke. J Cereb Blood Flow Metab 35:1711-1721.

29. Nakashima Y, Plump AS, Raines EW et al (1994) ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb Vasc Biol 14:133-140.

30. Andrés-Manzano MJ, Andrés V, Dorado B (2015) Oil Red O and Hematoxylin and Eosin Staining for Quantification of Atherosclerosis Burden in Mouse Aorta and Aortic Root. Methods Mol Biol Clifton NJ 1339:85–99.

31. Karunakaran D, Geoffrion M, Wei L, et al (2016) Targeting macrophage necroptosis for therapeutic and diagnostic interventions in atherosclerosis. Sci Adv 2:e1600224.

32. Ohshima S, Petrov A, Fujimoto S, et al (2009) Molecular imaging of matrix metalloproteinase expression in atherosclerotic plaques of mice deficient in apolipoprotein E or low-density-lipoprotein receptor. J Nucl Med 50: 612-617.

33. Tavakoli S, Razavian M, Zhang J, et al (2011) Matrix metalloproteinase activation predicts amelioration of remodeling after dietary modification in injured arteries. Arterioscler Thromb Vasc Biol 31:102-109.

34. Zhang J, Nie L, Razavian M, et al (2008) Molecular imaging of activated matrix metalloproteinases in vascular remodeling. Circulation 118:1953–1960.

35. Razavian M, Tavakoli S, Zhang J, et al (2011) Atherosclerosis plaque heterogeneity and response to therapy detected by in vivo molecular imaging of matrix metalloproteinase activation. J Nucl Med 52:1795–1802.

36. Fujimoto S, Hartung D, Ohshima S, et al (2008) Molecular Imaging of Matrix Metalloproteinase in Atherosclerotic Lesions: Resolution With Dietary Modification and Statin Therapy. J Am Coll Cardiol 52:1847–1857.

37. Schäfers M, Riemann B, Kopka K, et al (2004) Scintigraphic Imaging of Matrix Metalloproteinase Activity in the Arterial Wall In Vivo. Circulation 109:2554–2559.

38. Kiugel M, Hellberg S, Käkelä M, et al (2018) Evaluation of [68Ga]Ga-DOTA-TCTP-1 for the detection of metalloproteinase 2/9 expression in mouse atherosclerotic plaques. Molecules 23:3168.

39. Veseli BE, Perrotta P, De Meyer GRA, et al (2017) Animal models of atherosclerosis. Eur J Pharmacol 816:3-13.

40. Müller A, Krämer SD, Meletta R, et al (2014) Gene expression levels of matrix metalloproteinases in human atherosclerotic plaques and evaluation of radiolabeled inhibitors as imaging agents for plaque vulnerability. Nucl Med Biol 41:562–569.

41. Newby AC (2007) Metalloproteinases and vulnerable atherosclerotic plaques. Trends Cardiovasc Med 17:253-258.

42. Schierwagen R, Maybüchen L, Zimmer S et al (2015) Seven weeks of western diet in apoliprotein-E-deficient mice induce metabolic syndrome and non-alcoholic steatohepatitis with liver fibrosis. Sci Rep 12931.

43. Duarte S, Baber J, Fujii T, Coito AJ (2015) Matrix metalloproteinases in liver injury, repair and fibrosis. Matrix Biol 147-156.

44. Dalvie D, Cosker T, Boyden T et al (2008) Metabolism Distribution and Excretion of a Matrix Metalloproteinase-13 Inhibitor, 4-[4-(4-Fluorophenoxy)-benzenesulfonylamino]tetrahydropyran-4-carboxylic Acid Hydroxyamide (CP-544439), in Rats and Dogs: Assessment of the Metabolic Profile of CP-544439 in Plasma and Urine of Humans. Drug Metab Dispos 36:1869-1883.

45. Kuzuya M, Nakamura K, Sasaki T, et al (2006) Effect of MMP-2 Deficiency on Atherosclerotic Lesion Formation in ApoE-Deficient Mice. Arterioscler Thromb Vasc Biol 26:1120–1125.

46. Johnson JL, George SJ, Newby AC, Jackson CL (2005) Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. Proc Natl Acad Sci U S A 102:15575–15580.

47. Van Wart HE, Birkedal-Hansen H (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci USA 87:5578-5582.

48. Jackson CL (2007) Defining and Defending Murine Models of Plaque Rupture. Arterioscler Thromb Vasc Biol 27:973–977.

Figure Captions

Figure 1. Structure of target radiotracers. **(a)** [^{18}F]FMBP **(b)** [^{18}F]BR-351.

Figure 2. *In vitro* target specificity and co-localization with lipid content in atherosclerotic aortae. **(a)** Representative *ApoE*$^{-/-}$ aortic *en face in vitro* autoradiographs 1 h after incubation with 45 kBq [^{18}F]FMBP or [^{18}F]BR-351. Homologous blocking was performed by co-incubation of 2 µM and 10 µM non-radioactive FMBP or BR-351. Corresponding bright-field images of ORO stains are shown. Note the slight fold in the [^{18}F]BR-351 aorta under 10 µM blocking conditions. **(b)** [^{18}F]FMBP and [^{18}F]BR-351 aortic lesion uptake. Two-way ANOVA: ****P < 0.0001, ***P = 0.0001, **P < 0.0046, * P = 0.0363, n = 4–7 per group.

Figure 3. *Ex vivo* biodistributions 30 min after intravenous radiotracer administration (15 MBq) via the lateral tail vein. **(a)** [^{18}F]FMBP. Two-way ANOVA: ****P < 0.0001, ***P = 0.0007. **(b)** [^{18}F]BR-351.Two-way ANOVA: ****P <0.0001, **P = 0.0011, n = 6-7 per group (n = 2-3 for blood, heart, and muscle).

Figure 4. *Ex vivo* autoradiographic lesion uptake, specific binding, sensitivity to atherosclerotic tissue, and extent of lipid accumulation. **(a/b)** Representative aortic *en face ex vivo* autoradiographs 30 min after intravenous injection of 15 MBq [^{18}F]FMBP or [^{18}F]BR-351 in C57Bl/6 and *ApoE*$^{-/-}$ mice via the lateral tail vein. Blocking was performed with non-radioactive FMBP or BR-351 (5 mg/kg, IP, maximum FMBP solubility) 30 minutes prior to tracer administration. Corresponding bright-field images of ORO stains are shown. **(c)** [^{18}F]FMBP and [^{18}F]BR-351 aortic lesion uptake. Two-way ANOVA: ****P <0.0001, ns = not significant, n = 3–4 per group. **(d)** Quantification of

percentage positive ORO areas in C57Bl/6 and *ApoE*$^{-/-}$ (baseline + block) mice. Unpaired t-test: ***P = 0.0002, n = 7-15 per radiotracer.

Figure 5. Immunofluorescent staining of atherosclerotic lesions detected by [^{18}F]FMBP *ex vivo* autoradiography. **(a/b)** Selected [^{18}F]FMBP *ex vivo* autoradiographs in C57Bl/6 and *ApoE*$^{-/-}$ mice. Corresponding composite images of cross-sections following immunofluorescent staining for **(c/d)** Hoescht, **(e/f)** Mac-2, and **(g/h)** MMP-13. **(i/j)** Mac-2 and MMP-13 merge. Scale bar = 100 µm. **(k/l)** Quantification of percentage positive Mac-2 and MMP-13 areas in *ApoE*$^{-/-}$ and C57Bl/6 mice. Unpaired t-test: ***P = 0.0001, *P = 0.0127, n = 6–8 per group.

Tables

Table 1. Selectivity profiles of [18F]FMBP and [18F]BR-351

IC$_{50}$ (nM)*				
Radiotracer	MMP-2	MMP-8	MMP-9	MMP-13
[^{18}F]FMBP	>10^5	>10^5	>10^5	56 ± 2
[^{18}F]BR-351	4 ± 3	2 ± 1	50 ± 27	11 ± 0.3

*Values reported in Hugenberg *et al.* [22] and Wagner *et al.* [26] as the mean ± SD of three experiments.

Table 2. Plasma metabolite analysis of [^{18}F]FMBP and [^{18}F]BR-351.

Fraction*	[^{18}F]FMBP			[^{18}F]BR-351		
	C57Bl/6 Baseline	*ApoE*$^{-/-}$ Baseline	*ApoE*$^{-/-}$ Block	C57Bl/6 Baseline	*ApoE*$^{-/-}$ Baseline	*ApoE*$^{-/-}$ Block
Parent Radiotracer (%)	88	79	74	9	37	16
Polar Metabolites (%)	11	19	24	88	53	82
Nonpolar Metabolites (%)	1	2	2	3	10	2
Extraction Efficiency (%)	95	95	91	85	82	75

*Values expressed as % of total radioactivity obtained for pooled blood samples 30 min after intravenous radiotracer administration, *n* = 2-3.